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Construction of novel cytochrome b genes in yeast mitochondria by subtraction or addition of introns

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The mitochondrial *cob-box* gene coding for apocytochrome b in yeast has five introns and six exons or two introns and three exons depending on the wild-type strain considered. Some intron mutations in this gene affect not only its expression but also that of another mitochondrial gene: *oxi*3. To understand better the function of introns in gene expression, we have constructed a series of new strains that differ only by the presence or absence of one of the five wild-type introns in the cytochrome b gene, the rest of the mitochondrial and nuclear genome remaining unchanged. All constructions result from *in vivo* recombination events between *rho*$^-$ donor and *rho*$^+$ recipient mtDNA. The following genes have been constructed: I1$^\triangle$I2$^\triangle$I3$^\triangle$I4$^\triangle$I5$^\triangle$, I1$^+$I2$^+$I3$^\triangle$I4$^\triangle$I5$^\triangle$, I1$^\triangle$I2$^\triangle$I3$^+$I4$^\triangle$I5$^\triangle$, I1$^\triangle$I2$^\triangle$I3$^\triangle$I4$^+$I5$^\triangle$, I1$^+$I2$^+$I3$^\triangle$I4$^+$I5$^+$, IA$^\triangle$I2$^\triangle$I3$^+$I4$^+$I5$^+$ and I1$^+$I2$^+$I3$^+$I4$^+$I5$^+$ (where '+' symbolizes the presence of the wild-type intron and '$\triangle$' its absence). Interestingly, all the genes lead to the synthesis of cytochrome b, while only the genes having the intron bI4 allow the expression of *oxi*3. A nuclear gene, when mutated, can compensate for the absence of the intron bI4.

Key words: intron subtraction and addition/mitochondrial regulation/novel cytochrome b genes

Introduction

Splicing may be used as a control mechanism for gene expression, but experimental data pertaining to this problem are rather scarce. The mitochondrial genome of the yeast *Saccharomyces cerevisiae* (strain 777-3A) contains three split genes, two of which code for respiratory proteins, the cytochrome oxidase subunit I (gene *oxi*3, Van Ommen *et al.*, 1980; Bonitz *et al.*, 1980), and the apocytochrome b (gene *cob-box*, Slonimski *et al.*, 1978; Haid *et al.*, 1979; Hanson *et al.*, 1979; Lazowska *et al.*, 1980; Nobrega and Tzagoloff, 1980; Van Ommen *et al.*, 1980). Respiratory-deficient mutants mapping in the introns and the exons of this latter gene have recently been studied. Complementation analyses between exon and intron mutants (Lamouroux *et al.*, 1980), together with DNA sequence analyses of *trans*-recessive mutants in the bI2 and bI4 (introns of *cob-box* are denoted bI1, bI2....bI5, exons B1, B2.....B6 and introns of *oxi*3, aI1, aI2....aI7), known as *box*3 and *box*7 mutations, has led to the concept of mRNA maturase, a protein encoded within the long open reading frame present in these introns (Lazowska *et al.*, 1980; De La Salle *et al.*, 1982). The maturase is thought to be a *trans*-acting factor involved in splicing since RNA processing is blocked in these intron mutants (Church *et al.*, 1979; Halbreich *et al.*, 1980; Van Ommen *et al.*, 1980). Furthermore, all the *box*7 mutations display a pleiotropic phenotype: they prevent not only the processing of the cytochrome b pre-messenger but also that of the subunit I of cytochrome oxidase (*cox*I) (Church *et al.*, 1979; Van Ommen *et al.*, 1980; Dhawale *et al.*, 1981). These results, strengthened by the isolation of a strain lacking bI4 and bI5 and unable to synthetize *cox*I (Jacq *et al.*, 1982), suggest that bI4 and more precisely its maturase product (De La Salle *et al.*, 1982) controls the expression of two distant genes, *cob-box* and *oxi*3. Recently, it has been shown that the *box*7 splicing defect can be compensated by secondary mutations mapping in the intron aI4 of the *oxi*3 gene, *mim2-1* (Dujardin *et al.*, 1982), or in the nuclear genome, *NAM2-1* (Groudinsky *et al.*, 1981).

Therefore, it is apparent that some introns do play a role in the regulation of mitochondrial gene expression. However, several introns are dispensable since some wild-type strains (e.g., D273-10B) do not contain all the introns present in other wild-type strains (e.g., 777-3A), see Table I. Thus, if introns play a regulatory role, this role has to be optional and may reveal itself only under special conditions. To understand better the function of introns, the development of a general methodology able to reveal fine interaction would be useful. Beside the analysis of point mutations, which has already been largely exploited (*loc. cit.*), a systematic and precise removal of introns should provide interesting information. Such a strategy has been used by several investigators trying to relate RNA splicing to the problem of RNA stability. They have suggested that RNA splicing is required for the accumulation of stable mRNA in the cytoplasm (Hamer and Leder, 1979; Gruss and Khoury, 1980). Nevertheless, it seems that this requirement either is not absolute or can be bypassed (Gruss *et al.*, 1981).

Here we describe a methodology for adding or subtracting introns as a single DNA segment. We have constructed a series of isogenic yeast strains which differ one from another by the presence or absence of any one of the five wild-type introns of the *cob-box* gene. All the constructions result from *in vivo* homologous recombinations between *rho*$^-$ and *rho*$^+$ mtDNA and take advantage of the existence of polymorphic wild-type strains as well as of the strain lacking bI4 and bI5. From the ability or inability of the novel genomes to synthesize cytochrome b and cytochrome oxidase, we infer that no intron is essential for cytochrome b synthesis but that the intron bI4 is both necessary and sufficient for the expression of cytochrome oxidase subunit I gene. Moreover, the absence of intron bI4 can be relieved by a mutation in a nuclear gene.

