Nuclear and Mitochondrial Suppression of a Mitochondrially Inherited Cold-sensitive Mutation in *Aspergillus nidulans*

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Partial suppressors of a mitochondrially inherited mutation, [*cs-67*], conferring cold-sensitivity at 20 °C were identified. These mapped at one mitochondrial and four unlinked nuclear loci. Most suppressors partially restored the cytochrome *aa₃* deficiency of the cold-sensitive strain at 20 °C. Strains carrying two or more suppressors and [*cs-67*] showed considerably impaired growth. This effect was temperature-dependent, being more severe at 37 °C, and was not expressed in the presence of the [*cs-67*⁺] allele. The cytochrome oxidase activity of one of these strains was no more heat-sensitive than that of the wild-type implying that these mutations did not directly modify cytochrome oxidase. The wild-type strain grown in the presence of chloramphenicol and the cold-sensitive strain grown at 20 °C had similar cytochrome spectra and mitochondrial membrane protein profiles on sodium dodecyl sulphate gradient acrylamide gels. [*cs-67*] conferred pleiotropically a low level of resistance to paromomycin at 37 °C. It is suggested that [*cs-67*] and the suppressors act at the level of the mitochondrial ribosome.

INTRODUCTION

A cold-sensitive phenotype often results from the inability to assemble a multimeric complex, a classical example being found in mutations affecting the ribosome (for review, see Nomura *et al.*, 1977). In *Saccharomyces cerevisiae*, a cold-sensitive phenotype can be conferred by a number of mitochondrial mutations, including ones which almost undoubtedly map in the genes specifying the 21S mitochondrial ribosomal RNA (Bolotin-Fukuhara *et al.*, 1977; Devenish *et al.*, 1978; Singh *et al.*, 1978) and subunits of the ATPase (Trembath *et al.*, 1975*b*), cytochrome *b* and cytochrome oxidase (Bolotin-Fukuhara *et al.*, 1977). It has been established that mitochondrial mutations which confer erythromycin resistance and a cold-sensitive phenotype pleiotropically map in or close to the 21S rRNA gene and generally impair the assembly of the 50S mitoribosomal subunit (Singh *et al.*, 1978; Spithill *et al.*, 1978).

In *Aspergillus nidulans*, Waldron & Roberts (1973) isolated a cold-sensitive mutant whose phenotype was inherited cytoplasmically. Rowlands & Turner (1975) showed that the mutation responsible, [*cs-67*], was on the same linkage group as [*oliA1*], conferring resistance to oligomycin (Rowlands & Turner, 1973), and [*camA112*], conferring resistance to chloramphenicol (Gunatilleke *et al.*, 1975*b*). The linkage group almost undoubtedly represented the mitochondrial genome (Turner & Rowlands, 1977, and references therein; Lazarus & Turner, 1977). Turner & Rowlands (1976), using low temperature difference spectra, showed that the cold-sensitive strain grown at 20 °C had an excess of cytochrome *c*, a deficiency in cytochrome *aa₃* and a possible deficiency in cytochrome *b*. They suggested that [*cs-67*] affected mitochondrial protein synthesis, but from their spectral data could not

exclude the possibility that [*cs-67*] specifically impaired the synthesis of cytochrome oxidase.

Suppressors of mutations which adversely affect mitochondrial function have been identified in *Schizosaccharomyces pombe* (Goffeau *et al.*, 1976), *Neurospora crassa* (Mitchell & Mitchell, 1956; Kohout & Bertrand, 1976; Bertrand & Collins, 1978), *Paramecium tetraurelia* (Sainsard, 1975) and *Saccharomyces cerevisiae* (Storm & Marmur, 1975; Trembath *et al.*, 1975a). The latter two reports described suppressors of thermosensitive mutations.

We proposed to obtain suppressors of [*cs-67*] with two aims in mind. Firstly, we hoped to detect modifications by the suppressors to specific components of the mitochondrion which were of such a nature that the primary action of [*cs-67*] could be deduced. Secondly, as the biogenesis of the mitochondrion requires the co-ordinated activity of the nuclear and mitochondrial genomes, transcripts from which appear, in general, to be translated in the cytoplasm and mitochondrion respectively (for review, see Schatz & Mason, 1974), the identification and characterization of suppressor mutations should facilitate an understanding of the biogenesis of the mitochondrion – specifically, the interaction of the two genetic systems. A similar approach is being pursued in *N. crassa* by Kohout & Bertrand (1976) who have identified six nuclear suppressors of the mitochondrial mutation *poky*, which *inter alia* impairs mitoribosomal assembly.

METHODS

Strains. As well as markers in general use (Clutterbuck & Cove, 1974), *Aspergillus nidulans* strains carried any of the following: *oliC1* and [*oliA1*] – resistance to oligomycin (Rowlands & Turner, 1973); [*camA112*] – resistance to chloramphenicol (Gunatilleke *et al.*, 1975b); [*cs-67*] – cold-sensitivity (Waldron & Roberts, 1973). Gene symbols representing extranuclear markers are enclosed in square brackets. All strains used for biochemical work and growth rate studies on solid media carried the markers *yA2*, *pyroA4* and *cnxC3*. In order to facilitate heterokaryon formation, suppressor mutations were placed in both of the following two backgrounds: *biA1 luA1 cnxH3* and *yA2 pyroA4 cnxC3*. (*biA1*, *luA1* and *pyroA4* – nutritional requirements for biotin, leucine and pyridoxine, respectively; *yA2* – yellow conidial colour; *cnxC3* and *cnxH3* – inability to use nitrate or hypoxanthine as nitrogen source.) Strains which were isolated from heterokaryons were purified to ensure that they were homoplasmic.

