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GENETIC ANALYSIS OF MULTIPLE DRUG CROSS RESISTANCE IN *SACCHAROMYCES CEREVISIAE*: A NUCLEAR-MITOCHONDRIAL GENE INTERACTION

by

JOE D. COHEN

A dissertation submitted to
the Graduate Faculty in Biology
in partial fulfillment of the
requirements for the degree of
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1977

Joe Cohen

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

A mutant of the yeast Saccharomyces Cerevisiae, multiply resistant to the four antibiotics, chloramphenicol (CAP), tetracycline (TCN), oligomycin (OLI) and cycloheximide (CHX) was isolated. Tetrad analysis of diploids obtained from crosses between the resistant mutant and a sensitive wild type strain, as well as from selected back crosses, suggest that the pleiotropic phenotype is controlled by a single nuclear gene mutation, designated ant-r, interacting with several cytoplasmic loci. They also reveal that a strain or spore clone carrying the ant-r mutation can express resistance to an additional drug, rhodamine 6G (RHG), provided the correct cytoplasmic allele is present in the cells. Vegetatively growing diploid clones derived from crosses of the type ant-r [RHO$^+$] X + [RHO$^+$] show mitotic segregation of two phenotypic classes for the drugs OLI, RHG and TCN. Diploids derived from crosses of the type ant-r [RHO$^+$] X + [RHO^o] and ant-r [RHO^o] X + [RHO$^+$], fail to exhibit mitotic segregation. These results are consistent with our hypothesis concerning the involvement of cytoplasmic loci. In addition, they suggest that these loci are associated with mitochondrial DNA (mt. DNA).

Evidence for this association is provided by the demonstration of genetic linkage between the cytoplasmic loci involved in the pleiotropic phenotype and designated RHG-1, TCN-1 and OLI-5, and two well characterized mitochondrial mutations, ERY-R and CAP-R. In the case of locus OLI-5, additional evidence of its mitochondrial location has been obtained by analyzing the fate (loss or retention) of this locus in cytoplasmic petites of the [RHO$^-$] type.

We have mapped the nuclear ant-r mutation 3.3cM from the centromere-linked locus, leu-1 on chromosome VII. The order of the three markers, centrome VII, leu-1 and ant-r has also been determined.

Tetrad analysis of diploids derived from crosses between our mutant and two similar mutants described by others suggest a common genetic basis for all three.

Two partial revertants of the cross resistant mutant were isolated. Genetic analysis of these revertants indicates that both are due to single nuclear gene mutations, designated scr-1 and scr-2, and segregating independenly of the ant-r locus. The two revertant mutations were found to be heteroallelic and were mapped at approximately 16 cM from the ade-2 locus, on chromosome XV. The nuclear nature of these two revertant mutations is more in line with a nuclear mitochondrial interaction model, such as the one proposed here, than they are with the episomal model proposed by others.

The possible significance and usefulness of this kind of mutant, in terms of the interaction between the nuclear and mitochondrial genomes in specifying and regulating the biosynthesis of mitochondrial and other cellular components is discussed. The claims made by several authors regarding the episomal nature of mutations similar to the one described here are also reviewed.

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CHAPTER I

INTRODUCTION

Mitochondrial DNA has been found in a variety of eukaryotic cell types and organisms (Sager, 1972). In the case of the yeast, Saccharomyces cerevisiae, the study of the structure, physical organization and functions of mitochondrial DNA has been a particularly active field of research. A considerable amount of research has also been conducted in the fields of mitochondrial biogenesis and genetics. It is now well established that the mitochondrial DNA in Saccharomyces cerevisiae consists of a 25μm, double stranded molecule, very likely covalently circularized, with a base composition different fromthat of the nuclear DNA and with a molecular weight of approximately 50 X 10^6 (Rabinowitz et al., 1970). In addition, yeast mitochondria contain the complete enzymatic machinery necessary for the transcription and translation of genetic information (Ashwell et al., 1970). Mitochondrial ribosomes are distinguishable from cytoplasmic ones on the basis of their physical characteristics (Grivell et al., 1971) and specificity toward protein synthesis inhibitors (Clark-Walker et al., 1966; Lamb et al., 1968). Mitochondrial r-RNA species as well as several species of mitochondrial t-RNA have been shown to be transcribed exclusively from mitochondrial DNA sequences (Fukuhara, 1967; Faye et al, 1973). It is also widely believed that mitochondrial DNA also codes for several mitochondrial proteins, although, at the present time, only circumstantial evidence exists in support of this belief. It is, however, well established that the protein synthesizing systems in the cytoplasm and in the mitochondria both contribute specific protein components to several mitochondrial enzymatic complexes, such as the ATPase complex (Tzagoloff, 1971) and the cytochrome oxydase complex (Mason et al., 1973).