Results

Strategy for the construction of recombinant genes

As no reliable method exists for the transformation of mitochondria with extraneous DNA, we exploited *in vivo* recombination between homologous segments of mtDNA. In yeast, following the mating of two haploid strains, mitochondria fuse and multiple rounds of recombination take place between parental mtDNA. Within a few generations mitochondrial recombinant DNA molecules segregate finally to give rise to homoplasmic clones (for review, see Dujon,

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Table I. List of strains used for initial constructions

Name	Nuclear genotype	Mitochondrial genotype	*cob-box* introns	Ref.
777-3A	α *ade1 op1*	*rho*$^+$ *mit*$^+$	I1 to I5	(1)
D273-10B/A	α *met*	*rho*$^+$ *mit*$^+$	I4 and I5	(2)
D273-10B/A/M	α *met*	*rho*$^+$ *mit*$^-$	I4 and I5	(2)
KL14-4A	a *his1 trp2*	*rho*$^+$ *mit*$^+$	I1 to I5	(3)
KL14-4A/	a *his1 trp2*	*rho*$^-$		(3)
WR27-27/1	a *ura1*	*rho*$^+$ *mit*$^+$	I1 to I3	(4)
WR27-27/SR351	a *ura1*	*rho*$^-$		(4)
S912/50	a *his4 NAM2-1*	*rho*^{o}		(5)

(1) Lazowska *et al.* (1980); (2) Foury and Tzagoloff (1976); (3) Slonimski *et al.* (1978); (4) Jacq *et al.* (1982); (5) Dujardin *et al.* (1980).

1981). We have used this ability of mtDNA to recombine to generate new genes by recombination events between *rho*$^-$ and *rho*$^+$ mtDNA, where the *rho*$^-$ donates it sequence to a recipient *rho*$^+$ mtDNA.

Rho$^-$ and *rho*$^+$ were all derived from two wild-type strains, KL14-4A (which has all five introns) and D273-10B (which has only introns bI4 and bI5) or their respiratory-deficient mutants, as well as from a strain isolated by P. Pajot which lacks introns bI4 and bI5 (Jacq *et al.*, 1982), see Table I. The *rho*$^-$ are deletion mutants retaining very short segments of continuous sequences from the *rho*$^+$ mtDNA genome. They are a natural vehicle to clone introns and their flanking exons, or fused exons. By a genetic and molecular screening one can choose *rho*$^-$ clones retaining nothing but a fragment of the *cob-box* gene (see Figure 1). Because such *rho*$^-$ contain only the desired fragment, we can be certain that no rearrangements in other parts of the mitochondrial genome will occur. We therefore prefer this method to that of Perlman *et al.* (1980) in which the two non-isomitochondrial *rho*$^+$ strains are crossed. In such constructions, there is always a possibility that other regions of the mitochondrial genome become rearranged. The procedure used to mate strains and isolate homoplasmic clones is described in the legend of Figure 2 and in more detail in Dujardin *et al.* (1980).

Selection of recombinant genes

Screening and selection of recombinants was achieved by two different procedures. The first, most general one, is based on two successive molecular screenings (Figure 2A and 2B). The second is based on phenotypic selection and molecular screening (Figure 2C). Intron addition (Figure 2B and 2C) and intron subtraction (shown only for molecular screening in Figure 2A) are possible in both cases.

Molecular screening. We have used *in situ* colony hybridization (Grunstein and Hogness, 1975) with intron radioactive probes, followed by restriction and hybridization experiments carried out on small amounts of mtDNA (see minilysates in Materials and methods). The knowledge of the structure and sequence of the *cob-box* gene (Lazowska *et al.*, 1980 and in preparation; Nobrega and Tzagoloff, 1980) allowed us to choose among positive (for intron addition) or negative (for intron subtraction) colonies, those having gained (or lost) the intron. An example of colony hybridization is shown in Figure 3. This technique was reliable, as restriction and hybridization experiments showed that at least 90% of

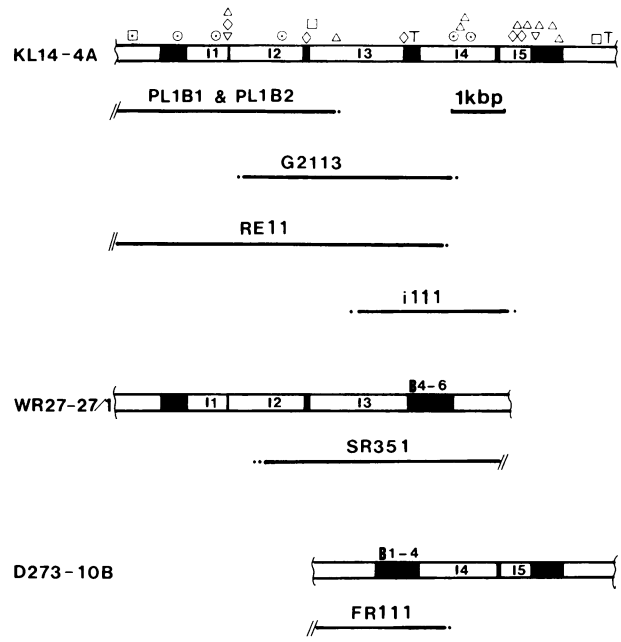

Fig. 1. Various *rho*$^-$ clones used for intron additions and subtractions. Cytochrome b exons are shown in black and introns are numbered I1 to I5. Each panel begins with the *rho*$^+$ strain from which the *rho*$^-$ clones are derived. The segments of the gene retained and amplified in the *rho*$^-$ are shown underneath. Restriction site symbols are as in Figure 4; in addition △ = *Rsa*I. PL1B1 and B2, RE11, SR351 and FR111 extend 3, 6, 20 and 1 kbp respectively beyond the gene (symbolized by a double slash).

colonies giving strong positive signals were true intron-plus recombinants (for intron addition). We have used this strategy when we had no *a priori* reason to predict whether or not the recombinant gene would confer a different phenotype from the parental one.