In vivo techniques. Growth testing and genetic techniques for *A. nidulans* have been described before (Arst & Cove, 1969, and references therein). Liquid and solid media were from Cove (1966) based on those of Pontecorvo *et al.* (1953). Urea (5 mM) or sodium nitrate (10 mM) were routinely used as the nitrogen source. Carbon sources were added at 1 % (w/v), except for acetate, which was added as the sodium salt, adjusted to pH 6.5 to give a final concentration of 144 mM. Chloramphenicol and oligomycin resistance were routinely scored on media containing 2.5 mg ml⁻¹ and 3.0 µg ml⁻¹, respectively, of the appropriate inhibitor. The marker [*cs-67*] was scored at 20 °C on complete medium. Oligomycin, pyrrolnitrin and carboxin were added as stock solutions, dissolved in methanol; as was diuron in acetone. KCN was always freshly prepared and added as an aqueous solution. Chloramphenicol, salicylhydroxamic acid, neomycin, erythromycin, spiramycin, streptomycin, lincomycin and paromomycin were added as solids to molten agar, cooled to 60 °C. Pyrrolnitrin, diuron, carboxin and paromomycin were gifts from Eli Lilly & Co. (Indianapolis, Indiana, U.S.A.), du Pont de Nemours & Co. (Wilmington, Delaware, U.S.A.), Uniroyal Ltd (Bromsgrove, Worcestershire) and Parke, Davis & Co. (Pontypool, Gwent), respectively. Sodium deoxycholate (Koch-Light) was used at a concentration of 0.8 % (w/v) to induce compact growth on plates (Mackintosh & Pritchard, 1963).

Growth of mycelium for in vitro studies. Two-litre flasks, each containing 800 ml of complete liquid medium, were inoculated with a conidial suspension to give a final concentration of 2×10^6 ml⁻¹. The flasks were shaken in an orbital shaker at 110 rev. min⁻¹. Growth times were set so that the mycelium was harvested towards the end of the exponential phase, these times being established from appropriate growth curves. Strains carrying the following mutations were grown for the number of hours indicated at 37 and 20 °C, respectively: wild-type (10.5, 36.0); [*cs-67*] (10.5, 64.0); [*cs-67*] and one of the suppressors *sumA31*, *sumA35*, *sumB19*, *sumB72*, *sumC40* or [*sumD16*] (10.5, 48.0); [*cs-67*] and *sumE83* (10.5, 56.0); [*cs-67*], *sumA31* and *sumB19* (15.0, 48.0); [*cs-67*], *sumA35* and *sumB19* (20.0, 56.0). The wild-type grown at 20 °C in the presence of 2.5 and 6.0 mg chloramphenicol ml⁻¹ was harvested after 48.0 and 64.0 h, respectively.

Heterokaryon formation. Strains carrying complementing nitrate non-utilizing markers and complementing auxotrophic markers were inoculated at exactly the same point on minimal medium containing nitrate as sole nitrogen source (Rever, 1965). Strains carrying *biA1 luA1 cnxH3* and *yA2 pyroA4 cnxC3* formed

balanced heterokaryon cores, giving good cytoplasmic mixing without the need for a transfer step to fresh nitrate medium (Gunatilleke *et al.*, 1975b).

Cytoplasmic inheritance. This was described fully by Gunatilleke *et al.* (1975b).

Analysis of mitochondrial recombination using random spore analysis. This was based on the method of Rowlands & Turner (1974b, 1975) with some modifications (R. B. Waring & C. Scazzocchio, unpublished results). In mitochondrial crosses where the phenotype of both strains was similar with respect to the mitochondrial markers (e.g. in heterokaryons between the wild-type and revertants of [*cs-67*] having a wild-type phenotype), reassortment of the mitochondrial genomes between the nuclear genomes was estimated using an independent marker such as [*camA112*].

Isolation of mutants. Strains were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine after the method of Alderson & Hartley (1969). Revertants of [*cs-67*] were selected on minimal media containing either glucose, ethanol or acetate as carbon source. Mutated conidia were plated at densities of 2×10^3 to 2×10^4 per plate and incubated at 18 °C.

Cytochrome spectra. Room temperature difference spectra were obtained using a Pye Unicam SP1800 spectrophotometer, with a turbid sample attachment. Reduction was achieved using sodium dithionite and oxidation by shaking the sample in air. Crude mitochondrial extracts were prepared as follows. Mycelium (1 to 2 g hand-pressed wet wt) was disrupted using an ice-cold pestle and mortar, with sand, in 1.5 vol. extraction medium (see below). The slurry was suspended in 30 ml extraction medium, vortexed for 20 s and then centrifuged twice at 800 *g* for 5 min to remove mycelial fragments. The supernatant was centrifuged at 48000 *g* to pellet the mitochondrial fraction; this was resuspended in extraction medium to give 2 to 3 mg protein ml⁻¹ and used at this concentration in the spectral analysis. Ratios of cytochrome *c*:*aa*₃ and *c*:*b* were calculated, based on the method of Lambowitz *et al.* (1972a). These calculations were actually estimates as absorption coefficients for *A. nidulans* were not known. Cytochrome concentrations were measured using the following pairs of wavelengths and absorption coefficients, based on Chance & Williams (1955): cytochrome *aa*₃, 608–630 nm, $\epsilon = 161 \text{ mmol}^{-1} \text{ cm}^{-1}$; cytochrome *b*, 560–575 nm, $\epsilon = 201 \text{ mmol}^{-1} \text{ cm}^{-1}$; cytochrome *c*, 551–540 nm, $\epsilon = 191 \text{ mmol}^{-1} \text{ cm}^{-1}$. As a straight and reproducible baseline was not always obtained, one was artificially derived by joining the reference wavelengths 540, 575 and 630 nm, these being virtually isosbestic points.

Thermolability of cytochrome oxidase. A crude mitochondrial extract was made as above, except that the mycelium was disrupted using a glass homogenizer in 10 vol. extraction medium (see below). The mitochondrial extract was layered on to a two-step gradient of 1.20 and 1.60 M-sucrose and centrifuged at 48000 *g* for 40 min. The brownish mitochondrial band at the 1.20/1.60 M interface was removed, and its protein concentration was adjusted to 4 mg ml⁻¹ (determined by Lowry's method). Samples were heated in a water bath for set times and then assayed for cytochrome oxidase activity at 25 °C using the method of Wharton & Tzagoloff (1967).

