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Long Range Control Circuits within Mitochondria and Between Nucleus and Mitochondria

I. Methodology and Phenomenology of Suppressors

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Summary. To uncover the functional circuitry both within the mitochondrial genome and between the mitochondrial and the nuclear genome, we have developed a general method for selecting and characterizing genetically suppressor mutations that restore the respiratory capacity of *mit*$^-$ mitochondrial mutants.

Several hundreds of pseudo-wild type revertants due to a second unlinked mutation which suppresses a target *mit*$^-$ mutation were isolated. The suppressor mutations were found located either in the nuclear (abbreviated NAM for 'nuclear accommodation of mitochondria') or in the mitochondrial genome (abbreviated MIM for 'mitochondrial-mitochondrial interaction').

The specificity of action of various suppressors upon some 250 different *mit*$^-$ mutations located in several genes was tested. According to this specificity of action, suppressors were subdivided into two major classes: allele specific or gene specific suppressors. Because the *cob-box* mitochondrial gene has a mosaic organization, we were able to find a novel third class of extragenic suppressors specific for *mit*$^-$ mutations within the introns of this gene.

Four examples of suppressors showing various specificities of action illustrate our approach. (1) a nuclear gene controlling specific alleles of different mitochondrial genes; (2) a nuclear gene controlling selectively one intron of a split mitochondrial gene; (3) a mitochondrial gene controlling specific alleles of different mitochondrial genes; (4) a region in one complex mitochondrial gene which controls selectively one intron of another split mitochondrial gene.

Different mechanisms of suppression are discussed stressing the alleviation of splicing deficiencies of intron mutations.

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Introduction

The organization of mitochondrial genes in *S. cerevisiae* has been the subject of numerous genetic and biochemical studies (for recent symposia see: Saccone and Kroon 1976; Bücher et al. 1976; Bandlow et al. 1977; Bacila et al. 1978). Hundreds of mutants have been mapped and assigned to different loci on the mitochondrial genome (cf. Dujon et al. 1977). The genetic map has been correlated with the physical map obtained by restriction endonuclease and hybridization analysis of mtDNA (cf. Borst and Grivell 1978). More recently, sequence data of some mitochondrial genes have been published (Hensgens et al. 1979; Coruzzi and Tzagoloff 1979; Fox 1979; Dujon 1980; Jacq et al. 1980).

Numerous products of mitochondrial transcription and translation have been identified. The mitochondrial genes code for rRNAs and tRNAs required for mitochondrial protein synthesis and for some polypeptides of the enzyme components of the inner mitochondrial membrane (cytochrome b, two at least (if not four) subunits of ATPase, three subunits of cytochrome oxidase).

Particularly extensive studies of the gene (*cob-box*) specifying cytochrome b have shown that this gene displays a mosaic organization of genetic information typical of many genes of higher organisms (Slonimski et al. 1978; Alexander et al. 1979; Haid et al. 1979; Grivell et al. 1979; Halbreich et al. 1980; Dujon 1979 for review; Crick 1979).

Although the nature of numerous mitochondrial genes has been well clarified, the interactions between different mitochondrial genes have, until now, been little studied. The fact that some *cob-box* mutations are pleiotropic and block the synthesis of both cytochrome b and subunit I of cytochrome oxidase (encoded

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by *oxi3*, another mitochondrial gene) suggests the existence of strong interactions between these two mitochondrial genes (Kotylak and Slonimski 1976; Church et al. 1979).

However, most genetic information necessary for the biogenesis of mitochondria is provided by the nuclear genome. Most, if not all, mitochondrial ribosomal proteins, many polypeptides of the enzyme complexes of the respiratory chain and the general enzymatic machinery of replication and transcription are encoded by the nuclear genome and synthesized on cytoplasmic ribosomes. Therefore it is logical to expect that interactions between the mitochondrial and the nuclear genome exist. Different authors have isolated in *S. cerevisiae* nuclear mutations modifying the expression of mitochondrial genes (Chen et al. 1950; Sherman and Slonimski 1964; Tzagoloff et al. 1975; Trembath et al. 1975; Schweizer et al. 1977; Waxman et al. 1979). Nuclear suppressors of mitochondrial mutations have also been described in other fungi such as *Neurospora crassa* (Mitchell and Mitchell 1956; Gillie 1970; Bertrand and Kohout 1977) and *Aspergillus nidulans* (Rowlands et al. 1977). But no systematic study of interactions between nuclear and mitochondrial genes has been reported until now.

To uncover the functional circuitry both within the mitochondrial genome and between the mitochondrial and the nuclear genome, we have undertaken a systematic search for suppressors of mitochondrial *mit*⁻ mutants. This paper describes the general methodology used to characterize genetically these suppressors. We shall subdivide these suppressors into two major classes: (1) suppressors called MIM (for 'mitochondrial-mitochondrial interactions') and located on the mitochondrial genome; (2) suppressors called NAM[1] (for 'nuclear accommodation of mitochondria') and located on the nuclear genome. Moreover, to get a comprehensive picture of various types of interactions, we have isolated several hundreds of suppressors from several dozens of specific *mit*⁻ mutations. We have developed rapid and easy experimental tests designed to: (1) discriminate against true back mutants and to retain only the revertants due to a second mutation at another site; (2) allocate these revertants to mitochondrial or nuclear genome and to discriminate between nuclear recessive and dominant mutations; (3) localize precisely the second mutation on the mitochondrial or on the nuclear genome; (4) test the specificity of action of the different suppressors on several hundreds of *mit*⁻ mutations.

Four examples of suppressors showing various specificities of action and providing a unique possibility of analyzing their specificity as a function of the location of suppressed mutations within introns and/or exons will illustrate our approach.

[1] this term has been proposed by Dr. D.C. Hawthorne

Abbreviations

EB ethidium bromide, EMS ethyl methane sulfonate, mtDNA mitochondrial DNA, rRNA ribosomal RNA.

Material and Methods

Strains

777-3A α *ade1 opl* [*rho*⁺] [*mit*⁺] (Kotylak and Slonimski 1977).

AB1-4A/8 *a his4 OP* [*rho*⁺] [*mit*⁺] (Kruszewska and Szczesniak 1980). AB1-4A/8 is isogenic to 777-3A except for mating type, the auxotrophy markers and for the *opl* allele.

D273-10B α [*rho*⁺] [*mit*⁺] (Sherman et al. 1968)

ID41-6/161 *a ade lys* [*rho*⁺] [*mit*⁺] $C^R_{321}O^R_1P^R_1$ (Alexander et al. 1979).

JC8 *a leu1 can*^{R} *kar1-1* [*rho*⁺] [*mit*⁺] (Clonde and Fink 1976).

JC8/55 *a leu1 can*^{R} *kar1-1* [*rho*°]

The original *mit*⁻ strains used were described by: Slonimski and Tzagoloff 1976; Kotylak and Slonimski 1977; Carignani et al. 1979; Alexander et al. 1979; Haid et al. 1979; Kruszewska et al. 1980. Some were obtained from Dr. A. Purtrament. They were derived from three non-isogenic strains: D273-10B, 777-3A, and ID41-6/161.

Series of *mit*⁻ strains of opposite mating types to the original ones were constructed: (1) as described in Slonimski and Tzagoloff 1976; (2) the original α *mit*⁻ strains were crossed to AB1-4A/8 (Lamouroux 1979). Diploids were sporulated and *mit*⁻ spores carrying the *a*, *his4*, and *OP* nuclear markers were selected. These *a mit*⁻ strains were isogenic to the original α *mit*⁻ strains selected in 777-3A; (3) the original α *mit*⁻ strains were crossed to JC8/55. Haploid cytoductants of *a* mating type, carrying the *OP* and *leu1* nuclear markers and the *mit*⁻ mutation were selected. These *a mit*⁻ strains were isomitochondrial but not isonuclear to the original α *mit*⁻ strains. *Rho*⁻ strains employed were derived from the strains KL14-4A (see Carignani et al. 1979) and IL8-8C (Lazowska et al. 1976).