Phenotypic selection followed by molecular screening. This mode of selection is applicable when the recombinant gene confers a phenotypic difference, for instance if parents are respiratory-deficient *box*$^-$ and *rho*$^-$, then recombinants can be selected for respiration. An appropriate choice of *rho*$^+$ *box*$^-$ mutants and *rho*$^-$ *box*$^+$ clones makes this selection possible. Indeed, consider a *rho*$^+$ strain that has a cytochrome b- or splicing-deficient *box*$^-$ mutation located within one of the exons adjacent to the intron to be added or withdrawn, or within the intron to be withdrawn. From a cross by a *rho*$^-$ clone derived from a *rho*$^+$ *box*$^+$ strain, respiratory-competent recombinants can be obtained. Three cases are to be considered. (1) The *rho*$^+$ *box*$^-$ strain carries an exon mutation and has no adjacent intron(s) while the *rho*$^-$ *box*$^+$ strain carries the wild-type exons and adjacent intron(s); among the *rho*$^+$ *box*$^+$ recombinants, some can acquire the intron(s) by homologous recombination in exons as shown in Figure 2C; the mechanism of this recombination is unknown and could either result from gene conversion or double crossing-over. (2) The *rho*$^+$ *box*$^-$ strain carries an exon mutation and does have adjacent intron(s) while the *rho*$^-$ *box*$^+$ does not have them; similarly, some *rho*$^+$ *box*$^+$ recombinants can loose the intron(s). (3) The *rho*$^+$ *box*$^-$ strain carries an intron mutation while the *rho*$^-$ *box*$^+$ strain lacks the corresponding intron; recombination within the flanking exons may remove the mutated intron and restore respiration.

After phenotypic selection, respiratory-competent strains were further analyzed with molecular probes as described

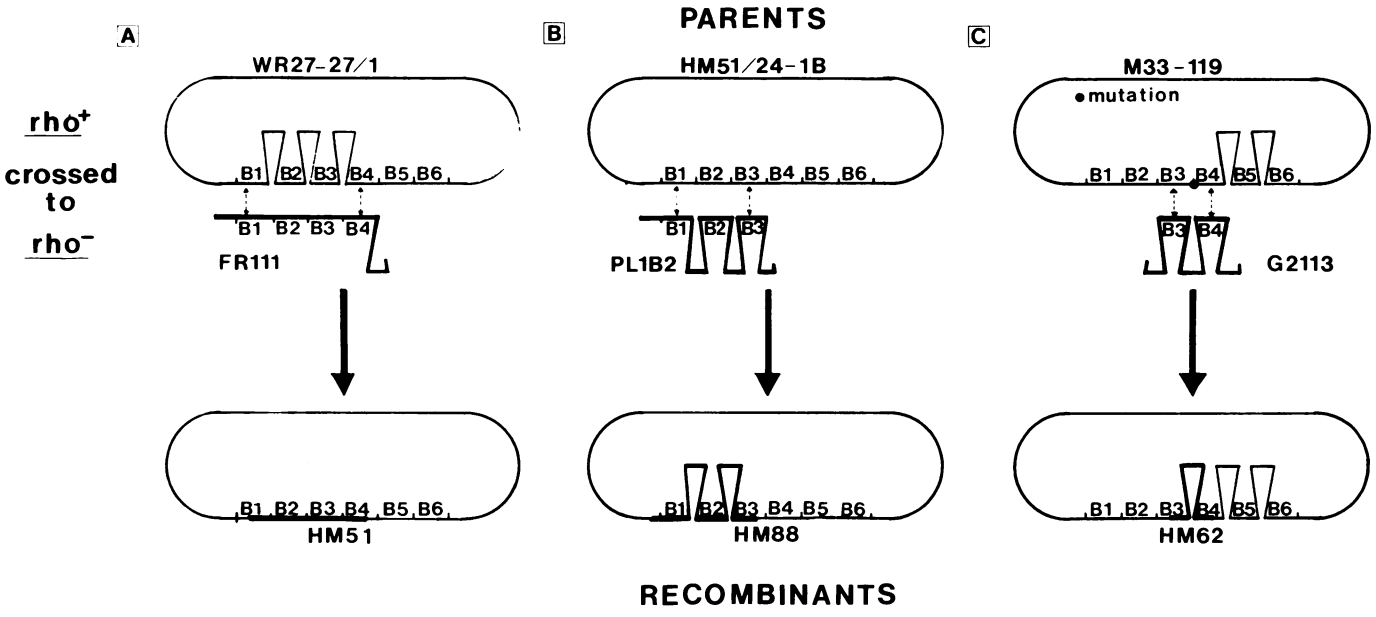

Fig. 2. Schematic representation of three strategies used to add or to withdraw intron DNA by the *in vivo* homologous recombination between exons of the cytochrome b gene. *Rho*$^+$ and *rho*$^-$ cells are mated synchronously and zygotes allowed to multiply for some 15 generations under non-selective conditions and thereafter homoplasmic *rho*$^+$ cells are screened and selected. **Panel A**, intron subtraction and construction of the intron-free gene: the *rho*$^-$ clone D273-10B/A/FR111, which contains the uninterrupted DNA sequence of exons B1 to B4 and is deleted for the 3′ part of intron bI4 as well as for the remaining exons, is crossed to the *rho*$^+$ strain WR27-27/1 which is devoid of introns bI4 to bI5; among the randomly isolated *rho*$^+$ descendants of the cross some clones were found to be devoid of all introns, as shown by restriction analysis of their mtDNA (Figure 4). **Panel B**, intron addition: the *rho*$^+$ intron-free strain (derived from HM51, see text) HM51/24-1B, is crossed to *rho*$^-$ clone KL14-4A/PL1B2 which contains the introns bI1 and bI2, the flanking exons B1, B2 and B3 and is deleted for the 3′ part of intron bI3 as well as for the remaining exons; among the randomly isolated *rho*$^+$ descendants of the cross some clones contained introns bI1 and bI2 as shown by colony hybridization (Figure 3) and restriction analysis of their mtDNA. **Panel C**, intron addition with phenotypic selection: the *rho*$^+$ strain D273-10B/A/M33-119, which carries a mutation in the exon sequence (shown as a black dot) and therefore does not grow on a non-fermentable medium, is crossed to *rho*$^-$ clone KL14-4A/G2113 which contains the intron bI3, the flanking exons B3 and B4 and is deleted for the distal parts of introns bI2 and bI4, as well as for the remaining exons. The progeny of the cross is plated on non-fermentable medium and respiratory sufficient colonies are isolated; restriction analysis of their mtDNA shows that they contain the intron bI3 (Figure 4). These subtractions or additions have most likely taken place by holomogous recombination in exons, which is symbolized by dotted arrows.

above (hybridization to mtDNA prepared by the minilysate procedure). This allows one to establish the organization of recombinant genes. In all cases studied, there was an addition or subtraction of introns in agreement with the predictions. Most likely, other genetic changes, like reversion or suppression, that could also account for the recovery of respiration, are much less frequent than recombination.