Preparation of mitochondria for gels and enzyme assays. This was based on the methods of Rowlands & Turner (1974a) and Gunatilleke *et al.* (1975b). Mycelium (30 to 40 g hand-pressed wet wt) was washed with deionized water and extraction medium and then suspended in 8 vol. pre-cooled extraction medium [0.44 M-sucrose, 10 mM-Tris, 2 mM-Na₂EDTA and 0.08 % (w/v) bovine serum albumin (BSA), fraction V; adjusted to pH 7.2 with HCl (Lambowitz *et al.*, 1972b; Gunatilleke *et al.*, 1975a)]. All subsequent procedures were carried out at 4 °C. The mycelial suspension was passed twice through a grind-mill (Weiss *et al.*, 1970), shaken in an MSE Atomix blender for 30 s at half-speed and then filtered through two layers of fine nylon crepe. The filtrate was centrifuged at 800 *g* for 10 min and the resulting supernatant was centrifuged at 21000 *g* for 30 min. The reddish-brown pellet obtained was resuspended in extraction medium using a ground glass homogenizer and loaded on to a discontinuous sucrose gradient, which usually consisted of four fractions: 0.90, 1.20, 1.35 and 1.60 M-sucrose, each containing 10 mM-Tris plus 2 mM-EDTA and adjusted to pH 7.0. After centrifugation at 38000 *g* for 70 min, the required bands were removed. The mitochondrial fraction banding at the 1.20/1.35 M interface was the purest on the criterion that it had the highest cytochrome *c*:cytochrome *b* ratio.

Preparation of mitochondrial membrane fractions. The purified mitochondrial fractions were washed in extraction medium and centrifuged at 21000 *g* for 30 min. A portion of each pellet was resuspended in 10 mM-Tris plus 2 mM-EDTA and sonicated at an amplitude of 8 to 12 μm for 15 s. These suspensions were centrifuged at 38000 *g* for 30 min to obtain membrane pellets. The pellets were dissolved in sodium dodecyl sulphate (SDS) sample buffer (0.05 M-Tris/HCl pH 6.8, 3 % SDS, 1 % mercaptoethanol, 0.24 M-sucrose and 0.05 % bromophenol blue) and heated for 5 min in a boiling water bath. The final protein concentration was 2 to 4 mg ml⁻¹.

SDS gradient acrylamide gels. The method was based on that of Laemmli (1970). The gradients were 8 to 20 % acrylamide. The gels were approximately calibrated for molecular weight using a preparation of beef heart cytochrome oxidase [a gift of W. Lalla-Maharajh of the Department of Chemistry, University of

Essex, prepared using a modified method of Yonetani (1960)] and the following standards: bovine serum albumin, fraction V (mol. wt 68 000), ovalbumin (43 000), aldolase (40 000), lactate dehydrogenase (36 000), carbonic anhydrase (29 000), chymotrypsinogen (25 700), myoglobin (17 200), ribonuclease A (13 700) and cytochrome *c* (11 700). Cytochrome *c* was obtained from Calbiochem and the other standards were from Sigma. It was considered that cytochrome *c* and ribonuclease A ran anomalously on the basis of the position of beef heart cytochrome oxidase subunits, whose molecular weights have been well characterized (Briggs & Capaldi, 1977), and the fact that myoglobin (mol. wt 17 200) ran to the same position as ribonuclease A (mol. wt 13 700), noting that cytochrome *c* and ribonuclease A, but not myoglobin, have previously been reported to run anomalously (Dunker & Rueckert, 1969; Lambin *et al.*, 1976).

Enzyme assays and protein determination. Cytochrome oxidase was assayed according to the method of Ferguson-Miller *et al.* (1976), employing an oxygen electrode. The reaction was started by adding the mitochondrial preparation. After 50 % of the oxygen had been consumed, KCN at a final concentration of 1 mM was added. The rate of cyanide-sensitive oxygen utilization was measured. Succinate dehydrogenase was assayed as described by Butcher (1970) and succinate cytochrome *c* reductase as described by King (1967). Protein concentrations were determined by the method of Lowry.

RESULTS

Revertants of [*cs-67*] were isolated at 18 °C from mutagenized strains carrying the markers *biA1*, *luA1*, *cnxH3* and [*cs-67*]. Each of the 43 isolated was tested for cytoplasmic inheritance of the revertant phenotype by being set in a heterokaryon with the strain *yA2 pyroA4 cnxC3* [*camA112 cs-67*] (see Methods). In 22 cases the revertant and cold-sensitive phenotype segregated in both of the two nuclear backgrounds, demonstrating that the revertant phenotype was cytoplasmically inherited. The remaining 21 revertants were sexually crossed to the strain *yA2 pyroA4 cnxC3* [*camA112 cs-67*]. Progeny with cold-sensitive and revertant phenotypes segregated in a 1:1 ratio in each cross, demonstrating that each of these revertants carried a nuclear suppressor.

Preliminary phenotypic and genotypic analysis of nuclear suppressors

None of the nuclear suppressors fully suppressed [*cs-67*] at 20 °C. The suppressors mapped at four unlinked loci, which were designated *sumA*, *sumB*, *sumC* and *sumE*. *sumA* had 16 alleles. Growth of strains carrying [*cs-67*] and *sumA35* or *sumA48* was slightly poorer than that of the wild-type at 37 °C on complete medium but not synthetic complete medium. The impaired growth phenotype was not separable from the suppressor phenotype in 100 and 36 progeny, respectively. These two mutants were indistinguishable from the other *sumA* mutants at 20 °C. *sumA35* and *sumA48* were not separable by recombination from *sumA23* in 1000 progeny tested, nor were any of the other *sumA* mutations in 50 to 800 progeny tested. *sumB* had two alleles, *sumB19* being a slightly better suppressor than *sumB72*. These two mutations were not separable by recombination in 351 progeny tested. The *sumC* and *sumE* loci had one representative each. *sumE83* was considerably less effective as a suppressor than the other mutations. The phenotypes of the suppressors are shown in Fig. 1.