Concomitantly with the biochemical approach to the problem of the biogenesis of mitochondria, a genetic approach has also been used. It has relied on the isolation and characterization of mutants affecting one or more mitochondrial functions. Numerous nuclear mutations of this type have been described (Beck et al., 1972; Ebner et al., 1973; Tzagoloff et al, 1975a). There also exist several classes of mitochondrially inherited mutations affecting various aspects of mitochondrial function. Among these is the ubiquitous "petite" mutation, first identified by Ephrussi, (1953) and which results in gross physical alterations (RHO^- petite) or lack of mitochondrial DNA (RHO^o petite) accompanied by multiple enzymatic defects (Sager, 1972).

Another class of mitochondrial mutants which played a particularly important role in the elucidation of mitochondrial genetics, are mutants resistant to inhibitors of mitochondrial protein synthesis, such as chloramphenicol and erythromycin (Linnane et al., 1968 and Thomas et al, 1968) or to inhibitors of oxydative phosphorylation, such as oligomycin (Avner et al., 1970).

Recently, a new class of mitochondrial mutants with specific lesions in various mitochondrial enzyme complexes has been reported (Tzagoloff et al., 1975b). These are known as mit$^-$mutants (Slominski et al., 1976) and differ from the pleiotropic [RHO$^-$] and [RHO^o] mutations in that they have retained most mitochondrial functions (including protein synthesis). Whereas the criteria for establishing the chromosomal (nuclear) location of a mutation are well known and will not be dealt with here, it may be useful to review those used in identifying a mutation as located on the mitochondrial DNA. These criteria, stated in numerous publications (Coen et al., 1970; Bollotin et al., 1971) are now widely accepted by workers in the field of mitochondrial genetics and can be summarized as follows:

a. Failure of the mutation to give characteristic mendelian segregation patterns during meiosis, these being replaced by meiotic segregation of the 0:4 and/or 4:0 types.

b. Mitotic segregation of the mutant and wild type phenotypes among vegetatively grown individual diploid clones, derived from crosses of the type: mutant [RHO$^+$] X wild type [RHO$^+$].

c. The elimination of the genetic determinant for the mutation, concomitantly with the elimination of mitochondrial DNA in petites of the [RHO^o] type. Evidence for this co-loss is obtained from tetrad analysis and the analysis of the phenotype of diploids derived from crosses of the type: mutant [RHO^o] X wild type [RHO^+] and mutant [RHO^+] X wild type [RHO^o]. Diploids from the former cross are expected to be all of the wild type phenotype and segregate 4 wild type: 0 mutant at meiosis, while diploids from the latter cross should all be of the mutant phenotype and segregate 0 wild type:4 mutant at meiosis.

d. Genetic linkage of the mutation under investigation to one or several well characterized mitochondrial genes. The evidence for such linkage is derived from genetic recombination data obtained by calculating the frequency of the recombinant phenotypes among the diploid progeny of crosses of the type: $mutant^x$ + [RHO^+] X + $mutant^M$ [RHO^+], where $mutant^x$ and $mutant^M$ refer to the mutation under study and to the tester mitochondrial mutation respectively.

While criteria a and b define the mutation as cytoplasmically inherited, criteria c and d demonstrate its mitochondrial location.

In general, mutations affecting mitochondrial functions have been unambiguously assigned either a nuclear or a mitochondrial location, on the basis of the criteria stated above. There also exists a number of yeast mutations, not directly involved in mitochondrial function, and which have been shown to be cytoplasmically, although non-mitochondrially, inherited. These are the "killer" character (Makower et al., 1963), the ψ (psi) suppressor mutation (Cox, 1965) and the [URE3] mutation (Lacroute, 1971; Aigle et al., 1975), which allows the uptake of ureidosuccinic acid by yeast cells.

However, during the past few years, several mutations of the drug resistance type, exhibiting ambiguous genetic characteristics, have been reported. These mutations display patterns of inheritance that are reminiscent of both nuclear and cytoplasmic genetics. Almost invariably, such mutants also present the peculiarity of being cross resistant to several drugs, often structurally and/or functionally unrelated, and in some cases, having a cellular target other than the mitochondrion.

The first report of such a mutant was made by Hughes et al. (1972). They studied a mutant resistant to the antibiotic tetracycline (TCN). Whereas the mutation segregates in a mendelian fashion at meiosis (2:2), indicating the involvement of a chromosomal genetic factor, it also displays mitotic segregation, a characteristic expected exclusively from a cytoplasmic mutation. Moreover, the genetic determinant for resistance to TCN was not eliminated in [RHO$^-$] petites induced by acriflavine, thus apparently precluding a mitochondrial location for this determinant. As a possible interpretation of their data, the authors proposed a model along the lines of bacterial episomal systems: an extrachromosomal element, alternating between a cytoplasmic location and a chromosomal association carries the mutation leading to TCN resistance.