Construction of an intron-free gene

Our first goal was to construct a haploid strain carrying a totally intron-free gene. With such a strain, subsequent additions of individual introns should be easy to realize.

This construction required two steps. A diploid HM51 was constructed as described in Figure 2A. The *rho*$^-$ clone FR111 was known to exhibit a high level of recombination with *rho*$^+$ ($\geq$80%). This property allowed us to screen a few independent clones (three) to isolate one intron-less recombinant on the basis of its restriction map. Further analysis proved that it was really lacking all the introns (see text and Figure 4, lane 7).

This diploid strain was, as its parent WR27-27/1 (Jacq *et al.*, 1982), respiratory deficient for the lack of bI4, and therefore did not sporulate. To bypass this requirement for sporulation, we plated cells on glycerol or lactate medium to select suppressor mutations compensating the deficiency (Dujardin *et al.*, 1980; Jacq *et al.*, 1982), true back-mutants were not expected. After a week at 28°C, individual colonies appeared at a frequency of 10^{-8}. Two revertants (HM51/15 and /24) were sporulated and subjected to tetrad analysis. In each case the revertant was found to be due to a single nuclear

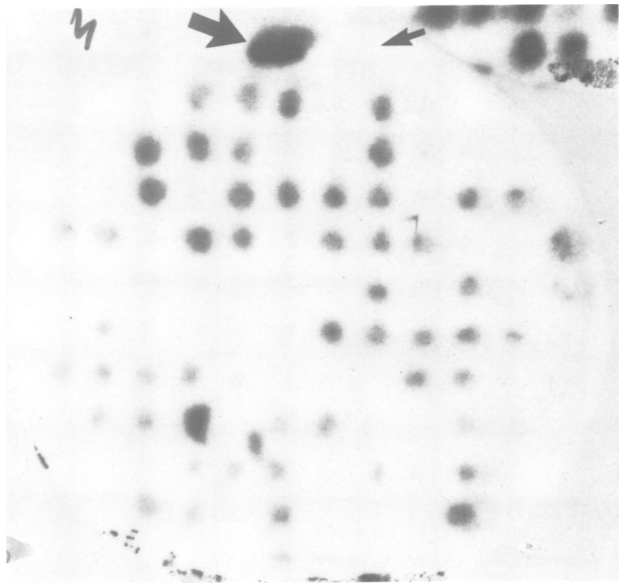

Fig. 3. Screening by colony hybridization of strains having acquired an intron. The results of the cross described in Figure 2B are shown. After mating and segregation, ~ 100 colonies, issued from homoplasmic diploid cells, were isolated and replica plated on a nitrocellulose filter which was subsequently hybridized to probes A plus B (Figure 4) which span introns bI1 and bI2, and autoradiographed. Thick and thin arrows point to the positive and negative controls, respectively.

gene (15 tetrads examined per revertant): (1) there was a 2:2 segregation for respiratory competency; (2) after conversion

of the spores to *rho*^{o} by ethidium bromide treatment, followed by test crosses to a *box7* mutation (Groudinsky *et al.*, 1981), the 2:2 segregation remained. A spore, HM51/24-12C, with the suppressor was also crossed to the strain S912/50 bearing the suppressor *NAM2-1* (Table I) and the hybrid sporulated and dissected. The results show that the suppressors are allelic. This new allele was designated *NAM2-6*, and for further studies a haploid strain HM51/24-1B (*α met NAM2-6*), was used.

Construction of other genes

The constructed genes with their parents are listed in Table II. Strains HM88 (see Figures 2B and 3 for its construction), HM89 and HM90 result from intron additions to strain HM51/24-1B (additions of bI1 and bI2, bI3 or bI4, respectively). They were selected after colony hybridization with the respective intron probes and analyses of mtDNA by the minilysate procedure. For these additions the fraction of cells giving positive signals by colony hybridization was 0.4, 0.01 and 0.1, respectively, Strains HM42 (addition of bI1 and bI2; frequency of glycerol^{+} cells *versus* glycerol^{-} cells: 4×10^{-2}), HM62 (addition of bI3, described in Figure 2C; 2×10^{-2}), HM75 (addition of bI1, bI2 and bI3; 3×10^{-1}), HM48 (subtraction of bI5; 7×10^{-5}) and HM64 (addition of bI3; 1×10^{-5}) were selected for their phenotype. These latter appeared at a frequency which was always significantly higher than the frequency of spontaneous reversion of the mutations: M17-162: $\leq 10^{-8}$; M33-119: $\leq 10^{-8}$ and M6-200: 10^{-7}.