Haploidization analysis (McCully & Forbes, 1965) located *sumA* on linkage group VII, *sumB* on group III, *sumC* on group IV and *sumE* on group I. None of the nuclear suppressors have been meiotically mapped as yet, although linkage of *sumA35* to the unmapped mutation *cbxB28*, which confers resistance to carboxin (Gunatilleke *et al.*, 1975*a*), was observed with a recombination frequency of 11 %.

sumA35 recombined freely with *oliC1*, a mutation conferring oligomycin resistance located in linkage group VII (Rowlands & Turner, 1973), while *sumB19* recombined freely with *plnA18* and *cbxA17*, also on linkage group III and conferring, respectively, pyrrolnitrin and carboxin resistance (Gunatilleke, 1974; Gunatilleke *et al.*, 1975*a*).

The nuclear suppressors were tested for dominance by constructing homozygous and heterozygous suppressor diploids in a [*cs-67*] background and comparing growth of the heterozygote with respect to that of the homozygote and a cold-sensitive diploid. In a

Fig. 1. Growth of suppressor mutants on complete medium at 37 °C (*left*) and 20 °C (*right*). Relevant genotypes are given below in the same pattern as on the plates.

1st row:	wild-type	[<i>cs-67</i>]	<i>sumA31</i> [<i>cs-67</i>]	<i>sumA35</i> [<i>cs-67</i>]
2nd row:	<i>sumB19</i> [<i>cs-67</i>]	<i>sumB72</i> [<i>cs-67</i>]	<i>sumC40</i> [<i>cs-67</i>]	<i>sumE83</i> [<i>cs-67</i>]
3rd row:	<i>sumA31 sumB19</i> [<i>cs-67</i>]	<i>sumA31 sumE83</i> [<i>cs-67</i>]	<i>sumA35 sumB19</i> [<i>cs-67</i>]	<i>sumA35 sumB72</i> [<i>cs-67</i>]
4th row:	<i>sumA35 sumC40</i> [<i>cs-67</i>]	<i>sumA35 sumE83</i> [<i>cs-67</i>]	<i>sumB19 sumC40</i> [<i>cs-67</i>]	<i>sumB19 sumE83</i> [<i>cs-67</i>]
5th row:		<i>sumC40 sumE83</i> [<i>cs-67</i>]		

[*cs-67*] background, at 20 °C, heterozygous diploids of *sumA23*, *sumA31*, *sumA35*, *sumB19*, *sumB72*, *sumC40* and *sumE83* all showed intermediate growth between that of their respective homozygous diploid (*sum/sum*) and the cold-sensitive diploid (*sum⁺/sum⁺*). The suppressor alleles mapping at the same locus were tested in heteroallelic diploids in a [*cs-67*] background for positive and negative complementation, but no indication of either of these was found.

From heterokaryons between wild-type strains and mutants carrying a suppressor mutation and [*cs-67*], strains carrying a suppressor and [*cs-67⁺*] were isolated. These were all phenotypically indistinguishable from the wild-type strain at 20 and 37 °C.

Genetic analysis of revertants with extranuclear mode of inheritance

Each revertant was set in a heterokaryon with a strain having the mitochondrial genome [*camA112*]. Using the random spore analysis technique (Rowlands & Turner, 1974*b*, 1975; R. B. Waring & C. Scazzocchio, unpublished results), the conidia of each heterokaryon were screened for the cold-sensitive phenotype, as this would indicate that the [*cs-67*] marker had segregated from a suppressor mutation. In the mitochondrial crosses involving 19 revertants, no cold-sensitive recombinants were observed in 100 to 1500 reassorted colonies. These mutants therefore carry either back-mutations or suppressor mutations which are extremely tightly linked to [*cs-67*], the former explanation being more probable as the phenotype of each mutant was indistinguishable from the wild-type at 37 and 20 °C. Between 3 and 10 % of reassorted colonies from heterokaryons involving revertants 12, 16 and 74 were cold-sensitive recombinants, inferring that the revertants carried suppressor mutations. This conclusion was consistent with the observation that these three mutations only partially suppressed [*cs-67*]. As the suppressor mutations were not easily separable by recombination from [*cs-67*], they almost undoubtedly map in the mitochondrial genome.

To see if the suppressor mutations themselves were separable by recombination, mitochondrial crosses were conducted using a marker selection technique, the use of which

facilitates the detection of rare recombinational events. Heterokaryons were set in order to carry out mitochondrial crosses such as [*camA112 cs-67 sum-16*] to [*oliA1 cs-67 sum-74*]. Conidia were plated on media containing chloramphenicol and oligomycin in order to select for [*camA112 oliA1*] recombinants (the growth temperature was non-restrictive). These were then screened for the occurrence of a recombinational event between [*sum-74*] and [*sum-16*] by looking for cold-sensitive colonies. The advantage of this method can be seen if one considers the frequency with which recombination between two unlinked markers will be observed. As at least one mitochondrial recombinational event will have occurred in every [*camA112 oliA1*] recombinant, two unlinked markers will be likely to show recombination in 50 % of the colonies analysed. By comparison, in random spore analysis recombination between two unlinked markers will only be observed in about 5 % of all colonies analysed (Rowlands & Turner, 1975).