Avner _et_ _al_. (1973a, b) reported the isolation and characterization of two classes of oligomycin (OLI) resistant mutants. Class II mutants are resistant to OLI only and show classical mitochondrial inheritance patterns. Class I mutants, on the other hand, are cross resistant to a series of other drugs: aurovertin, triethyltin and "1799", all affecting energy conservation mechanisms, and chloramphenicol, mikamycin and spiramycin, all inhibitors of mitochondrial protein synthesis. Upon genetic analysis, mutants in this latter class produce 2:2 segregations at meiosis but _apparently_ exhibit mitotic segregation as well:

> "Quantitative replica plating analysis of diploids...has shown three types of colonies to be present: resistant (i.e. virtually confluent), mixed (consisting of both resistant papillae and sensitive cells) and sensitive cells." (Avner _et_ _al_., 1973b).

However, conversion of the resistant mutants to the [RHO$^-$] state does not lead to the loss of the genetic determinant responsible for resistance to oligomycin, suggesting that it does not reside on the mitochondrial DNA. The possibility of an interaction between a nuclear determinant and a cytoplasmic, but non-mitochondrial, element is advanced. An alternative, non-genetic explanation is also proposed, in which it is postulated that the anomalies of the system could be the result of the distribution and interaction patterns of the products of nuclear genes in heterozygous diploid cells where these products are randomly distributed to cell organelles such as the mitochondrion.

Somewhat similar observations were reported by Mitchell et al. (1973) in their study of a mutant initially selected for its resistance to oligomycin. This mutant was found to be also resistant to chloramphenicol, mikamycin, lincomycin and carbomycin. Here again meiotic segregation (2:2) but also mitotic segregation was observed. But, in contradistinction to the two preceding reports, the genetic determinant for cross resistance is eliminated in petites induced by ethidium bromide treatment, suggesting the involvement of mitochondrial genes in determining the mutant phenotype. In a subsequent publication (Howell et al., 1974), the genetic analysis of the mikamycin resistance part of the phenotype is reported. Strong evidence in favor of a nuclear-mitochondrial synergistic gene interaction is given, at least as far as resistance to mikamycin is concerned.

Guerineau et al., (1974) reported the isolation of a single step mutation leading to resistance to the drugs oligomycin, venturicidin, triethyltin, chloramphenicol and cycloheximide. Although no genetic data are given in this publication, the authors claim that the multiple drug resistance in their mutant results from the interaction of two genetic factors showing both chromosomal and episomal characteristics. The factors, designated by them π (pi) and τ (tau), are said to confer resistance to oligomycin ($\tilde{\pi}$) and to all other drugs (τ). Episome π, it is claimed, can be lost spontaneously while τ can be completely eliminated by treatment with ethidium bromide. Loss of the factors is said to be independent of the loss of mitochondrial DNA, therefore excluding a mitochondrial location. Finally the authors claim that episome π is identical to a yeast DNA species, independent from either nuclear or mitochondrial DNA, and known as the 2μ DNA. This DNA species has been isolated and characterized by Clark-Walker, (1972 and 1973). It consists of covalently closed circles, ranging in size from 1.9μm to 5.8μm and in molecular weight from 4.02 X 10^6 to 12.04 X 10^6. These characteristics are similar to those of the DNA of many bacterial plasmids and episomes (Clowes, 1972). In addition, a series of recent studies using electron microscopic and restriction nuclease mapping techniques (Guerineau *et al.*, 1976a; Hollenberg *et al.*, 1976; Royer *et al.*, 1977; Livingston *et al.*, 1977) have demonstrated that the 2μ circles contain inverted repeat sequences similar to those described in bacterial plasmids (Bukhari *et al.*, in press). In the latter case these sequences promote the insertion and translocation of genes into other plasmids or into the host chromosome (Kleckner *et.* *al.*, 1975). It must be pointed out that the Guerineau, *et.* *al.*, group did not present any meaningful genetic data in support of their claim, either in the paper cited earlier or in a subsequent publication (Guerineau *et al.*, (1976b) dealing with this mutation. However, one pertinent observation is apparently supported by data: in seven clones which have lost resistance to oligomycin, no detectable 2μ DNA is found in cellular extracts subjected to equilibrium centrifugation in CsCl propidium diiodide gradients (Guerineau _et al._, 1974, 1976b). Lancashire _et al._, (1975) described the isolation and characterization of mutants resistant to venturicidin (VEN), an inhibitor of oxydative metabolism in yeast. A certain proportion of these mutants are also cross resistant to other inhibitors and uncouplers of oxydative phosphorylation, such as oligomycin (OLI), and triethyltin (TET). These workers can unambiguously ascribe a mitochondrial location to the mutants of phenotype VEN^R and VEN^R OLI^R, and a nuclear location to those of phenotype VEN^R OLI^R TET^R. But two mutants of phenotype VEN^R TET^R do not fall in either one of these categories. These mutants do show mitotic segregation, but give high recombination values (45%) relative to several mitochondrial loci. Although no meiotic segregation data are presented for this latter class of mutants, and although the loss of resistance to VEN and TET is always accompanied by the conversion to the petite state, the authors conclude from the recombination data that the genetic determination for the VEN^R TET^R phenotype in these two mutants is cytoplasmic and located on a DNA molecule other than mitochondrial DNA. They propose the 2μ DNA as a possible candidate.