Media

YPGA: 1% yeast extract (OLHY), 1% peptone (DIFCO), 2% glucose, 20 μg/ml adenine; YPD: as YPGA but with 2% glycerol and 0.1% glucose and without adenine; YP10: as YPGA but with 10% glucose; WO: 0.67% yeast nitrogen base without amino acids (DIFCO), 2% glucose; W10: as WO but with 10% glucose; N3: 1% yeast extract (OLHY), 1% peptone (DIFCO), 2% glycerol, 0.05M sodium potassium phosphate pH 6.25; NE: same as N3 but with 2% ethanol added after autoclaving instead of glycerol; NL: 0.75% yeast extract (DIFCO), 0.75% peptone (DIFCO), 0.2 M K/Na phthalate, pH 4.6 and 0.5% DL lactate (Sigma); NOEB20: 1% yeast extract (DIFCO), 1% peptone (DIFCO), 2% glucose, 0.05 M Na/K phosphate pH = 6.25 and ethidium bromide added after autoclaving to a final concentration of 20 μg/ml; NOEB40: same as NOEB20 but ethidium bromide added to a final concentration of 40 μg/ml.

We found it preferable to use yeast extract from OLHY (2 Hamburg 70, Postfach 10889 RFA) rather than from DIFCO

because residual growth of [*rho*$^-$] cells was observed on N3 medium with some batches of DIFCO yeast extract.

EMS Mutagenesis and Selection of Revertants

The parental *rho*$^+$ *mit*$^-$ strains were grown in 20 ml YP10 liquid medium to late logarithmic phase. The cells were harvested and resuspended in 4 ml saline solution. 1 ml of the cell suspension was inoculated into the following solution: 4 ml 0.25 M Sörensen buffer pH 8, 4.8 ml 4% glucose, 0.2 ml EMS and incubated for 1 h at 28° C. Cells were harvested, washed three times, inoculated into 20 ml of YP10 liquid medium and incubated overnight at 28° C. After appropriate dilution the cells were plated on non-fermentable media (for the selection of revertants) and on YPGA (for a viable cell count).

Construction of rho^0 *Strains from Revertants*

The *rho*$^+$ revertants were patched on YPGA master plates (25 *rho*$^+$ revertant clones per plate) and grown for 2 days at 28° C. Plates were replicated on NOEB40 medium and incubated for 2 days, then replicated onto NOEB40 again, three times successively. To reduce the number of cells, the plates were stamped three times with velvet which was discarded before each replica plating on NOEB40 medium. After the fourth passage, the NOEB40 plates were replicated onto YPGA plates and incubated for two days at 28° C. These YPGA plates constitute the *rho*^{0} revertant master plates. This procedure is useful for constructing numerous *rho*^{0} strains simultaneously.

Obtention of rho$^-$ *Clones from rho*$^+$ *Revertant Strains*

EB mutagenesis was performed as follows: cells of the [*rho*$^+$] revertant strain grown in YPGA liquid medium until early stationary phase were diluted to a final density of 10^7 cells/ml in NOEB20 liquid medium and incubated for 1 h at 28° C. The cells were washed and plated on YPGA plates. These *rho*$^-$ primary clones were then subcloned to give *rho*$^-$ secondary clones (Schweyen et al. 1976).

Synchronous Crosses and Sorting out of Mitochondrial Homoplasmons

The parental strains were grown at 28° C in N3 (for the *rho*$^+$ *mit*$^+$ strains) or YP10 (for the *rho*$^+$ *mit*$^-$ or *rho*$^-$ strains) liquid medium to late logarithmic phase. Cells of the two parents were mixed to a final density of 10^7 cells/ml each in 10 ml of YP10 liquid medium and shaken for 1.5 h at 28° C. The cells were then harvested by centrifugation and incubated 30 min at 28° C without shaking. The cells were resuspended and shaken for 2 h at 28° C again. The cells were washed twice, resuspended in 10 ml of saline and 0.5 ml of this supension was inoculated into 10 ml of W10 liquid medium. After 2 days' incubation at 28° C without shaking, 0.1 ml of the suspension was inoculated into 10 ml of fresh W10 liquid medium and incubated for 2 days again under the same conditions. Aliquots of this culture were diluted in saline and plated onto WO medium. After three days' incubation the colonies were replicated onto N3 medium and incubated for 4 days. Glycerol positive and negative colonies were scored. As pointed out in Kotylak and Slonimski (1976), the selective advantage of the glycerol positive cells over the glycerol negative ones is minimized under those conditions.

Results

Isolation of Revertants from mit$^-$ *Mutants*

Mit$^-$ mutants are respiration deficient and consequently do not grow on non-fermentable substrates such as glycerol, ethanol, or lactate. Revertants from *mit*$^-$ mutants were screened by plating concentrated suspensions of cells on non-fermentable media containing either glycerol or ethanol or lactate as energy source. The plates were incubated at 28° C, 18° C, and 36° C. The lawn did not grow but a small number of respiratory sufficient colonies appeared after variable times. These colonies were picked up as presumed revertants and each revertant was subcloned on a selective medium. Revertants were selected either spontaneously or after EMS mutagenesis (see Materials and Methods). After EMS mutagenesis, the frequency of revertants was increased only for some specific *mit*$^-$ mutants: for example, in the case of the *box7-1* mutant, the frequency was 6×10^{-8} before and 2×10^{-7} after EMS mutagenesis and plating on glycerol medium at 28° C. This frequency of revertants was similar at 18° C and 28° C but was lower at 36° C. No major difference between the three substrates was observed in this particular case.

We have isolated several thousands of revertants from specific *mit*$^-$ mutants of different mitochondrial regions.

Different classes of revertants are to be expected depending on the nature and the genetic location of the second mutation leading to restoration of respiratory capacity: (1) A true back mutant where the second mutation is at the site of the forward mutation and restores the wild-type mtDNA; (2) A mutant where the second mutation is also at the site of the forward mutation but does not restore the original wild-type mtDNA sequence; (3) A closely linked suppressor where the second mutation is located close to the forward *mit*$^-$ mutation; (4) A remote mitochondrial suppressor where the second mutation is on the mtDNA but far away from the *mit*$^-$ mutation; (5) A dominant nuclear suppressor where the second mutation is on the nuclear DNA and is dominant in a heterozygous diploid; (6) A recessive nuclear suppressor where the second mutation is on the nuclear DNA and is recessive in a heterozygous diploid.

Only classes (1) to (5) are expected when the revertants are isolated from a diploid *mit*$^-$ strain. Some physiologic and biochemical characteristics of revertants originating from diploid strains have already been reported (Pajot et al. 1977; Wambier-Kluppel 1977). A more general procedure concerning revertants originating from haploid strains is described

below. The first step aims at eliminating the class (1) revertants which are of no interest.

Selection of Pseudo-wild Type Revertants

To discriminate between true back mutants and other types of revertants, we reasoned that a true back mutation had to restore the exact wild-type phenotype of the original *mit*$^+$ strain while a pseudo-wild type phenotype (i.e., partial restoration of the ability to grow on nonfermentable substrates) could be displayed by the other classes of revertants. This assumption was confirmed by subsequent studies.