The frequency of recombinants varies greatly among dif-

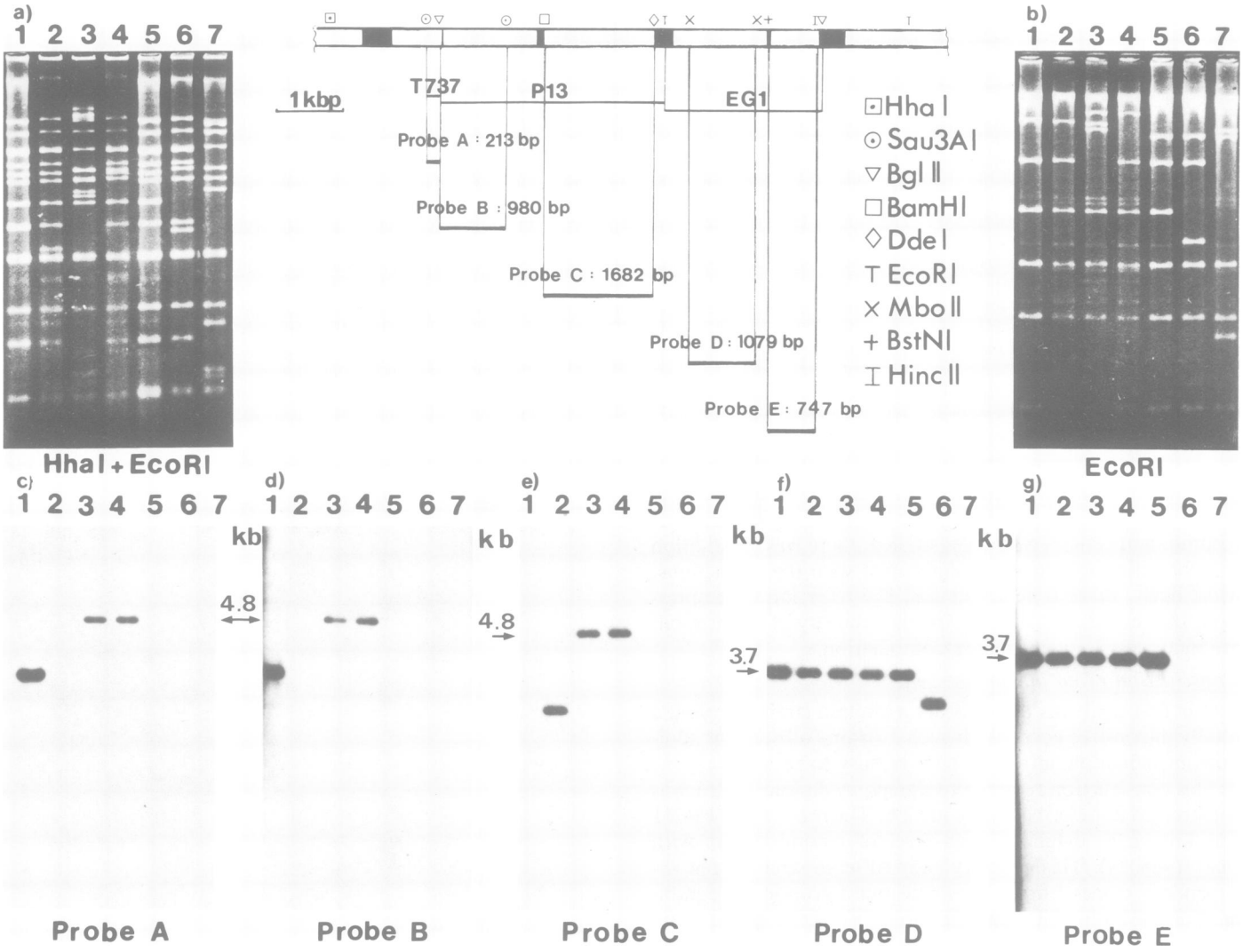

Fig. 4. Structure of constructed novel cytochrome b genes as shown by hybridization with intron probes. The central panel presents the longest gene (six exons shown in black space and five introns shown in empty space). The map is compiled from Lazowska *et al.* (1980, and unpublished data); and Nobrega and Tzagoloff (1980) and indicates some of the restriction sites used. Molecular probes used for hybridization are shown underneath and are derived from three recombinant plasmids T737, P13 and EG1 that have inserted into pBR322 the restriction fragments *Sau3*AI-*Bgl*II, *Bgl*II-*Eco*RI and *Eco*RI-*Bgl*II, respectively. The probe A is T737 and the other probes are restriction fragments isolated from agarose gels after digestion of plasmid DNA with the indicated enzymes. Probe A contains only intron bI1 sequence, Probe B contains 950 bp of intron bI2, 14 bp of exon B2 and 14 bp of intron bI1, Probe C contains only intron bI3 sequence, Probe D only intron bI4 sequence and Probe E contains 714 bp of introns bI5 and 33 bp of exon B5. The total mtDNA was purified from seven strains, restricted either with *Hha*I + *Eco*RI (**panels a, c, d, e**) or with *Eco*RI alone (**panels b, f, g**), electrophoresed through 1.2% agarose gels, stained with ethidium bromide (**panels a** and **b**), then transferred to nitrocellulose filters and hybridized with ^{32}P-labelled nick-translated probes and autoradiographed (**panels c – g**). In each case the **lane 1** corresponds to the strain HM42 (constructed I3$^{\Delta}$), **lane 2** to the strain HM62 (constructed, I1$^{\Delta}$12$^{\Delta}$), **lane 3** to the strain KL14-4A (wild-type, all introns present), **lane 4** to the strain HM75 (constructed, all introns present), **lane 5** to the strain D273-10B (wild-type, I1$^{\Delta}$I2$^{\Delta}$I3$^{\Delta}$), **lane 6** to the strain HM48 (constructed, I1$^{\Delta}$I2$^{\Delta}$I3$^{\Delta}$ I5$^{\Delta}$) and **lane 7** to the strain HM51 (constructed, all introns absent). As shown in **panels (a)** and **(b)** comparable amounts of mtDNA were loaded in different lanes.

ferent constructions. We believe that it reflects the recombination levels between the *rho*$^-$ and *rho*$^+$ strains, and is roughly related to the length of the sequences involved in recombination. In some cases a heterogeneity among the size of recombinant colonies was observed, for unknown reasons.