In a subsequent paper (Griffiths et al., 1975) dealing with these VEN^R TET^R mutants (which are now reported to be also resistant to rhodamine 6G and "1799") an attempt to establish that the loss of resistance to VEN is accompanied by the loss of 2μ DNA, is reported. But this attempt fails to yield any positive results, as all the VEN^S clones analyzed still contain 2μ DNA. A more recent publication, originating from the same laboratory (Carignani et al., 1977) describes the isolation and characterization of mutants selected for their resistance to rhodamine 6G (RHG), and which exhibit cross resistance to VEN, TET, "1799", bongkrekic acid and to cycloheximide, but sensitive to oligomycin and to inhibitors of mitochondrial protein synthesis. One of the mutants studied, when mated with a sensitive strain, displays mitotic segregation among its diploid progeny. But meiotic segregation is also obtained among random spores derived from the diploid progeny (no tetrad anlaysis is reported). As was the case with the VEN^R TET^R mutants described by this group (Griffiths et al., 1975) the loss of resistance to all or any of the drugs is always accompanied by conversion to the petite state. Conversely, the induction of the $[RHO^-]$ or $[RHO^o]$ mutation, either spontaneously or following treatment with ethidium bromide, does not necessarily lead to the loss of resistance (although in some petites, resistance to one or more of the inhibitors is lost). It is concluded from these data that both nuclear and cytoplasmic (but non-mitochondrial) genetic factors may be involved in the determination of the cross resistance mutation. Once again, 2μ DNA is proposed as the possible location of the cytoplasmic genes involved.

Finally, Rank *et al.*, (1973, 1975 and 1976) have described the physiological and genetic analysis of a mutant cross resistant to 13 inhibitors (OLI, VEN, TET, CAP, TCN, CHX etc...) and collaterally sensitive to 5 other drugs (ethidium bromide, paromomycin, neomycin, etc...). In this case, the cross resistant phenotype is attributed to a single nuclear gene mutation.

The preceding and rather exhaustive review of the literature available on the subject of cross-resistant mutants in yeast reveals the great deal of confusion and conflicting interpretations that resulted from the study of such mutants. However, some of the interpretations proposed to explain the anomalous genetic behavior of many of the mutants, if confirmed, would clearly open very exciting prospects. Among them are: the possibility of assigning a function or functions to the 2μ DNA; the further possibility that this DNA species may behave as an episome and, much like bacterial eposomes, may integrate and/or excise into and from chromosomal and/or mitochondrial location; the opportunity to study the molecular events involved in the translocation of genes from one genome to another in a eukaryotic organism eminently amenable to biochemical and genetic investigations. Alternatively, the demonstration that these mutants are the result of the interaction between nuclear and mitochondrial genes, although less glamorous than the episomal theory, would be of great interest to the work in the field of mitochondrial biogenesis. It could provide new insight and a new genetic approach to the problem of the integration and cooperation of the nuclear and mitochondrial genomes in the specification of mitochondrial function and structure.

Unfortunately, many of the claims made and interpretations proposed have not been supported by appropriate data, particularly genetic data, crucial in the present case. In several instances, tetrad analysis is lacking or replaced by much less informative random spore analysis (Lancashire _et al._, 1975; Griffiths _et al._, 1977). In other cases, genetic data are completely lacking (Guerineau _et al._, 1974) or so fragmentary as to make them worthless (Guerineau _et al._, 1976b). Finally, in some instances, there are major flaws in the interpretation of existing data (see our discussion).

The goal of the work described in this thesis was to subject a cross resistant mutant we had isolated in our laboratory to a thorough genetic analysis. In many respects, our mutant appeared to be similar to several of the mutants described in the literature. Therefore, the work we decided to undertake was of interest as it could have been expected to contribute to a better understanding or to the solving of the problem of the genetic nature of this class of mutants.