Revertants were grown on three media containing three different nonfermentable substrates: glycerol, ethanol, and lactate and at three different temperatures 18° C, 28° C, and 36° C. Table 1 shows some examples of this phenotypic discrimination between the different revertants isolated from the *box7-1* mutant. Wild type revertants (such as 8-7) restoring the exact *rho*$^+$ *mit*$^+$ phenotype, could, on the basis of this test, be true back mutants and were not further analyzed. In contrast, the growth of pseudo-wild type revertants (such as 12-15, 9-12, or 9-11) depends on the temperature and on the substrate. It is interesting to notice that in this case all the revertants were able to grow on a glycerol medium at 28° C. The pseudo-wild type revertants were presumed not to be true back mutants; i.e., to be any of classes (2) to (6) above. They were retained for further analysis.

In these pseudo-wild type revertants the original *mit*$^-$ mutation will be referred to as the 'target' mutation and the second mutation will be referred to as the 'suppressor' mutation. When the suppressor mutation is located in another gene from the one carrying the target *mit*$^-$ mutation, this gene will be designated as the 'suppressor' gene. In such a case, the revertant carries the active allele of the suppressor gene and the original wild-type strain carries the inactive allele of the suppressor gene.

Table 1. Examples of phenotypic discrimination between wild-type and pseudo wild-type revertants obtained from the *box7-1* mutant

Strains	Temperature								
	18° C			28° C			36° C		
	Substrates								
	N3	NE	NL	N3	NE	NL	N3	NE	NL
rho$^+$ *mit*$^+$	+	+	+	+	+	+	+	+	+
rho$^+$ *mit*$^-$	−	−	−	−	−	−	−	−	−
rho$^+$ revertants									
8–7	+	+	+	+	+	+	+	+	+
12–15	−	−	−	+	+	+	−	−	−
9–12	−	−	−	+	+	+	+	−	−
9–11	+	−	−	+	+	+	+	−	+

rho$^+$ revertants were grown on glucose master plates at 28° C for 2 days and replicated onto three different media containing glycerol (N3), ethanol (NE), and lactate (NL) as carbon source. Replicas were incubated at 18° C (6 days), 28° C and 36° C (4 days). + = growth; − = no growth. The first line shows the phenotype of a wild-type *rho*$^+$ *mit*$^+$ strain. The second the phenotype of the *rho*$^+$ *mit*$^-$ strain *box7-1*. The four following lines show the phenotypes of four different revertants obtained from the *box7-1* mutant

Genetic Nature of the Suppressor Mutations Present in Pseudo Wild-Type Revertants

To establish the genetic nature of the second mutation present in a revertant, we adopted the following strategy: For every revertant, a *rho*^{0} clone was produced by EB mutagenesis by growing the *rho*$^+$ revertant on NOEB40 solid medium (see Material and Methods). The *rho*$^+$ revertant and the *rho*^{0} derivative were crossed to the tester *rho*$^+$ strain carrying the target *mit*$^-$ mutation as described in the legend of Table 2. Diploid progeny of these crosses were tested for the ability to grow on glycerol at 28° C since all the revertants were able to grow under these conditions. Table 2 presents different theoretical possibilities. When the second mutation is nuclear, the two test-crosses give the same result: the diploid cells are always heterozygous for the suppressor mutation. If the active allele of the suppressor is dominant, all the diploid progeny grow on glycerol medium. If it

Table 2. Characterization of the genetic nature of the suppressor mutation present in a revertant

Test-crosses		Diploid progeny		
Revertant	× Tester			
rho$^+$ [*gly*$^+$]	*rho*$^+$ *mit*$^-$ [*gly*$^-$]	+	+	−
Revertant	× Tester			
rho^0 [*gly*$^-$]	*rho*$^+$ *mit*$^-$ [*gly*$^-$]	+	−	−
Genetic nature of the second mutation		Nuclear dominant	Mitochondrial	Nuclear recessive

A *rho*$^+$ revertant and the *rho*^{0} obtained from it were grown on YPGA master plates and cross-replicated onto the tester *rho*$^+$ *mit*$^-$ strain lawn on WO medium. After 3 days' incubation at 28° C, the mating plates were replicated onto N3 medium and incubated 4 days at 28° C. + = growth on N3 medium, − = no growth

Table 3. Examples of the genetic nature of the second mutation present in pseudo wild-type revertants from different *mit*$^-$ mutants

mit$^-$ mutant	Genetic nature			
	Mitochondrial	Nuclear		Total
		Dominant	Recessive	
box2-2	26	–	54	80
box2-4	8	–	–	8
box3-4	44	–	–	44
box3-5	6	–	–	6
box7-1	32	83	–	115

The first column gives the initial *mit*$^-$ mutants from which the revertants were selected. The second column indicates the number of revertants for which the second mutation is located in the mtDNA; the third and the fourth give the number of revertants for which the suppressor mutation is in the nuclear DNA

is recessive, no diploid cells grow on glycerol medium in the two test crosses. When the second mutation is mitochondrial, mitotic segregation of the active and inactive alleles of the suppressor occurs only in the cross with the *rho*$^+$ revertant strain. The diploid cells carrying the active allele grow on glycerol. In the rho^o derivatives the active allele located on the mtDNA has been lost and thus the test-crosses give no suppression of the *mit*$^-$ mutation in the diploid cells; i.e., there are no diploid cells able to grow on glycerol.

The revertants were classified into three groups depending on the genetic nature of the second mutation: nuclear dominant, mitochondrial, and nuclear recessive.

Table 3 shows some examples of the genetic nature of the second mutation present in pseudo wild-type revertants we have isolated from different *mit*$^-$ mutants. The number of revertants as well as the genetic nature of the second mutation depends on the original *mit*$^-$ mutation. The second mutation was always found to be located on the mitochondrial genome in the revertants from the *box3-4, box3-5,* and *box2-4* mutants. In contrast, for the *box7-1* and *box2-2* mutants, the second mutation was assigned either to the mitochondrial or to the nuclear genome. The nuclear suppressor mutation was found to be dominant for the revertants from the *box7-1* mutant and recessive for the ones from the *box2-2* mutant.

To confirm the assignment of the second mutation in the case of some particularly interesting revertants the mitotic and meiotic segregation of the glycerol positive and glycerol negative cell types were studied. Diploid progeny of the previous cross (see Table 2) between the *rho*$^+$ revertant and the *rho*$^+$ *mit*$^-$ strain were quantitatively analyzed. We have found that when the second mutation was nuclear, there was no mitotic segregation: all the diploids were glycerol positive, when the suppressor was dominant, and glycerol negative when the suppressor was recessive (data not shown). When the second mutation was mitochondrial, we observed a mitotic segregation of glycerol positive and negative cell types. In a further analysis we sporulated the diploid cells and dissected tetrads. A meiotic segregation of glycerol positive: glycerol negative cell types was observed only for a nuclear mutation. For a mitochondrial one a 4:0 segregation for the glycerol positive: glycerol negative cell types was observed.

Thus, the genetic nature of the suppressor mutation present in many different revertants originating from specific *mit*$^-$ mutants has been established. For some particularly interesting revertants, this mutation was precisely localized either on the nuclear or on the mitochondrial genome.

Localization of the Suppressor Mutation on the Nuclear Genome

When the suppressor was nuclear and a 2:2 segregation of glycerol positive: glycerol negative cell types indicating monogenic inheritance was observed, chromosome mapping of this suppressor gene was undertaken. Crosses between the *rho*$^+$ revertant strain and several strains carrying many chromosomal markers (kindly provided by Dr. D.C. Hawthorne) were performed. Diploid cells were sporulated and tetrads dissected. Analysis of the recombinant frequencies between the suppressor gene and different chromosomal markers should permit us to place this suppressor gene on the linkage map of *S. cerevisiae*. Detailed results of the chromosome mapping of one nuclear suppressor called NAM2 will be reported in a separate publication (Dujardin, in preparation).