Physical analysis of the constructed genes

MtDNA of a representative clone from each cross was purified, its restriction map established and the presence or absence of introns analyzed by hybridizations with specific molecular probes. The results are shown in Figures 4 and 5

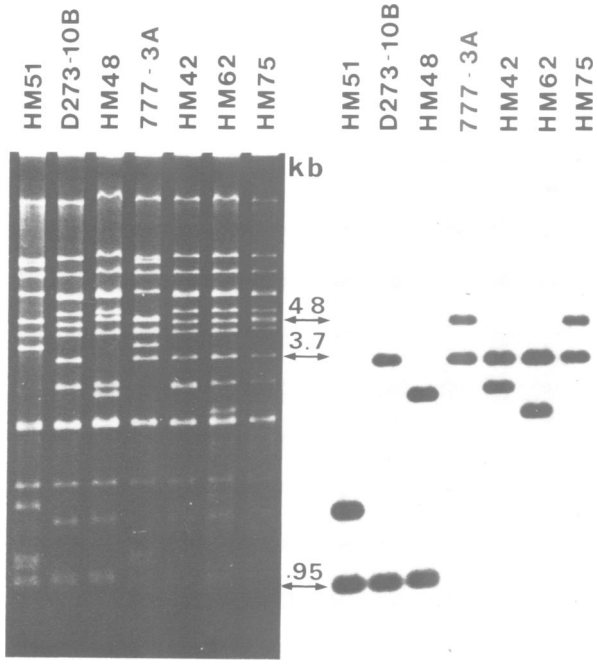

Fig. 5. Structure of constructed novel cytochrome b genes as shown by hybridization with an exon probe. Conditions are as in Figure 4 except that the mtDNAs were digested with *Hha*I + *Eco*RI and then hybridized with the exon probe constituted by the plasmid p14.

(data not shown for strains HM64, HM88, HM89 and HM90).

First, by restriction mapping, it appears that there are no major changes anywhere in the genome (Figure 3 panels a and b, Figure 5 left panel, compare the strains that are isomitochondrial as indicated in Table II). Second, the hybridization data establish clearly the presence of the introns or their absence. Third, hybridization with intron probes (Figure 4) or exon probes (Figure 5) shows that the addition or the subtraction of intron(s) is precise. Consider, for instance, the construction HM42 (Figure 4 lane 1 and Figure 5): (1) it hybridizes with probes A (bI1), B (bI2), D (bI4), E (bI5) and not with probe C (bI3) and (2) the hybridization with the exon probe p14 (Figure 5) shows that the lower band of D273-10B has a length of 950 bp, the upper band of 777-3A a length of 4800 bp and the lower band of HM42 a length of 3100 bp. These differences account exactly for the presence of introns bI1 and bI2 (765 and 1406 bp, respectively, the sum of which is close to the difference 3100 − 950) and for the lack of intron bI3 (1691 bp, a length which is close to the difference 4800 − 3100). HM42 has therefore an I1$^+$ I2$^+$ I3$^\triangle$ I4$^+$ I5$^+$ *cob-box* gene (where '+' symbolizes the presence of the intron and '△' its absence). In an analogous manner the correct structure of the other genes can be deduced. In particular, we can see that the mtDNA of the strain HM51 does not hybridize to any intron probe (Figure 4 lane 7) but presents two bands hybridizing to an exon probe (Figure 5). The size of these bands agrees perfectly with what we can expect for an intron-free gene from the sequence data of Nobrega and Tzagoloff (1980). Table II summarizes the physical organization of various genes.

Cytochrome synthesis by novel genes

The main question that we can ask is: are the new genes expressed? It appears from Table II and Figure 6 that all of the genes, including the intron-free gene, are able to lead to the synthesis of cytochrome b. On the contrary, only strains having the intron bI4 are able to synthesize cytochrome oxidase, and the presence of other introns is not necessary.

Table II. A summary of constructed intron-plus and intron-less cytochrome b genes

Parental genes		Constructed genes			Properties		
rho$^+$ introns 1 2 3 4 5	*rho*$^-$ introns 1 2 3 4 5	Introns 1 2 3 4 5	Mitochondrial background	Strain number	Cytochromes b	Cytochromes aa_3	Growth on glycerol
△△△+ + (1)	+ +ϵ− − (6)	+ +△+ +	D	HM42	+	+	+
△△△+ + (1)	+ + +ϵ− (7)	+ + + + +	D	HM75	+	+	+
△△△+ + (2)	−ϵ+ϵ− (8)	△△+ + +	D	HM62	+	+	+
△△△+ + (2)	−ϵ+△△ (9)	△△+ + +	D	HM64	+	+	+
△△△+ + (3)	−ϵ+△△ (9)	△△△+△	D	HM48	+	+	+
+ + +△△ (4)	△△△ϵ− (10)	△△△△△	W	HM51	+	−	−
△△△△△ (5)	+ +ϵ− − (11)	+ +△△△	W	HM88[a]	+	−	−
△△△△△ (5)	−ϵ+ϵ− (8)	△△+△△	W	HM89[a]	+	−	−
△△△△△ (5)	− −ϵ+ϵ (12)	△△△+△	W	HM90[a]	+	+	+

Symbols are as follows: (+): presence of a full-length intron with its adjacent exons; (ϵ): presence of that part of intron which is adjacent to its neighbouring exon; (△): absence of intron but presence of its adjacent exons; (−): absence of intron and its adjacent exons.
(1): D273-10B/A/M17-162; (2): D273-10B/A/M33-119; (3): D273-10B/A/M6-200; (4): WR27-27/1; (5): HM51/24-1B; (6): KL14-4A/PL1B1; (7): KL14-4A/RE11; (8): KL14-4A/G2113; (9): WR27-27/SR351; (10): D273-10B/A/FR111; (11): KL14-4A/PL1B2; (12): KL14-4A/i111. The *rho*$^-$ clones are shown in Figure 1.
D: mitochondrial genome of D273-10B, except for the *cob-box* gene. W: mitochondrial genome of WR27-27/1, except for the *cob-box* gene.
Cytochrome spectra of derepressed cells were determined as in Groudinsky *et al.* (1981). [a]The strains HM88, HM89 and HM90 have a nuclear background heterozygous for the gene *NAM2-6*, as they result from a cross between HM51/25-1B (which is *NAM2-6*) and KL14-4A (which is *nam*$^+$), and they grow on glycerol. Therefore, to establish the properties of their mitochondrial genes, we analyzed the *nam*$^+$ meiotic segregants of heterozygotes.