CHAPTER II

MATERIALS AND METHODS

A. STRAINS

The strains of saccharomyces used in this work, all belonging to the species cerevisiae, are listed below, along with their genotypes:

STRAIN	GENOTYPE
RD35	a ade-2 leu-1 pet-ts*
RD35-CR	a ade-2 leu-1 pet-ts ant-r
Z1EK27-D	α lys-1
S3B-CR	a ade-2 leu-1 pet-ts ant-r
DRS8-CR	a ade-2 leu-1 pet-ts ant-r
I1C-CR	α lys-1 leu-1 ant-r
M9-3**	α
M5-121**	α
M5-60**	α
M6-239**	α
M6-200**	α
M7-40**	α
28D-CR	α leu-1 ant-r
332-7c	a his-6 met trp-1 oli^{PR1}
KL9D	a his-1
RD35-S1	a ade-2 leu-1 pet-ts ant-r scr-1
DRS8-S2	a ade-2 leu-1 pet-ts ant-r scr-2
T18D-S1	a ade-2 leu-1 ant-r scr-1
T32C-S2	α lys-1 leu-1 ant-r scr-2

---

*The pet-ts (our designation) mutation was characterized by us as a temperature conditional single nuclear gene mutation. Cells carrying pet-ts are converted to the petite [RHO$^-$] state upon growth at 37°C (our unpublished results). A more detailed description of the phenotype of this mutation appears in chapter V.

**These strains are cytoplasmic petite mutants of the mit$^-$ type (Slonimski, P.P. and A. Tzagoloff, 1976), the isolation and description of which is given by Tzagoloff <u>et al.</u>, (1975b). They were kindly made available to us by Dr. R.B. Needleman.

B. MEDIA

Complete media: The standard rich broth medium used for general purposes was YEPD, 1% yeast extract (Difco), 1% peptone (Difco), 2% glucose. When 3% glycerol + 2% ethanol is substituted to the glucose this medium becomes YEPGE.

Minimal medium: The medium lacking amino acids and nitrogenous bases, used for the selection and subsequent growth of prototrophic diploids was YNBD, containing .7% yeast nitrogen base w/o amino acids (Difco) and 2% glucose. In a few instances it was necessary to supplement this medium with 20 µg/ml leucine, in order to select and/or culture homozygous leu-1/leu-1 diploids.

Sporulation medium: Sporulation was induced on KAC agar plates containing 1% potassium acetate, .1% dextrose and .25% yeast extract. In the case of leu-1/leu-1 homozygous diploids, this medium was supplemented with 20 µg/ml leucine.

Drop out media: These media, used in the determination of auxotrophic requirements of spore clones in tetrad analysis, consisted in YNBD medium supplemented with the appropriate additions of amino acids and nitrogenous bases, as described by Sherman et al., (1970).

Drug media: Unless otherwise specified, these media were: YEPD-CHX, YEPD + .5 µg/ml cycloheximide; YEPGE-OLI, YEPGE-CAP, YEPGE-ERY, YEPGE-RHG and YEPGE-TCN are YEPGE supplemented with 3 µg/ml oligomycin, 4 mg/ml chloramphenicol, 2 mg/ml erythromycin, 15 µg/ml rhodamine, 6G and 3 mg/ml tetracycline, respectively. The drugs were added to the media, after autoclaving and cooling to 55°C, as ethanol solutions, except cycloheximide and tetracycline hydrochloride, which were added as aqueous solutions. Oligomycin, chloramphenicol, erythromycin, cycloheximide and tetracycline hydrochloride were purchased from Sigma Chemical Co., St. Louis, Mo.. Rhodamine 6G was a gift from Dr. J. Marmur.

When required, the media were solidified by the addition, before autoclaving, of 2% agar (Difco).

C: INDUCTION OF THE CYTOPLASMIC PETITE STATE:

Cytoplasmic petites mutants were obtained by growing cells of the appropriate strain in YEPD liquid medium containing 50 μg/ml ethidium bromide, for 48 hours. This first treatment usually resulted in an almost quantitative conversion of the culture to the petite state. A purified petite clone was then subjected to a second round of growth in YEPD + 50 μg/ml ethidium bromide. A purified single clone isolate from this second treatment was used for subsequent work. We have made the assumption that all the ethidium bromide petites used in this work, and induced in the manner described above were of the [RHO^{o}] type, i.e. lacking detectable mitochondrial DNA. Although no biochemical determination of the mitochondrial DNA of these petites was performed, we feel that this assumption is justified in view of the extent of the ethidium bromide treatment used to generate them (for a discussion of the effect of ethidium bromide treatment on mitochondrial DNA see Goldring *et al.*, 1970). Additional justification of this assumption comes from our determination that, in crosses to [RHO$^+$] cells, all these petites are of the "neutral" type (Michaelis *et al.*, 1971; Nagley *et al.*, 1970).

D: MANGANESE CHLORIDE MUTAGENESIS:

The method described by Putrament *et al.*, 1975, was used, with the following modifications: the cells to be mutagenized were pre-grown to stationary phase in YEPGE medium. An aliquot from this culture was inoculated into 10 ml of YEPD medium, to a final titer of approximately 1 X 10^6 cells/ml. Manganese chloride was added to a final concentration of 8mM. The cultures were incubated at 25°C in a rotary shaker until stationary phase was reached. The cells were then washed with sterile water and resuspended in water. The appropriate serial dilutions were made and cells were plated on selective (drug) medium at concentrations ranging from 1 X 10^5 to 1 X 10^7 cells/plate.