Localization of the Suppressor Mutation in the Mitochondrial Genome

The suppressor mutation present in a mitochondrial revertant can be either closely linked or far away from the target mutation. In the second case, a *rho*$^-$ clone carrying only this suppressor mutation and deleted for the target mutation can be isolated. Starting from different revertants, the screening of such a *rho*$^-$ clone was undertaken to determine whether the suppressor mutation was closely linked to the target mutation or not. In the present study, the analysis of remote mitochondrial suppressors is of principal interest. Closely linked suppressors are of interest too as has already been shown by the work of Cabral

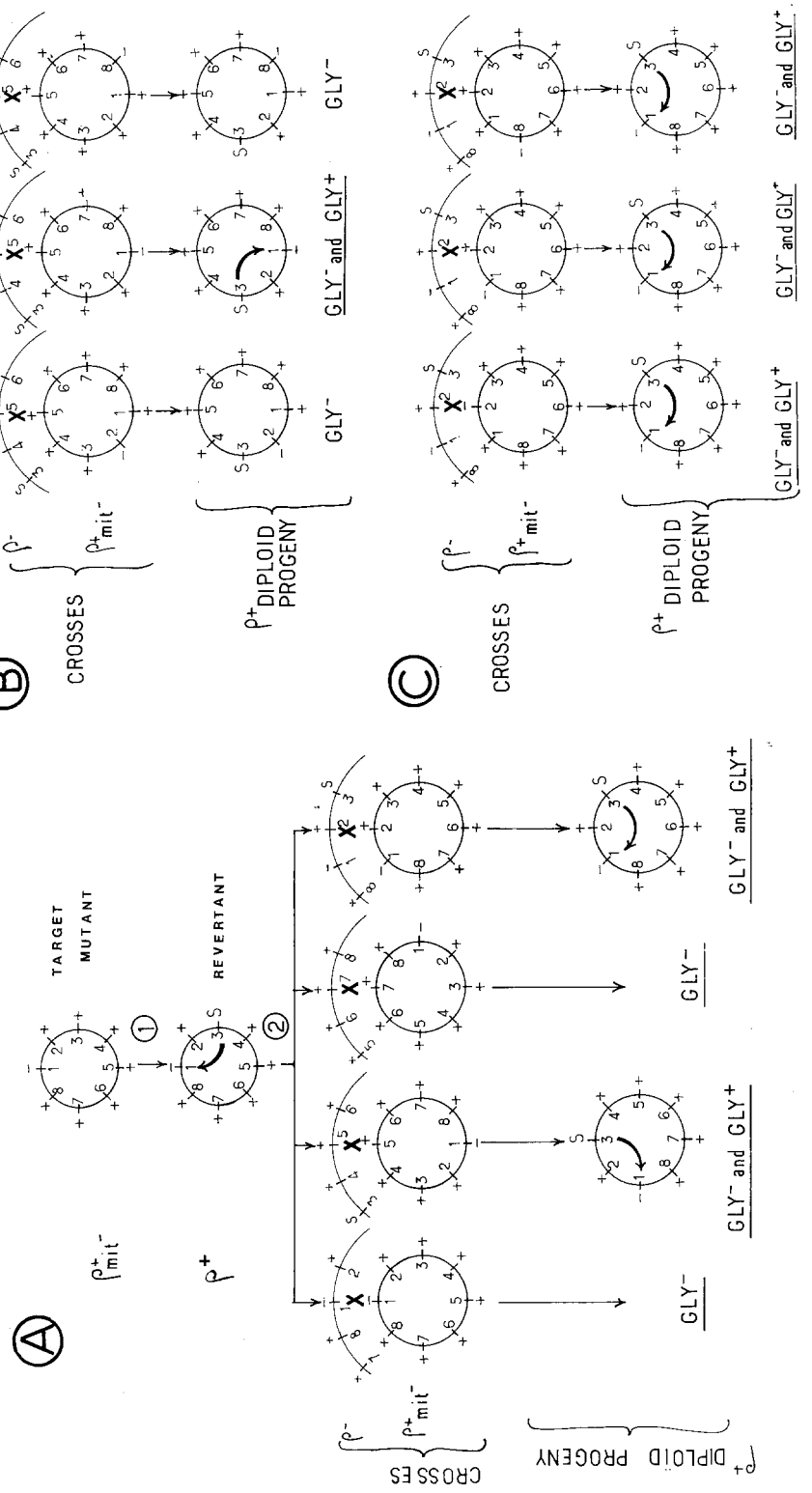

Fig. 1 A–C. Isolation of the suppressor mutation and selection of *rho*⁻ clones carrying the active suppressor allele. The circle represents the mtDNA of a *rho*⁺ strain. The arc of the circle represents the segment of mtDNA retained in a *rho*⁻ strain. 1, 2, 4, 5, ... 8 represent *mit*⁻ mutation sites in different mitochondrial genes. + = *mit*⁺ allele, − = *mit*⁻ allele. S and + are the mutated active and the wild-type inactive alleles of the suppressor gene. The arrow schematises the effect of the suppressor on the mutation 1. GLY⁻ = no growth on N3 medium. GLY⁺ = growth on N3 medium. The isolation of suppressors is schematized in **A**, above. A random collection of *rho*⁻ secondary clones is obtained by ethidium bromide mutagenesis of the revertant. Clones carrying the active suppressor are selected as shown at the bottom of Fig. 1A. Further screening of such *rho*⁻ clones permits separation of those that are deleted for the target mutation (**B**) from those that still have it (**C**)

et al. (1978) and Fox (1979) but their mechanisms of action may be quite different.

1. Selection of Remote Suppressors. *Rho*$^-$ mutants were produced by EB mutagenesis (see Materials and Methods) of the *rho*$^+$ revertants. The resulting *rho*$^-$ clones were tested for the retention of the suppressor mutation and of the target mutation. This analysis was done in two steps. In the first step, all the *rho*$^-$ clones carrying the suppressor mutation were selected. To do this, all *rho*$^-$ clones were crossed with the *rho*$^+$ *mit*$^-$ strain carrying the target mutation. These *rho*$^-$ clones were grown on YPGA master plate (2 days) and cross replicated onto the tester rho$^+$ lawn on WO medium. After 3 days' incubation, the mating plates were replicated onto N3 medium and incubated for 4 days. As shown on Fig. 1 A, if the retained segment of the *rho*$^-$ clone carries the suppressor mutation (called S on the figure), recombination can occur and this segment can be integrated into the *rho*$^+$ genome. The *mit*$^-$ mutation is then suppressed leading to the formation of glycerol positive diploid cells. These diploid cells display the pseudo wild-type phenotype.

The next step was to select from the *rho*$^-$ clones carrying the suppressor mutation those which are deleted for the target *mit*$^-$ mutation. The *rho*$^-$ clones were crossed with two *rho*$^+$ *mit*$^-$ strains each carrying a different *mit*$^-$ mutation flanking and linked to the target mutation but not suppressed by the suppressor mutation. *Rho*$^-$ clone master plates were cross replicated onto the tester *rho*$^+$ *mit*$^-$ strain lawns on WO medium. After 3 days' incubation, the mating plates were replicated onto N3 medium and incubated for 4 days. As shown in Fig. 1 C, if the retained segment of the *rho*$^-$ clone is allelic to the mutated region of the DNA where the two *mit*$^-$ mutations are located, this segment can be integrated by recombination in the *rho*$^+$ genome leading to the formation of glycerol positive diploid cells. In contrast, when a *rho*$^-$ clone has lost this region, no diploid cells able to grow on glycerol are obtained (Fig. 1 B). In this case, the *rho*$^-$ clone was supposed to have lost all this region and in particular the target *mit*$^-$ mutation.