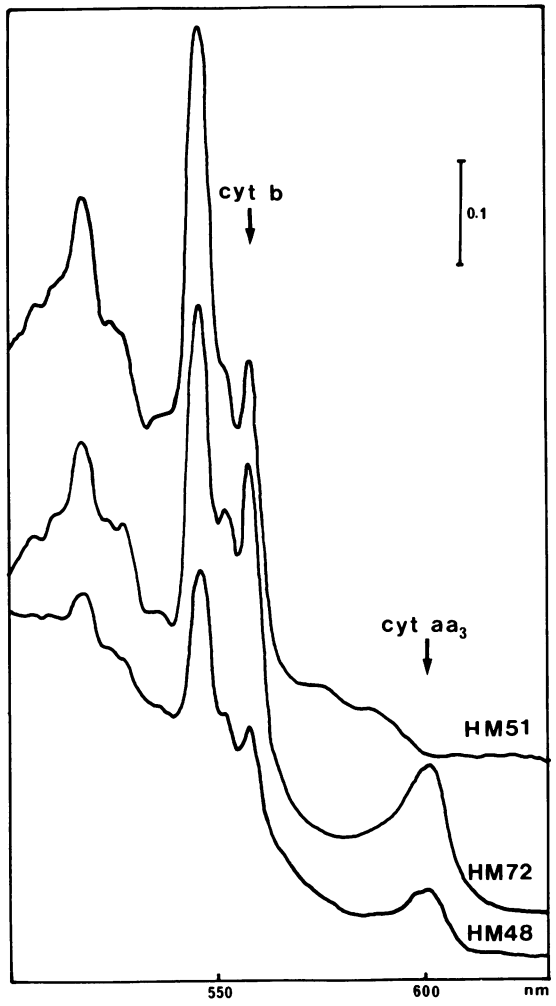

Fig. 6. Intron-free gene allows the synthesis of cytochrome b while the intron bI4 is necessary and sufficient for that of cytochrome aa_3. Isonuclear strains having two introns (bI4, and bI5, strain HM72 with the wild-type mitochondrial genome of D273-10B), one intron (bI4, strain HM48) and no introns (strain HM51) in the *cob-box* gene were grown and analyzed as in Groudinsky *et al.* (1981).

Discussion

Construction of novel cytochrome b genes

We have constructed a series of new yeast strains that differ one from another only by the presence or the absence of one or several of the wild-type introns of the mitochondrial *cob-box* gene. For these constructions we have taken advantage of four phenomena: (1) the existence of a natural polymorphism between various wild-type strains, some of which contain the optional introns bI1, bI2, bI3, while others do not (Lazowska *et al.*, 1980; Nobrega and Tzagoloff, 1980); (2) the isolation of revertants of intron mutations which lack introns bI4 and bI5 (Jacq *et al.*, 1982); (3) the possibility of cloning well defined segments of mtDNA in *rho*$^-$ mutants; and (4) the facility with which cloned segments of *rho*$^-$ mtDNA can be introduced by homologous recombination into the *rho*$^+$ genome. With this method introns can be added or removed by homologous recombination in flanking exons. Theoretically the method has no limits, but in practice it may be restrained by the length of the sequences involved in recombination. For instance we have been able to add (or withdraw) introns bI3 and bI4 alone, for which the flanking exons are, respectively, 77 and 250 bp (B3 and B4) or 250 and 51 bp (B4 and B5), while we were unable to manipulate singly the intron bI2 for which its flanking exon B2 is only 14 bp long. Genetic and molecular characterization of the constructed strains show that they breed true to type and are homoplasmic. Restriction and hybridization analyses of their mtDNA show that introduction or withdrawal of introns are precise, with regard to both the length and the position of the manipulated intron. Although the techniques used do not allow the detection of a few base changes and the final proof can be obtained only by sequencing the intron-exon or exon-exon junctions, we believe that the manipulations have an accuracy of a single base pair since all strains synthesize a normal, physiologically active cytochrome b.

Expression of the cob-box gene

We conclude that none of the *cob-box* introns, nor splicing *per se* are necessary for expression of this gene. Moreover, the data presented in Table II show that introns which code for a mRNA maturase (e.g., bI2, Lazowska *et al.*, 1980; bI4, De La Salle *et al.*, 1982; bI3, Lazowska *et al.*, in preparation) are self-sufficient, i.e., the maturase of bI2 can be expressed and splicing of bI2 can occur in the absence of introns bI3, bI4 and bI5, that of bI3 in the absence of introns bI1, bI2, bI4 and bI5, etc.... This conclusion has to be confronted with previous observations of Church *et al.* (1979), Halbreich *et al.* (1980), Van Ommen *et al.* (1980) and Schmelzer *et al.* (1981). They have shown that mutants located in bI2 accumulate RNA precursors containing unspliced bI2, bI3 and bI4. Thus, a mutation in an upstream intron prevents splicing of a downstream intron. This is comprehensible, if we remember that for the synthesis of the bI4 maturase, a message comprising the bI4 open reading frame fused in the same register with all fused upstream exons has to exist (De La Salle *et al.*, 1982). Such a scheme implies that all upstream introns have to be excised to allow the synthesis of a downstream maturase and subsequent excision of a downstream intron, but it does not imply that splicing of a given intron encoding a maturase (bI2 or bI3 or bI4) requires in addition to the activity of its own maturase that of the other two maturases. In summary, we can say that all *cob-box* introns are optional for expression of this gene, but when present, they must be in their wild-type form.

Genes with optional introns have been found in other organisms. The rat preproinsulin gene I lacks an intron compared to the rat preproinsulin gene II (Lomedico *et al.*, 1979). Although both can be expressed, nothing is known about the quantitative aspects of their expression. A few cases of intron-free variants of intron-containing genes have also been reported, but they are silent pseudo-genes that probably arose by reverse transcription of mature mRNA followed by integration of the cDNA (Lueders *et al.*, 1982). Optional introns may play a role as a control mechanism in differential gene expression. A few examples of what can be interpreted as differential expression under the control of splicing have already been described (Segal *et al.*, 1979; Shaw and Ziff, 1980; Young *et al.*, 1981). Our series of isogenic strains provides a unique opportunity to study the role of introns of a gene which is known to be expressed at different levels under different physiological conditions. Yeast displays a variety of integrated regulations which comprise respiratory adaptation, fermentative repression, sporulation and meiosis. We are currently investigating the quantitative aspects of the expression of intron-less and intron-plus genes under such conditions.