E: GENETIC ANALYSIS:

Crosses, selection and sporulation of diploids, microdissections and tetrad analysis were performed by standard techniques, described in detail by Mortimer and Hawthorne, (1969).

F: DETERMINATION OF THE DRUG RESISTANCE PHENOTYPE OF INDIVIDUAL DIPLOID CLONES:

Random diploids were allowed to grow for 30 to 40 generations, by successive subculturing in liquid YNBD* medium. They were then diluted and plated on YNBD* agar plates (100-150 colonies per plate). Single colonies were picked and used to make masters by spreading each colony into a patch, on YNBD* plates. Each master contained 50 individual diploid patches. After 4 to 5 days of incubation, the masters were replicated onto YEPGE and onto drug containing media. Drug plates were scored after 6 to 12 days of incubation, depending on the particular drug tested (see chapter IV for further details). Care was taken in trying to use inocula of approximately equal sizes when making patches on master plates. This was done in order to obtain patches of comparable cell densities at the time of replica plating to the drug media.

G: GROWTH CONDITIONS:

All plates and liquid cultures were routinely incubated at 25°C, since many of the strains used in this study carry the temperature sensitive pet-ts mutation, described elsewhere (see footnote in page 14. See also chapter V.

---

*Supplemented with 20 μg/ml leucine when necessary.

CHAPTER III

MEIOTIC SEGREGATION OF THE CROSS RESISTANCE MUTATION IN STRAIN RD35-CR

Strain RD35 had been used in our laboratory for the study of a nuclear temperature sensitive "petite" mutation, pet-ts. In the course of this study, mutations to erythromycin resistance (ERY-R) and chloramphenicol resistance (CAP-R) were introduced in strain RD35 by two independant and successive treatments with manganese chloride ($MnCl_2$), reportedly a specific mitochondrial mutagen (Putrament *et al*. 1975). Upon genetic analysis, these two mutations conformed to the standard criteria for *bona fide* mitochondrial genes, as proposed by Bolotin *et al*. (1971). The doubly resistant strain was then subjected to a third $MnCl_2$ treatment with the aim of introducing an additional mitochondrial mutation to oligomycin resistance (OLI-R). One of the several OLI-R mutants obtained following this treatment, strain RD35-CR, showed ambiguous genetic characteristics upon preliminary analysis; i.e. the pattern of inheritance of this OLI-R mutation was suggestive of both chromosomal (Mendelian) and non-chromosomal (cytoplasmic) inheritance. Such genetically ambiguous mutants had already been reported in the literature, notably by Mitchell *et al*. (1973), Avner *et al*. (1973), Rank *et al*. (1973 and 1975), Guerrineau et al. (1974) and Lancashire *et al*. (1975). In all cases, the mutants reported on by these workers exhibited cross resistance to several drugs in addition to oligomycin resistance. The similarity between these mutants and the one we had isolated extended also to the domain of cross resistance. Further analysis of strain RD35-CR revealed that it had also acquired resistance to the drugs cycloheximide (CHX-R) and tetracycline (TCN-R).

As we mentioned in the introduction to this work, conflicting claims, often unsubstantiated by appropriate genetic data, had been made in the literature, concerning the possible genetic basis of these mutations. It was therefore of interest to conduct a thorough genetic analysis of our mutant. This was initiated by performing a detailed study of the meiotic segregation patterns of the cross resistance mutation in strain RD35-CR.

In view of the published claims concerning the possible involvement of cytoplasmic genetic factors and the fact that the nature (episomal or mitochondrial or others) of these cytoplasmic factors had not been clearly established, we performed a cross between the mutant and wild type sensitive strain (Z1EK27-D) in the following combinations:

CROSS 1A: RD35-CR [RHO$^+$] X Z1EK27-D [RHO$^+$]
CROSS 1B: RD35-CR [RHO$^+$] X Z1EK27-D [RHO°]
CROSS 1C: RD35-CR [RHO°] X Z1EK27-D [RHO$^+$]

Random diploids from each of the three crosses were sporulated, dissected and subjected to tetrad analysis.

When one is dealing with a <u>bona fide</u> mitochondrial mutation, let us say "a" (wild type "A"), this series of crosses yields characteristic meiotic segregation patterns: The diploids from the [RHO^+] X [RHO^+] type cross yield a mixture of 4a : 0A and 0a : 4A tetrads in variable proportions. Crosses of the [RHO^+] X [RHO°] and [RHO°] X [RHO^+] types on the other hand, yield exclusively either 4A : 0a or 0A : 4a tetrads. These results obviously differ from those expected of a mutation controlled by a Mendelian (chromosomal) gene and are well explained in terms of genes located on the mitochondrial genome or mitochondrial DNA (mt. DNA).