In mitochondrial crosses of [*oliA1 cs-67 sum-16*] to [*camA112 cs-67 sum-12*] and [*camA112 cs-67 sum-74*], no cold-sensitive colonies were observed in 545 (<0.2 %) and 353 (<0.3 %) [*camA112 oliA1*] recombinants, respectively. As a control, the linkage between [*cs-67*] and [*sum-16*] was also estimated using double marker selection analysis. In a mitochondrial cross such as [*oliA1 cs-67 sum-16*] to [*camA112*], recombination between [*cs-67*] and [*sum-16*] took place in 8 to 15 % of [*camA112 oliA1*] recombinants. As these two markers are loosely linked (R. B. Waring & C. Scazzocchio, unpublished results), the three mitochondrial suppressors almost undoubtedly map in the same gene. This was named [*sumD*]. As the above results strongly suggest that these three mutations are allelic and as they confer the same phenotype at 37 and 20 °C, only [*sumD16*] was used for further studies.

In the mitochondrial cross [*cs-67 sumD16*] to [*cs-67⁺ sumD⁺*], no novel phenotype corresponding to the [*sumD16*] genotype was observed and so it was initially assumed that such a strain would have a wild-type phenotype. Strains carrying [*sumD16*] were isolated from four-point mitochondrial crosses (R. B. Waring & C. Scazzocchio, unpublished results). Putative [*sumD16*] strains were checked by establishing heterokaryons with strains carrying [*cs-67*] and scoring for the presence of strains showing the suppressed phenotype. Two of the putative [*sumD16*] strains yielded such suppressed colonies, establishing that [*sumD16*] single mutants have the wild-type phenotype.

Interaction of suppressor mutations with other mutations affecting mitochondrial function

In a [*cs-67*] background, the suppressors *sumA35*, *sumB19* and [*sumD16*] did not interact with the following mutations, which are supposed to affect mitochondrial function: *plnA18*, *cbxA17*, *cbxB28* and *oliC2*, conferring pyrrolnitrin, carboxin and oligomycin resistance, respectively (the action of the inhibitors is described below). The effect of the suppressors upon the mitochondrial mutations [*camA112*] and [*oliA1*] was tested by transferring these two markers into each single suppressor strain carrying [*cs-67*] and one of the suppressors *sumA31*, *sumA35*, *sumB19*, *sumB72*, *sumC40*, *sumE83* or [*sumD16*]. Strains were tested on media with and without chloramphenicol and oligomycin at 37 and 20 °C. No significant effects were seen. At 20 °C [*camA112*] slightly reduced the growth rate of all strains carrying [*cs-67*], including the cold-sensitive strain itself.

Phenotypes of strains containing more than one suppressor mutation

When strains containing suppressor mutations at more than one chromosomal locus were constructed in a [*cs-67*] background, a severe reduction in the rate of growth at 37 °C occurred, this result being more striking on complete medium than on synthetic complete medium. The effect on growth rate of the combinations of different suppressors is shown in Table 1 and selected examples are shown in Fig. 1. It must be stressed that this reduction in the rate of growth was strictly dependent on the presence of [*cs-67*] and was absent in a [*cs-67⁺*] background. Moreover, for *sumA* and *sumB*, where more than one allele was available, it was clear that growth impairment was allele-specific, *sumA35* and *sumB19*

Table 1. *Impairment of growth in mutants carrying [cs-67] and two nuclear suppressor mutations*

Every double suppressor mutant carried the [cs-67] mutation. Each mutant was scored on the scale 1 to 10 for its growth on solid complete medium at 37 °C. The wild-type and [cs-67] strain were both scored as 10.

Suppressor mutation	Growth score					
	<i>sumA35</i>	<i>sumB19</i>	<i>sumE83</i>	<i>sumA31</i>	<i>sumC40</i>	<i>sumB72</i>
<i>sumA35</i>		1	2	—	2	6
<i>sumB19</i>	1		3	6	10	—
<i>sumE83</i>	2	3		8	7	10
<i>sumA31</i>	—	6	8		9	10
<i>sumC40</i>	2	10	7	9		10
<i>sumB72</i>	6	—	10	10	10	

reducing growth more drastically than *sumA31* and *sumB72*, respectively. The results from Table 1 established the following general order in the degree of impairment in growth: *sumA35* > *sumB19* > *sumE83* > *sumA31* > *sumC40* > *sumB72*. All strains in Table 1 that appeared as exceptions to this order carried *sumC40*.

Strains containing one suppressor and the mitochondrially inherited [*sumD16*] were also impaired in growth at 37 °C when [cs-67] was present, the one exception being *sumB72* [cs-67 *sumD16*], where the impairment was marginal. [*sumD16*] further reduced the rate of growth of *sumA31 sumB19* [cs-67], *sumA35 sumB19* [cs-67] and *sumA35 sumC40* [cs-67] (other combinations were not tested). Even this very drastic reduction in growth was dependent on the presence of the [cs-67] allele.

For all combinations of nuclear suppressors, the impairment of growth at 20 °C was consistent with, but less obvious than, that at 37 °C because the effects of suppression and impairment of growth acted in opposite directions. Impairment of growth at 20 °C was scarcely noticeable in strains carrying a nuclear suppressor together with [cs-67] and [*sumD16*].

In vivo characterization of suppressor mutations of [cs-67]

These studies were performed on solid media to test the behaviour of the suppressor mutants under a variety of conditions. The strains tested included those shown in Fig. 1 and strains carrying a single suppressor, having the mitochondrial genome of, firstly, [cs-67⁺] and, secondly, [cs-67 *sumD16*]. Strains were tested at 37 and 20 °C.

Carbon source. The effects of glucose, ethanol, acetate and galactose were compared. No significant differences were observed.

Temperature. The strains were grown at 44, 37, 25, 20 and 15 °C. All strains could still grow at 15 °C. The growth test at 44 °C revealed that strains carrying [cs-67] and one of the suppressors *sumA35*, *sumB19* or *sumC40* were slightly heat-sensitive.