The revertants for which a *rho*$^-$ clone carrying the suppressor mutation and deleted for the target mutation could be easily selected, were classified as remote suppressors. Further studies on such mitochondrial suppressors (their localization and specificity of action) were performed as described below.

2. Localization of Mitochondrial Suppressors. Two methods, both using the properties of *rho*$^-$ mutants, were adopted for mapping of suppressor mutations on the mitochondrial DNA. The principles of these two methods are illustrated in Fig. 2. In the first, crosses of the *rho*$^-$ clone carrying the suppressor mutation and deleted for the target *mit*$^-$ mutation with various *rho*$^+$ *mit*$^-$ strains were performed. These *mit*$^-$ mutations were already well mapped in the four regions *cob-box, oxi1, oxi2,* and *oxi3* (Slonimski and Tzagoloff 1976; Kotylak and Slonimski 1977; Carignani et al. 1979; Alexander et al. 1979; Haid et al. 1979; Kruszewska et al. 1980).

The different *rho*$^+$ *mit*$^-$ strains were grown on YPGA master plates for 2 days at 28° C, cross-replicated onto the *rho*$^-$ strain lawn on WO medium and replicated onto N3 medium. If the retained segment of the *rho*$^-$ clone is allelic to the region mutated in the *rho*$^+$ *mit*$^-$ strain, this segment carrying the *mit*$^+$ allele, can be integrated in the *rho*$^+$ genome by recombination leading to the formation of glycerol positive cells. This test shows which *mit*$^-$ mutants are restored by the *rho*$^-$ clone carrying the suppressor and in consequence which *mit*$^-$ mutations are close to the suppressor mutation. As these *mit*$^-$ mutations are well mapped on the *rho*$^+$ genome, it is easy to locate the suppressor on the mitochondrial genome. The shorter the segment retained in the *rho*$^-$ clone the more precise is the suppressor mapping. However, short *rho*$^-$ segments carrying the suppressor are sometimes difficult to obtain. Moreover, the suppressor can be located in a region where there are no *mit*$^-$ mutations available as genetic markers. For these two reasons we have developed a second method of locating the suppressor mutation.

This method employs *rho*$^-$ mutants which have retained various short regions of mtDNA and were derived from a *rho*$^+$ *mit*$^+$ strain which carries the *inactive* allele of the suppressor. These *rho*$^-$ mutants are physically well characterized by restriction mapping or by electron-microscopic analysis (Lazowska and Slonimski 1976; Slonimski et al. 1978). The *rho*$^-$ clones were crossed with the *rho*$^+$ revertant strain according to the synchronous cross method (see Material and Methods). Quantitative analysis by replica plating on N3 medium was performed. If the retained segment of one of these *rho*$^-$ clones carries the inactive allele of the suppressor gene, this allele can be integrated in place of the active allele present in the *rho*$^+$ revertant. The target *mit*$^-$ mutation is then no longer suppressed leading to the formation of glycerol negative diploid cells. Precautions have to be taken in order to discriminate between *rho*$^+$ *mit*$^-$ and *rho*$^-$ colonies among the diploid progeny because both display a glycerol negative phenotype. Depending on the original *rho*$^+$ *mit*$^-$ mutation used, two characteristics were employed. First, the cytochrome spectra of some *rho*$^+$ *mit*$^-$ mutants are different from those of *rho*$^-$ mutants (Slonimski and Ephrussi 1949; Pajot

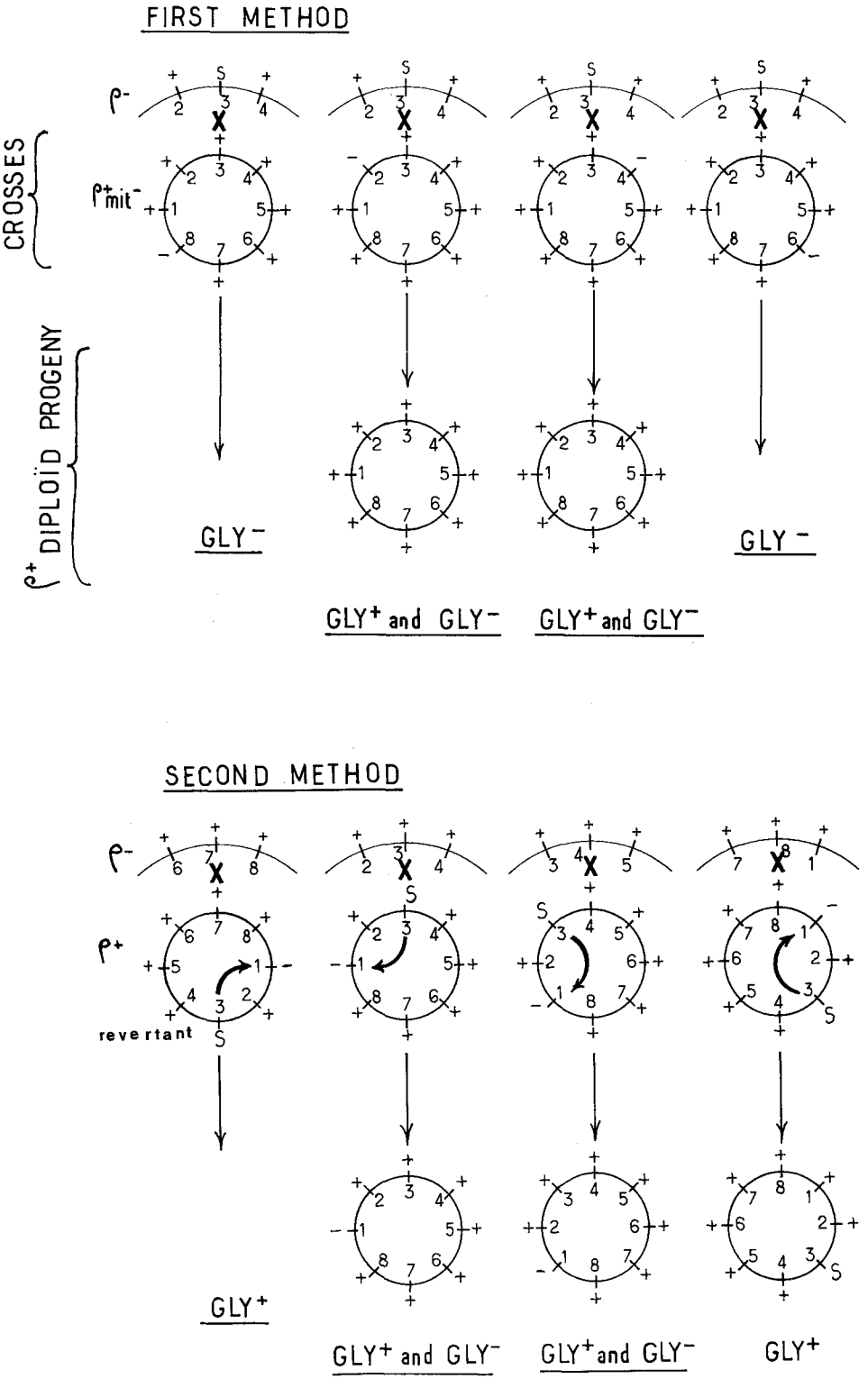

Fig. 2. Localization of suppressor mutation in a given segment of mtDNA. The suppressor was isolated on the target mutation 1 (as in Fig. 1) and found inactive on the mutations in 2, 4, 6, and 8 (as in Fig. 3). Its localization in 3 is deduced from the fact that: a *rho*⁻ clone carrying the active allele of suppressor can transmit its flanking wild-type markers 2 and 4 leading therefore to a *GLY*⁺ progeny (first method); the inactive wild-type suppressor allele can be transmitted by a *rho*⁻ clone leading therefore to *GLY*⁻ progeny in a cross to the revertant (second method)