Interactions between cob-box and other genes

The new constructions have also allowed us to stress the importance of intergenic communications between the *cob-box* and *oxi*3 genes. We provide the final proof that, as previously hypothesized (Lazowska *et al.*, 1980; Dhawale *et al.*, 1981; Groudinsky *et al.*, 1981; De La Salle *et al.*, 1982), the presence of intact intron bI4 is both necessary and sufficient for the synthesis of cytochrome oxidase subunit I. Furthermore, it is of interest that nuclear compensations, previously uncovered in the study of point mutations or deletions of the bI4 (Groudinsky *et al.*, 1981; Jacq *et al.*, 1982) can also be realized in the case of a cytochrome b gene devoid of all the introns. We have shown that the nuclear mutation *NAM2-6* isolated in an intron-free strain is allelic to the nuclear suppressor *NAM2-1* isolated in a nonsense mutant in the bI4 maturase (*loc. cit.*). The mechanism of compensation, the function (if any) of the wild-type allele *nam2+* and the reason for the presence in the nuclear genome of a potential compensator gene are intriguing. The nature of the relays that connect nuclear genes with intron-encoded mitochondrial maturases and/or other elements of the splicing machinery are obviously a challenging problem. A battery of genes with or without introns should be useful to answer questions about the role of specific nuclear genes involved in the expression of split mitochondrial genes.

Materials and methods

Yeast strains

The strains used for initial constructions are listed in Table I. Other strains are described in the text.

Media and genetic techniques

Media and genetic techniques are described elsewhere (Dujardin *et al.*, 1980).

MtDNA preparation

For minilysates, yeast strains were grown overnight in 1.5 ml YPGA at 28°C with shaking. The cells were lysed with zymolyase 60 000 (Kirin Brewery) and the DNA was prepared as described by Dujon and Blanc (1980). The yield was ~1 μg mtDNA /culture and ~0.1 μg was used for blotting experiments. For large scale preparations mtDNA was purified as described in De La Salle *et al.* (1982).

Molecular probes and hybridization experiments

Four recombinant plasmids were used as a source of mtDNA fragments for molecular hybridizations. The plasmid p13 was constructed by ligating a total *Bgl*II-*Eco*RI digest of KL14-4A mtDNA to a *Bam*HI-*Eco*RI cleaved pBR322, and transforming the *Escherichia coli* strain HB101 with the ligation mixture (Bolivar and Backman, 1978). Recombinant plasmids were selected first for ampicillin resistance, tetracycline sensitivity, then by colony hybridization (Cami and Kourilsky, 1979) to a fragment prepared from a *rho*$^-$ mtDNA and covering the second intron (fragment *Bgl*II-*Sau*3AI, Figure 4). This plasmid contains a 3252 bp long fragment constituted mainly of introns bI2 and bI3 sequences of the *cob-box* gene (Lazowska *et al.*, 1980, and in preparation). The plasmid p14 was constructed by inserting the cytochrome b intron-free gene of strain HM51 (see text) in the *Bam*HI site of pBR325 (Bolivar, 1978). Total mtDNA of HM51 was cleaved by *Hind*II and ligated to *Bam*HI linkers (BRL). The ligation mixture was then digested with *Bam*HI and linkers were separated from mtDNA by electrophoresis through 5% polyacrylamide gel. After recovery from the gel, the mtDNA was cloned in *Bam*HI-cleaved pBR325 as described for plasmid p13 and screened by restriction analysis of recombinant plasmids. The intron-free gene is therefore cloned through the *Hind*II site lying some 300 bp from the AUG initiation codon and the *Bam*HI site lying 851 bp downstream of the termination codon (Nobrega and Tzagoloff, 1980). The plasmid T737 was a gift of A. Halbreich and it contains a 213-bp fragment of the intron bI1 (Figure 4). The plasmid EG1 was a gift of C. Jacq and it contains a 2554-bp fragment representing the 3′ part of the gene (Figure 4).

Recombinant plasmid DNAs were prepared according to Birnboim and Doly (1979). When necessary, DNA fragments were purified from recombinant plasmids by electrophoresis through agarose gels after cleavage with appropriate restriction enzymes and were recovered from gels by the method of Dretzen *et al.* (1981). DNA fragments or recombinant plasmids were made radioactive by nick-translation (Maniatis *et al.*, 1975) with [α-^{32}P]dATP (410 Ci/mmol, Amersham International). Specific activities of 1–2 x 10^8 c.p.m./μg were routinely obtained with native plasmids and slightly less with isolated fragments.

Restriction endonucleases were purchased from New England Biolabs and Boehringer, Mannheim and used in the recommended buffers. Restriction fragments were separated by electrophoresis, through horizontal agarose gels containing 40 mM Tris acetate pH 7.8, 5 mM sodium acetate and 1 mM EDTA. The electrophoresis was carried out overnight at 1–2 V/cm. DNA fragments were transferred after electrophoresis to nitrocellulose filters (BA85 Schleicher and Schuell) following the procedure of Southern (1975). The filters were hybridized to radioactive probes for 16–20 h at 60°C in heat-sealed plastic bags containing 3 x SSC, 1 x Denhardt solution (Denhardt, 1966), 100 μg/ml denatured sonicated calf thymus DNA. The filters were subsequently washed at 60°C in 3 x SSC, 1 x Denhardt for 2–3 h with five changes (first wash for 1 h) and then twice for 15 min in 0.1 x SSC/0.5% SDS at 60°C. The filters were finally dried before autoradiography.

Screening of mitochondrial recombinants by in situ colony hybridization

Individual colonies were gridded on yeast extract 1%, bactopeptone 1%, galactose 2%, glucose 0.1% plates (100 coloines/plate), incubated for a day at 28°C and replicated onto nitrocellulose filters (BA85 Schleicher and Schuell) which were then treated essentially as described by Blanc *et al.* (1978).

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