In our analysis, we scored the phenotypes of the spore clones for all the drugs to which the mutant strain RD35-CR was resistant. In addition, resistance or sensitivity to the lipophilic dye, rhodamine 6G (RHG), a potent inhibitor of oxydative phosphorylation in rat liver mitochondria (Grear, 1974), was also scored. The inclusion of this drug in our analysis was prompted by a personal communication from Dr. R. B. Needleman, that cross resistant mutants under study in other laboratories exhibited resistance to RHG.

The results of the tetrad analyses are presented in Table 1. These results clearly do not conform to the patterns of segregation expected either from a Mendelian gene (or genes) or from mitochondrial (or cytoplasmic) genes, although they present characteristics of both genetic systems. Their salient features can be stated as follows:

a. In any given tetrad the spores are simultaneously resistant or simultaneously sensitive to all the drugs exhibiting a 2:2 segregation for resistance:sensitivity.

TABLE 1

MEIOTIC SEGREGATION OF DRUG RESISTANCE IN CROSSES
OF RD35-CR BY Z1EK27-D

	Number of tetrads analyzed	Tetrad classes	Segregation of resistance: sensitivity for each of the drugs tested						Number of tetrads in each class
			ERY	CAP	OLI	RHG	TCN	CHX	
CROSS 1A	21	I	0:4	2:2	0:4	2:2	2:2	2:2	13
		II	4:0	4:0	2:2	0:4	2:2	2:2	4
		III	4:0	2:2	2:2	2:2	2:2	2:2	1
		IV	0:4	2:2	2:2	2:2	2:2	2:2	2
		V	4:0	4:0	0:4	0:4	2:2	2:2	1
CROSS 1B	9	II	4:0	4:0	2:2	0:4	2:2	2:2	9
CROSS 1C	10	I	0:4	2:2	0:4	2:2	2:2	2:2	10

b. In all crosses, all tetrads exhibit a 2:2 segregation for CHX and TCN resistance:sensitivity.

c. Both 2:2 and 0:4 segregations are observed for OLI and RHG resistance:sensitivity among the tetrads derived from the [RHO$^+$] x [RHO$^+$] cross, whereas only 2:2 or 0:4 patterns are obtained in the [RHO+] x [RHO^o] and [RHO^o] x [RHO$^+$] crosses, with the patterns for OLI and RHG being reversed in the two latter crosses.

d. 2:2 and 4:0 segregations for CAP resistance:sensitivity are obtained among the tetrads derived from the [RHO$^+$] x [RHO$^+$] cross whereas the [RHO$^+$] x [RHO^o] and [RHOo] x [RHO$^+$] crosses yield only 4:0 and only 2:2 segregations, respectively.

e. The segregation patterns for ERY resistance:sensitivity are unambiguous and consistent with those expected from a mitochondrial mutation.

As a working hypothesis, we propose the following model to explain these apparently inconsistent results:

a. A single nuclear gene mutation, designated ant-r (wild type +) is responsible and necessary for the expression of resistance to the drugs CHX, TCN, CAP, RHG and OLI.

b. Whereas the nuclear mutation alone is necessary and sufficient for the expression of resistance to CHX, TCN and CAP, expression of resistance to RHG and OLI requires the additional presence of two cytoplasmic mutations, designated RHG-1R and OLI-5R (wild type alleles RHG-1S and OLI-5S). Only strains of genotypes ant-r [RHG-1R] and ant-r

[OLI-5R] will express resistance to RHG and OLI, respectively. All other genotypes, ant-r [RHG-1S], +[RHG-1R], +[RHG-1S], ant-r [OLI-5S], +[OLI-5R] and +[OLI-5S] will express sensitive phenotypes.

c. The cytoplasmic loci OLI-5 (R or S) and RHG-1 (R or S) are sensitive to ethidium bromide (E.B.) and, upon treatment with this drug, are physically altered or eliminated in much the same way as, or concomitantly with, mitochondrial DNA.

d. Superimposed on this genetic system, but independent from it, are the two mitochondrial mutations, ERY-R and CAP-R, present in strain RD35-CR.

e. The parental strains involved in these three crosses (and those involved in subsequent ones) can be assigned nuclear and cytoplasmic genotypes as shown in table 2.

Given the features of the proposed model, the results shown in table 1 can be readily interpreted as follows:

The diploids derived from cross 1B (RD35-CR [RHO$^+$] x Z1EK27-D [RHO^o]) have the genotype ant-r/+ [ERY-R CAP-R OLI-5R RHG-1S] and as a result exhibit the following segregation patterns (resistance:sensitivity): 4:0 for ERY and CAP, 2:2 for OLI, 0:4 for RHG and 2:2 for CHX and TCN.