Inhibitors of respiration and oxidative phosphorylation. The following inhibitors were tested: oligomycin – an inhibitor of ATPase (Lardy *et al.*, 1958); carboxin – an inhibitor of succinate dehydrogenase (Gunatilleke *et al.*, 1975*a*); pyrrolnitrin – a multiple site inhibitor between ubiquinone and cytochrome *b* (Warden & Edwards, 1976); diuron – an inhibitor of complex III (Inoue *et al.*, 1967); salicylhydroxamic acid – an inhibitor of the alternative oxidase pathway (Henry & Nyns, 1975); KCN – an inhibitor of cytochrome oxidase. The inhibitors were used at a variety of concentrations in synthetic complete and complete media but none of the strains was either resistant or hypersensitive at either 37 or 20 °C.

Inhibitors of protein synthesis. Many inhibitors of protein synthesis in *E. coli* are known to affect yeast mitochondrial ribosomes (Grivell *et al.*, 1973). In *E. coli*, some of these, at low concentration, can suppress the phenotypes conferred by mutations in protein synthesis (Kirschmann & Davis, 1969; Gorini, 1974). Suppressor strains were screened for hypersensitivity and resistance to a number of inhibitors, looking especially for phenotypic

Fig. 2. Room temperature difference spectra of suppressor mutants grown at 37 and 20 °C: A, wild-type; B, [*cs-67*]; C, [*cs-67 sumD16*]; D, *sumA31* [*cs-67*]; E, *sumA35* [*cs-67*]; F, *sumB19* [*cs-67*]; G, *sumC40* [*cs-67*]; H, *sumE83* [*cs-67*]; I, *sumA31 sumB19* [*cs-67*]; J, *sumA35 sumB19* [*cs-67*].

suppression of impaired growth. Tests were carried out on complete and synthetic complete media at 20 and 37 °C. Inhibitors and the concentrations used were: chloramphenicol (1.2, 2.5 and 4.0 mg ml⁻¹), neomycin (0.5, 0.8, 1.0 and 2.0 mg ml⁻¹), paromomycin (0.2 to 1.0 at 0.1 intervals, 2.0 and 5.0 mg ml⁻¹), erythromycin (5.0 and 10.0 mg ml⁻¹), lincomycin (5.0 mg ml⁻¹), streptomycin (5.0 and 10.0 mg ml⁻¹) and spiramycin (5.0 and 10.0 mg ml⁻¹). Only the first three drugs strongly inhibited the wild-type strain.

[*cs-67*] conferred a low level of resistance to paromomycin at 37 °C, the cold-sensitive phenotype co-segregating with paromomycin resistance in conidia of heterokaryons hetero-

Table 2. Mitochondrial cytochrome ratios in strains carrying [*cs-67*] and suppressor mutations

Cytochrome ratios $\pm$ standard error of the mean were calculated as described in Methods from room temperature difference spectra (Fig. 2). Three to five independent measurements of the same mitochondrial sample were made. Only in the case of the wild-type, [*cs-67*], *sumA35* [*cs-67*] and *sumA35 sumB19* [*cs-67*] strains was more than one independent sample prepared.

Strain (relevant genotype)	Cytochrome ratios					
	Grown at 37 °C			Grown at 20 °C		
	<i>c:aa₃</i>	<i>c:b</i>	<i>b:aa₃</i>	<i>c:aa₃</i>	<i>c:b</i>	<i>b:aa₃</i>
Wild-type	1.30 $\pm$ 0.06	0.95 $\pm$ 0.03	1.37 $\pm$ 0.06	1.61 $\pm$ 0.09	1.01 $\pm$ 0.08	1.59 $\pm$ 0.18
[<i>cs-67</i>]	1.35 $\pm$ 0.09	0.97 $\pm$ 0.03	1.39 $\pm$ 0.10	4.65 $\pm$ 0.23	2.14 $\pm$ 0.16	2.15 $\pm$ 0.17
[<i>cs-67 sumD16</i>]	1.40	1.03	1.39	3.00 $\pm$ 0.15	1.40 $\pm$ 0.11	2.14 $\pm$ 0.17
<i>sumA31</i> [<i>cs-67</i>]	1.86 $\pm$ 0.08	1.08 $\pm$ 0.02	1.78 $\pm$ 0.16	3.23 $\pm$ 0.21	1.53 $\pm$ 0.08	2.11 $\pm$ 0.23
<i>sumA35</i> [<i>cs-67</i>]	2.56 $\pm$ 0.19	1.13 $\pm$ 0.03	2.24 $\pm$ 0.19	2.80 $\pm$ 0.18	1.33 $\pm$ 0.09	2.10 $\pm$ 0.15
<i>sumB19</i> [<i>cs-67</i>]	3.09 $\pm$ 0.03	1.27 $\pm$ 0.04	2.44 $\pm$ 0.06	4.01	1.56	2.61
<i>sumC40</i> [<i>cs-67</i>]	2.06 $\pm$ 0.09	1.14 $\pm$ 0.04	1.80 $\pm$ 0.07	4.05 $\pm$ 0.21	1.75 $\pm$ 0.06	2.31 $\pm$ 0.14
<i>sumE83</i> [<i>cs-67</i>]	2.08 $\pm$ 0.04	1.12 $\pm$ 0.02	1.86 $\pm$ 0.03	4.65 $\pm$ 0.22	2.10 $\pm$ 0.20	2.21 $\pm$ 0.20
<i>sumA31 sumB19</i> [<i>cs-67</i>]	3.50 $\pm$ 0.05	1.52 $\pm$ 0.12	2.30 $\pm$ 0.15	4.42	1.61	2.73
<i>sumA35 sumB19</i> [<i>cs-67</i>]	3.80 $\pm$ 0.21	1.53 $\pm$ 0.05	2.48 $\pm$ 0.08	4.62 $\pm$ 0.25	1.73 $\pm$ 0.20	2.67 $\pm$ 0.16

zygous for the [*cs-67*] gene. This was only clearly seen on complete medium over a narrow concentration range (0.4 to 0.8 mg ml⁻¹). The conferment of resistance to paromomycin by [*cs-67*] was more striking in strains carrying *sumA35*, *sumB19* and *sumC40*. This was because the mutations resulted *per se* in hypersensitivity to paromomycin, while *sumA31*, *sumE83* and the mitochondrially inherited [*sumD16*] were indistinguishable from wild-type. [*cs-67*] was epistatic to all suppressor mutations *vis-à-vis* paromomycin resistance.