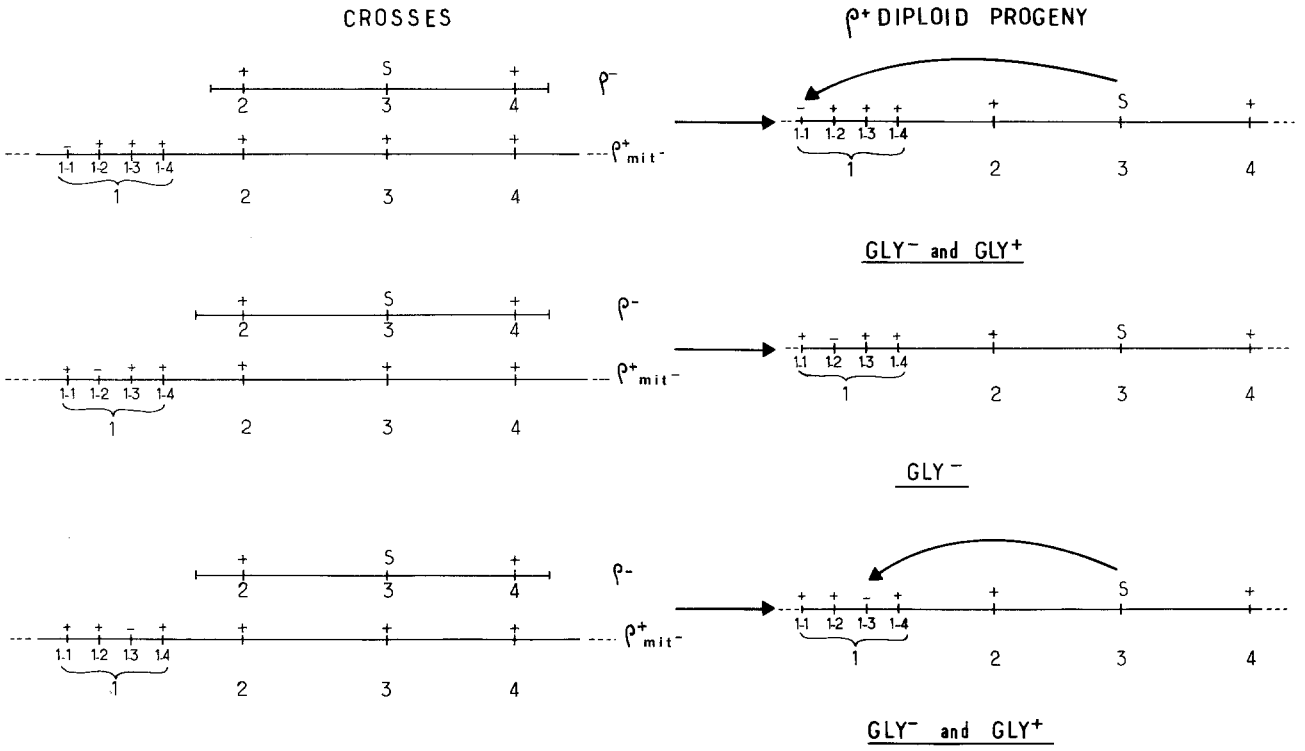

Fig. 3. Determination of the action spectrum of a mitochondrial suppressor. The suppressor was isolated on the target mutation 1-1 and its activity on different alleles (1-2, 1-3, 1-4, etc...) of the same mitochondrial gene tested. It was found inactive on the allele 1-2

et al. 1977). For these *rho*⁺ *mit*⁻ mutants, it was sufficient to record the absorption spectra of the glycerol negative cells in order to distinguish *rho*⁺ *mit*⁻ from *rho*⁻ mutants. Second, revertants from some *rho*⁺ *mit*⁻ mutants can be selected while the *rho*⁻ mutants never revert. Glycerol negative clones were therefore grown on YPGA medium, replicated on YPD medium and incubated for 2 weeks. When glycerol positive papillae appear, one can conclude that the initial glycerol negative phenotype was due to a *rho*⁺ *mit*⁻ mutation and not to a *rho*⁻ mutation.

Two mitochondrial suppressors have been precisely located by the combination of these two methods: *MIM1-1* is located in the gene coding for the 23S rRNA and *MIM2-1* is in the middle of the *oxi3* region. Detailed results of these mapping studies will be presented elsewhere (Pajot in preparation, Dujardin, in preparation).

Determination of the Action Spectra

As the first step in the investigation of the mechanism of action of these suppressors, we have tested the activity of each suppressor upon a set of some 250 different *mit*⁻ mutants in the *cob-box, oxi1, oxi2,* and *oxi3* regions of mtDNA.

To test the activity of a suppressor upon the various *mit*⁻ mutations distinct from the original target mutation, a strain carrying the active allele of the suppressor but not the target mutation is necessary. For each active nuclear suppressor, the *rho*° strain derived from the original revertant was used. This *rho*° strain was individually crossed with the complete set of *rho*⁺ *mit*⁻ strains. Diploid progeny of these crosses were then tested for the ability to grow on glycerol medium, positive growth meaning that the *mit*⁻ mutation tested was suppressed. For a recessive nuclear suppressor, the active allele is not expressed in diploid cells heterozygous for the suppressor mutation. In this case, we have used two methods. The first (suggested by D.C. Hawthorne and E. Petrochilo) consists of the induction of mitotic recombination in the heterozygous diploids cells by U.V. leading to diploid cells homozygous for the suppressor mutation. When the *mit*⁻ mutation tested is suppressed, these diploid cells grow on glycerol medium. The second (suggested by A. Kruszewska), takes advantage of the cytoduction phenomenon: when karyogamy does not immediately follow plasmogamy, there can be segregation of haploid cells which contain a nucleus from the *rho*° parent and mitochondria from the *rho*⁺ *mit*⁻ parent. In such haploid cytoductant cells,

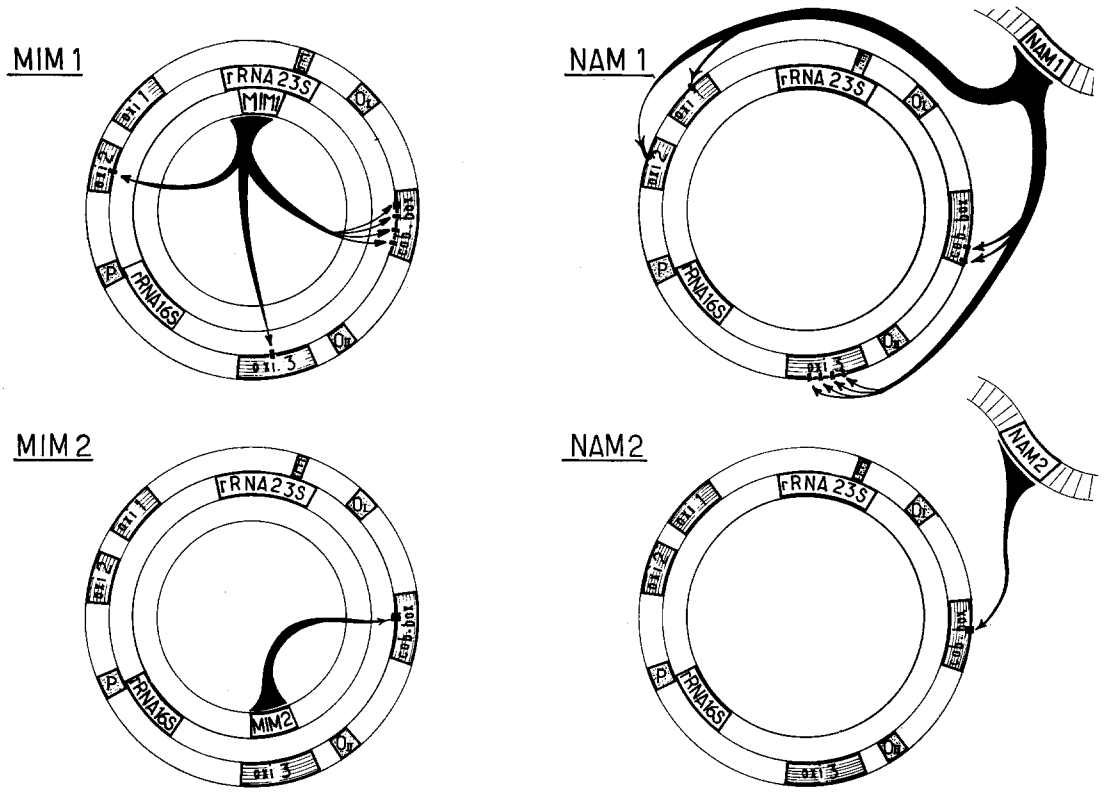

Fig. 4. Action spectra of two mitochondrial (MIM1 and MIM2) and two nuclear (NAM1 and NAM2) suppressors. The concentric circles schematize the mtDNA. On the external one, the main genes affecting drug resistance or respiratory function have been placed. The second circle shows the genes of rRNAs and the third the suppressor mutations. The arrows represent the suppression effect of the suppressor on different *mit*⁻ mutations. *NAM1* and *NAM2* genes are located on the nuclear DNA

the recessive allele of the suppressor is expressed and, when it suppresses the *mit*⁻ mutation tested, these haploid cells can grow on glycerol medium. The percentage of cytoductants can be increased by U.V. treatment or by the use of the mutant karl-1 (Wright and Lederberg 1957; Conde and Fink 1976; Zakharov and Stepanova 1977).

For each mitochondrial suppressor, a *rho*⁻ clone, carrying the active suppressor allele and deleted for the target *mit*⁻ mutation, was crossed individually to all the *rho*⁺ *mit*⁻ strains. As shown in Fig. 3, the retained segment of the *rho*⁻ clone can be integrated into the *rho*⁺ genome leading to glycerol positive diploid cells if the *mit*⁻ mutation is suppressed. It is difficult, however, to test the action of the suppressor upon the *mit*⁻ mutations located close to itself: the *mit*⁺ alleles of these mutants are retained by the *rho*⁻ clone carrying the suppressor mutation and these *mit*⁺ alleles can be integrated in the *rho*⁺ genome, leading to the formation of glycerol positive diploids indipendantly of suppressor action.

Figure 4 represents the action spectra of four typical suppressors. *NAM1* and *NAM2* are nuclear dominant suppressors. *NAM1* was isolated from an *oxi3* *mit*⁻ mutant and it presents a relatively large spectrum of action: it suppresses six mutations of the *oxi3* region, 13 mutations of the *box7* and *box9* regions, one of the *oxi1*, and one of the *oxi2* region (Groudinsky, in preparation). Conversely, *NAM2* has a very restricted spectrum: it suppresses only *mit*⁻ mutations located in or close to the *box7* region (Dujardin, in preparation). *MIM1* is a mitochondrial suppressor located in the gene coding for the 23S rRNA. It suppresses five *mit*⁻ mutations spread out along the *cob-box* region, one in *oxi3* and two in *oxi2* (Pajot, in preparation). *MIM2* is a mitochondrial suppressor located in the *oxi3* region and which is specific for the *box7* *mit*⁻ mutations (Dujardin, in preparation).

The study of the action spectra of different suppressors has permitted us to establish a classification and to propose some hypotheses about the mechanisms of action of the different classes of suppressors.

Discussion

In this paper, we have described a general method for selection and genetic analysis of suppressor mutations that restore the respiratory capacity of *mit*⁻ mutants. Two nuclear (*NAM1* and *NAM2*) and two

mitochondrial (*MIM1* and *MIM2*) suppressors illustrate this study. Mitochondria depend on two sources of genetic information: nuclear and mitochondrial and strong interactions should exist between mitochondrial components specified by these two genomes. Thus, it is not surprising to find nuclear suppressors of mitochondrial mutations. In this case, the suppressor mutation is in different cellular compartment from the mutation on which it acts and all hypotheses concerning the mechanism of suppression must take this physical separation into account.

According to the specificity of action of the suppressors, two principal mechanisms of extragenic suppression have been proposed in other systems: informational and functional (see Hawthorne and Leupold, 1974; Gorini and Beckwith 1966, for review). Informational suppressors correspond to mutations which modify one of the factors controlling the mechanism of transfer of genetic information from DNA to protein: e.g., a mutation in a tRNA gene producing an anticodon change in a tRNA, a mutation in a ribosomal protein affecting the accuracy of reading of mRNA or in one of the enzymes involved in protein synthesis. Functional suppressors correspond to mutations which modify, e.g., the structure or interactions of the mutated product, or provide an alternative metabolic pathway or a by-pass of the mutational block. Functional suppressors are essentially gene specific whereas the informational ones are allele specific: they suppress different alleles of different genes. In the present study, we have been able to classify suppressors according to their specificity of action upon some 250 different *mit*$^-$ mutations located in several different genes, into two major groups: allele- or gene-specific suppressors. Because of the fact that some of mitochondrial genes have a mosaic organization characteristic of higher eucaryotes, composed of intron and exon sequences (Gilbert 1978) we were able to find *a novel third class of extragenic suppressors* undiscovered until now. These suppressor mutations are specific for intron alleles and thus presumably modify splicing and maturation of mRNA.

1. Allele Specific, Gene Non-specific Suppressors

By analogy with other systems, allele specificity suggests an informational suppression mechanism. If a suppressor of *mit*$^-$ mutations is nuclear, the simplest hypothesis is that the second mutation is in a mitoribosomal protein gene. The majority of mitoribosomal proteins are coded for by nuclear genes while the mtDNA specifies most if not all of the tRNAs and all rRNAs necessary for mitochondrial protein synthesis (cf. Gillham 1978, for discussion). If the suppressor is mitochondrial, the simplest hypothesis is that this mutation is located in a tRNA or rRNA gene. All such suppressors are expected to alleviate only those *mit*$^-$ mutations which are located within the mitochondrial DNA sequences coding for a protein. Figure 4 shows two examples of allele specific suppressors of *mit*$^-$ mutations located in different mitochondrial genes.

MIM1-1 has been selected to suppress the *box1-1* mutation which is clearly located in a protein-coding region or exon of the *cob-box* gene (Slonimski et al. 1978; Claisse et al. 1978). *MIM1-1* has a large spectrum of action and no gene specificity. These characteristics suggest that it is an informational suppressor. Interestingly, we found that *MIM1-1* is on the mtDNA in the gene coding for the large rRNA. The exact mechanism by which a mutation in the gene coding for the large rRNA which presumably changes the structure of the ribosome, can alleviate a mutation in a protein structural gene is unclear. Several possibilities may exist: e.g., a misreading of a missense or a nonsense codon or a reading frame correction. Helser et al. (1971) have shown that the presence of modified nucleotides in the small rRNA of *E. coli* clearly influences the properties of the ribosome.