Mitochondrial cytochrome spectra

Spectra of mutants carrying [*cs-67*] and one or more nuclear suppressors (Fig. 2) showed that none of these mutants had normal cytochrome complements at either 37 or 20 °C. The spectra, which were obtained from samples of comparable protein concentration, and the ratios of cytochrome *c:aa₃* (Table 2) suggested that many of the suppressors partially restored the low level of cytochrome *aa₃* found in the cold-sensitive strain at 20 °C. Given this and the fact that the ratio of cytochrome *b:aa₃* in each strain carrying [*cs-67*] and a suppressor, grown at 20 °C, was never significantly less than that of the cold-sensitive strain at 20 °C (Table 2), it seemed that these suppressors were modifying the level of cytochrome *b* at the same time as that of cytochrome *aa₃*. This was distinct from the situation found at 37 °C where mutants carrying [*cs-67*] and a nuclear suppressor showed a deficiency in cytochrome *aa₃* with respect to the cytochrome *c* level, which varied with each suppressor, but a minimal deficiency or no alteration in cytochrome *b* with respect to the cytochrome *c* level.

The mitochondrially inherited suppressor [*sumD16*] partially suppressed the modifications in the cytochrome spectra of [*cs-67*] at 20 °C, while the spectrum of the double mutant [*cs-67 sumD16*] was indistinguishable from that of the wild-type and cold-sensitive strains at 37 °C (Fig. 2 and Table 2).

At 20 and 37 °C the growth rate of suppressor mutants on solid medium did not correlate with the degree to which the cytochrome spectra were modified. For example, at 37 °C, the mutant *sumB19* [*cs-67*] had more abnormal cytochrome ratios than *sumA35* [*cs-67*], despite the fact that the latter grew less well on solid complete medium.

Thermolability of cytochrome oxidase

As some of the strains carrying [*cs-67*] and two suppressors were partially heat-sensitive, the cytochrome oxidase activity of these strains should have been more heat-sensitive than

Fig. 3. Heat inactivation of cytochrome oxidase from the wild-type strain ($\square$ — $\square$) and mutant *sumA35 sumB19* [*cs-67*] ($\triangle$ — $\triangle$). The enzyme activity was E_0 at time 0 and E_t at time t , where t was the duration of heat treatment. Open and filled symbols represent heat treatment at 40 and 60 °C, respectively.

Table 3. *Assay of respiratory enzymes of wild-type, cold-sensitive and chloramphenicol-treated wild-type strains*

Mycelium was grown at 20 °C, in the presence of chloramphenicol where indicated, and mitochondrial samples were prepared using a grind-mill (see Methods). Values are the means of assays performed on mitochondrial fractions banding on discontinuous sucrose gradients at the 1.20/1.35 and 1.35/1.60 M interfaces.

Strain	Chloramphenicol (mg ml ⁻¹)	Cytochrome oxidase (nmol O min ⁻¹ mg ⁻¹)	Succinate- cytochrome <i>c</i> reductase (nmol min ⁻¹ mg ⁻¹)	Succinate dehydrogenase (ΔA min ⁻¹ mg ⁻¹)
Wild-type	—	240	980	7.6
[<i>cs-67</i>]	—	56	890	8.7
Wild-type	2.5	155	2530	10.9
Wild-type	6.0	84	955	8.3

that of the wild-type if [*cs-67*] and the suppressors directly modified cytochrome oxidase. However, the cytochrome oxidase activity of the mutant with the most extreme heat-sensitive phenotype, *sumA35 sumB19* [*cs-67*], was similar to that of the wild-type (Fig. 3) indicating that the mutations involved did not directly modify cytochrome oxidase subunits.

Analysis of components of the respiratory chain

The results in Table 3 show that the cold-sensitive strain grown at 20 °C has a reduced cytochrome oxidase activity but normal succinate dehydrogenase and succinate-cytochrome *c* reductase (this involves the cytochrome *bc₁* complex – Complex III). As three subunits of cytochrome oxidase and one subunit of Complex III are synthesized in the mitochondria of yeast and *N. crassa* (Katan *et al.*, 1976; Lin *et al.*, 1978; Weiss, 1972), this suggested that [*cs-67*] directly affects cytochrome oxidase rather than mitochondrial protein synthesis. This conclusion became less tenable as a result of a control experiment in which it was shown that the succinate-cytochrome *c* reductase activity of the wild-type strain was not decreased when grown in the presence of the mitoribosomal inhibitor chloramphenicol (Table 3).

Figure 4 shows that the cytochrome content of the cold-sensitive strain grown at 20 °C was very similar to that of the wild-type grown in the presence of chloramphenicol. Both had a cytochrome *aa₃* deficiency but the effect on cytochrome *b* was not clear.

Fig. 4. Room temperature difference spectra: A, wild-type; B, [*cs-67*]; C and D, wild-type grown in the presence of 2.5 and 6.0 mg chloramphenicol ml⁻¹, respectively. Mycelium was grown at 20 °C, and mitochondria were isolated using a grind-mill (see Methods). Spectral studies were performed on a fraction banding at the 1.20/1.35 M interface of a discontinuous sucrose gradient.

Protein profiles of mitochondrial membrane preparations on gradient gels

Membrane preparations were made from the wild-type, the cold-sensitive strain, a strain having the mitochondrial genotype [*cs-67 sumD16*] and the wild-type grown in the presence of 2.5 and 6.0 mg chloramphenicol ml⁻¹. (At 20 °C in the presence of 6.0 mg chloramphenicol ml⁻¹, the wild-type grew at approximately the same rate as the cold-sensitive strain.)