The *MIM1-1* mutation is the first case in our knowledge of a mutation in a ribosomal RNA gene which gives rise to an informational suppressor of mutations in protein-coding genes.

Further analysis of the action of this suppressor on various alleles of the *cob-box* region and of the nature of the suppressible *mit*$^-$ mutations will be discussed in a separate paper (Pajot, in preparation).

NAM1-1 has been selected to suppress an *oxi3 mit*$^-$ mutation. The fact that *NAM1-1* has a large spectrum of action and no gene specificity suggests equally an informational mechanism, the suppressor mutation being located in a gene coding for a mitochondrial ribosomal protein for example. The *oxi3 mit*$^-$ mutations suppressed by *NAM1-1* are still not assigned to exon or intron segments although there is some biochemical evidence that this gene is split like the *cob-box* region (Church et al. 1979; van Ommen et al. 1979). *NAM1-1* suppresses several mutations all located within the *box3* intron and within the *box9* cluster (for which the assignment as exon or intron is still uncertain) but not a single mutation located with certainty in an exon. This result is unexpected for an informational suppressor. As intron sequences are necessary for correct splicing to occur (Church et al. 1979; Halbreich et al. 1980), one can hypothesize that *NAM1* could be a nuclear gene specifying a factor involved in the splicing mechanism. Splicing requires cooperation between RNA sequences located at the exon-intron junctions. It is

therefore conceivable that *NAM1-1* could suppress mutations both within the introns (like *box3*) and at the exon-intron junctions (like *box9*).

An even more interesting possibility comes from recent evidence presented by Jacq et al. (1980) in favor of the idea that the *box3* intron codes for a protein 'RNA maturase' involved in splicing. It was proposed that this mitochondrially encoded maturase is part of a general splicing complex, the other parts of which are encoded by nuclear genes. Our data strengthen this idea by showing that a diffusible product, presumably a protein coded by a nuclear gene, corrects splicing deficiences arising from a mutation in a mitochondrial gene. A mutual quaternary readjustment between a mutated subunit of the *box3* maturase and a mutated subunit of the *NAM1-1* counterpart in the splicing complex may result in the restoration of activity and gene expression.

Alternatively, a weak informational suppression of a protein required in small amounts, like the cytochrome b mRNA maturase (cf. Jacq et al. 1980), may be phenotypically detectable, while the same degree of suppression in cytochrome b itself would not.

2. *Gene Specific Suppressors*

By comparison with other systems, gene specificity suggests a functional suppression mechanism. If a suppressor were found acting on all *mit*⁻ alleles of a given gene, it would have indicated that the suppressor has provided an alternative metabolic pathway. No such suppressor was found in the present study.

If a suppressor was found acting on only some *mit*⁻ mutations of a single gene, one could imagine that the suppressor mutation was located in the gene coding for a protein interacting with the first mutated protein. It is well established that different polypeptides of enzyme complex III (cytochrome c-reductase) and IV (cytochrome c-oxidase) are synthesized by the cytoplasmic and the mitochondrial ribosomes (Katan et al. 1976; Schatz and Mason 1974) and coded by either the nuclear or the mitochondrial genome. One can easily imagine that functional interactions between two mutated polypeptides of such enzymatic complexes could at least partially restore respiration. One would then expect to find nuclear suppressors which could display a specificity for certain mutated alleles of a gene coding for a mitochondrially encoded subunit and which would not act upon mutated alleles of a gene coding for another subunit of the complex. This kind of suppressor would be very interesting for the study of assembly of mitochondrial membrane components. No such suppressors were found in the present study, but a more complete search starting with exon mutations in *oxi1, oxi2, oxi3,* and *cob-box* regions should be continued.

Completely different mechanisms of suppression can be proposed when the suppressible *mit*⁻ mutations are within the introns. Two examples of such suppressors are shown on Fig. 4.

The mitochondrial suppressor *MIM2-1* acts selectively upon nine *mit*⁻ mutations (out of 250 examined) all located in the *box7* intron. The fact that *box7* mutants affect both the synthesis of subunit I of cytochrome oxidase and of cytochrome b (Pajot et al. 1977; Claisse et al. 1978) and that they can be suppressed by a suppressor located in the *oxi3* region shows the existence of interactions between these two mitochondrial genes. Already Church et al. (1979) have shown that pleiotropic mutants (where cytochrome c-reductase and oxidase activities were absent) located in *box7,* and *oxi3* regions interfere with both pre-mRNA splicing patterns. One can imagine that the interactions between two sequences of two mitochondrial gene primary transcripts would be necessary for correct RNAs splicing to occur.

The nuclear suppressor *NAM2-1* is also very specific. It suppresses eight mutations (out of 250 examined) all located in the *box7* intron and those are the same ones that are suppressed by *MIM2-1*. Different active heteroalleles (*NAM2-2, NAM2-3, NAM2-4*) of the *NAM2* have been obtained and a full description of their properties will be presented elsewhere (Dujardin, in preparation).

The present work demonstrates the existence of *intron specific, allele non-specific suppressors* of mutations located in one particular intron (*box7*). This is the first time, to our knowledge, that such suppressors have been isolated and characterized. This finding is susceptible to two kinds of interpretation which are not mutually exclusive, both of which suggest the existence of novel mechanisms of regulation of gene expression.

The first is to suppose that these genes code for protein factors acting in trans and involved in the splicing of one specific intron: one of these factors would be specified by the mitochondrial genome and the second by the nuclear genome. The action of these genes has to be different from that of *NAM1-1* which is allele specific and presumably *intron non-specific*. We interpret it as indicating that the trans-acting product of *NAM1* is involved in the general splicing machinery while the products of *NAM2* and *MIM2* are involved in the selective choice of the intron to be spliced.

The second interpretation is based upon the guide RNA model (Lamouroux et al. 1979; Church et al. 1979; Halbreich et al. 1980) which proposes that RNAs corresponding to introns (and possibly exon-

intron junctions) either as precursors or as free introns serve as a guide to direct a splicing enzyme correctly. The guide RNA sequence would be mutated and inefficient in *box7* mutants and the suppressor mutation would provide a new efficient guide RNA. In the case of *NAM2*, the new guide RNA would be transcribed from a nuclear gene and must have been transported from the nucleus to the mitochondria through the mitochondrial membrane. This hypothesis is unlikely. In the case of *MIM2*, the new guide RNA would be provided by another mitochondrial region: the *oxi3* one and the second mutation in *oxi3* would create a new sequence which could interact with the mutated guide RNA of the *box7* mutant.

Whatever the exact mechanism could be the present study demonstrates two kinds of suppressor distinguished by their specificity of action. The first kind is allele specific (as *NAM1*) and the second one is gene specific and even more, intron specific (as *NAM2* or *MIM2*). That suggests the existence of at least two elements in the splicing mechanisms, one involved in the selection of a particular intron and another common to different introns.

In conclusion, the study of suppressors of *mit*⁻ mutations provides information on the problem of interactions between different gene products encoded either by the mitochondrial or by the nuclear genome. In particular, one can hope to understand better the mitochondrial translation mechanisms by the study of informational suppressors and the mitochondrial splicing mechanisms by the study of intron-specific suppressors and hopefully the interactions between these two mechanisms.

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