SDS gradient acrylamide gels exposed a few reproducible differences in these preparations, for proteins of molecular weight between 60000 and 10000 (outside these limits, the gels were less reproducible). The bands on the gels have been numbered arbitrarily beginning with a band of apparent molecular weight 52000. Under the experimental conditions used here, the following band differences were observed in the mitochondrial membrane preparations from the cold-sensitive strain with respect to those from the wild-type: a decrease in bands 5, 10a, 18 and 24 and an increase in bands 8, 9 and 23 (Fig. 5). The mitochondrial membrane preparations from the wild-type strain grown in the presence of chloramphenicol were virtually identical to those from the cold-sensitive strain grown at 20 °C except for the following differences: a decrease in band 8, a slight decrease in band 9 and a slight increase in band 23 (Fig. 5). It appeared that increasing the concentration of chloramphenicol in the growth medium from 2.5 to 6.0 mg ml⁻¹ only significantly affected band 8.

Although a mitochondrial membrane sample was only made once from a strain carrying [*cs-67 sumD16*], the results (not shown) suggested that, with respect to the alterations caused by [*cs-67*], [*sumD16*] fully suppressed the increase in bands 9 and 23, partially suppressed the increase in band 8, partially suppressed the decrease in band 18 and had no effect on the decrease in bands 5 and 24 (band 10a was not resolved on a gel containing this preparation).

By calibrating the gels with standard proteins, as described in Methods, the following bands had the apparent molecular weights indicated: 5 (43000), 8 (35000), 9 (34000), 10a (33000), 18 (20000), 23 (15000), 24 (14000).

Fig. 5. Mitochondrial membrane protein profiles on SDS gradient acrylamide gels: A and B, wild-type grown in the presence of 2.5 and 6.0 mg chloramphenicol ml⁻¹, respectively; C, wild-type; D, mutant [*cs-67*]. Mycelium was grown at 20 °C and membranes were prepared from the mitochondrial fractions which banded at the 1.20/1.35 M interface of a discontinuous sucrose gradient. The scans are of the tracks shown in the photograph.

DISCUSSION

Suppressors of the mitochondrially inherited mutation [*cs-67*] mapped in four nuclear genes and one mitochondrial gene. The most obvious hypothesis to account for this pattern was that the [*cs-67*] gene and five suppressor genes participated in specifying the assembly of a mitochondrial component. However, it should be stated clearly that there was no evidence that any of the genes participated directly in coding for a subunit of the component, rather than in a number of possible indirect ways. Indeed, as only the [*cs-67*] allele was available we could not exclude rigorously the seemingly far-fetched hypothesis that one or more of the suppressors were translational suppressors.

The spectral properties of [*cs-67*] suggested cytochrome oxidase as the possible primary product of the [*cs-67*] gene. The results presented here made this hypothesis unlikely. The triple mutant *sumA35 sumB19* [*cs-67*] resulting in a heat-sensitive phenotype did not result in a heat-sensitive cytochrome oxidase. This did not exclude that [*cs-67*] might be involved specifically, but indirectly, in the synthesis or assembly of cytochrome oxidase, in a post-translational or regulatory capacity or perhaps, as in the *box* mutations in *S. cerevisiae*, in the regulation of both cytochrome oxidase and cytochrome *b* (for review, see Borst & Grivell, 1978). However, the lack of correlation of growth rate with cytochrome content suggested that in [*cs-67*] strains cytochrome deficiencies were not primarily the factor limiting growth.

Two lines of evidence pointed to the mitoribosome as the primary target of the [*cs-67*] mutation. Firstly, the alterations in the pattern of mitochondrial membrane protein by the [*cs-67*] mutation mimicked those produced by sub-lethal concentrations of chloramphenicol, an inhibitor of mitochondrial protein synthesis in yeast (Davey *et al.*, 1969). *N. crassa* (Kuntzel, 1969) and, almost undoubtedly, *A. nidulans* (Lazarus & Turner, 1977). Some caution, however, should be exercised before giving this analogy too much weight. Growth in the presence of chloramphenicol did not seem to decrease the activity of succinate-cytochrome *c* reductase (a possible reason for this may be found in the work of Beattie *et al.*, 1974). Chloramphenicol inhibition of the synthesis of cytochrome *b* was ambiguous and the effect on the protein profile of the mitochondrial membrane was limited. It must be stressed that in the chloramphenicol treatment, total protein rather than *de novo* synthesized protein was detected in the gels. Only experiments following the specific incorporation of label into the chloramphenicol sensitive bands can clarify this point.

The second line of evidence concerned the action of paromomycin. This antibiotic inhibits protein synthesis at the level of the ribosome in *E. coli* (Weisblum & Davies, 1968) and causes mistranslation in an *in vitro* system employing the cytoplasmic ribosomes of *Tetrahymena* (Palmer & Wilhelm, 1978). In *S. cerevisiae*, paromomycin inhibits mitochondrial protein synthesis (Davey *et al.*, 1970) and mutations resistant to paromomycin map in the mitochondrial genome. The suppressor mutations, while suppressing the cold-sensitivity, did not suppress the paromomycin resistance of [*cs-67*]. Incidentally, this argued against the *sum* mutations representing translational rather than physiological suppressors.

It should be mentioned that, while the evidence pointed to the mitoribosome as the primary target of the [*cs-67*] mutation, other interpretations were still possible, for example, [*cs-67*] could have been affecting a component of the mitochondrial membrane, resulting in both defective integration of cytochrome oxidase and cytochrome *b* at 20 °C and decreased permeability to paromomycin at all temperatures.

Mutations in the mitochondrially inherited suppressor [*sumD*] recombined with [*cs-67*] at a frequency sufficient to suggest that these two phenotypes represented mutations in different genes. However, the recent data pointing to the presence of introns one or more kilobases long in mitochondrial genes of yeast (for review, see Borst & Grivell, 1978) calls for caution in interpreting recombination data.

The results presented in this paper constitute the genetic characterization of a system of nuclear-mitochondrial interactions. Further clarification of the nature of the system can only be achieved by biochemical studies of the components of the mitochondrial membrane and the mitoribosome.

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