The diploids derived from cross 1C (RD35-CR [RHO^o] x Z1EK27-D [RHO$^+$]) have the genotype ant-r/+ [ERY-S CAP-S OLI-5S RHG-1R] and consequently segregate 0:4 for ERY, 2:2

TABLE 2

GENOTYPES OF STRAINS, INFERRED FROM THE MODEL PROPOSED IN THE TEXT

STRAIN		NUCLEAR GENOTYPE	CYTOPLASMIC GENOTYPE				
RD35-CR	[RHO^{+}]	ant-r	[ERY-R	CAP-R	OLI-5R	RHG-1S	RHO^{+}]
RD35-CR	[RHO^{o}]*	ant-r	[ERY^{o}	CAP^{o}	OLI-5^{o}	RHG-1^{o}	RHO^{o}]
Z1EK27-D	[RHO^{+}]	+	[ERY-S	CAP-S	OLI-5S	RHG-1R	RHO^{+}]
Z1EK27-D	[RHO^{o}]	+	[ERY^{o}	CAP^{o}	OLI-5^{o}	RHG-1^{o}	RHO^{o}]
S3B-CR	[RHO^{+}]	ant-r	[ERY-R	CAP-R	OLI-5R	RHG-1S	RHO^{+}]
S3B-CR	[RHO^{o}]	ant-r	[ERY^{o}	CAP^{o}	OLI-5^{o}	RHG-1^{o}	RHO^{o}]
DRS8-CR	[RHO^{+}]	ant-r	[ERY-S	CAP-S	OLI-5S	RHG-1R	RHO^{+}]
DRS8-CR	[RHO^{o}]	ant-r	[ERY^{o}	CAP^{o}	OLI-5^{o}	RHG-1^{o}	RHO^{o}]

*Superscript o indicates the postulated physical absence of the genetic locus concerned, following treatment with Ethidium Bromide.

for CAP, CHX and TCN, 0:4 for OLI and 2:2 for RHG.

The diploids derived from cross 1A (RD35-CR [RHO$^+$] x Z1EK27-D [RHO$^+$]), originate from zygotes having a genetically heterogeneous cytoplasm and therefore the genotype ant-r/+ [ERY-R CAP-R OLI-5R RHG-1S/ERY-S CAP-S OLI-5S RHG-1R].

Assuming random assortment and/or recombination between the cytoplasmic alleles, and subsequent purification of the cytoplasmic types among the vegetative diploid progeny (as is the case for mitochondrial genes), 32 (2^5) different genotypes are theoretically possible among the random diploids derived from this cross. It would be tedious and of questionable usefulness to list here all 32 genotypes and the segregations expected from each of them. Rather, as it is clear by now that the two drugs for which ambiguous segregations are obtained are OLI and RHG, we will restrict ourselves to listing the four possible genotypes having relevance to these two drugs:

ant-r/+ [OLI-5R RHG-1S]
ant-r/+ [OLI-5S RHG-1R]
ant-r/+ [OLI-5R RHG-1R]
ant-r/+ [OLI-5S RHG-1S]

These diploids will produce the tetrad classes labelled in table 1 as class II, class I, class III and IV and class V, respectively.

As expected, 4:0 and 2:2 tetrads are observed for CAP, depending on whether the CAP-R or CAP-S allele has been inherited by a particular diploid. Similarly only 2:2 segregations for CHX and TCN are seen, as they are dependent solely upon the segregation of the ant-r and + alleles of the nuclear gene.

Finally typical mitochondrial segregations (4:0 and 0:4) are obtained for ERY.

To test further the validity of our model, we performed two series of backcrosses involving cross resistant meiotic segregants derived from crosses 1B and 1C. The following crosses were performed and random diploids from each cross were sporulated, dissected and subjected to tetrad analysis:

Cross 2A: S3B-CR [RHO^{+}] x Z1EK27-D [RHO^{+}]
Cross 2B: S3B-CR [RHO^{+}] x Z1EK27-D [RHO^{o}]
Cross 2C: S3B-CR [RHO^{o}] x Z1EK27-D [RHO^{+}]

and,

Cross 3A: DRS8-CR [RHO^{+}] x Z1EK27-D [RHO^{+}]
Cross 3B: DRS8-CR [RHO^{+}] x Z1EK27-D [RHO^{o}]
Cross 3C: DRS8-CR [RHO^{o}] x Z1EK27-D [RHO^{+}]

Strain S3B-CR is a meiotic segregant from cross 1B (table 1), resistant to ERY, CAP, CHX, TCN and OLI, but sensitive to RHG, and therefore having the putative genotype, ant-r [ERY-R CAP-R OLI-5R RHG-1S], and strain DRS8-CR is a meiotic segregant from cross 1C (table 1) resistant to RHG, TCN, CHX and CAP and therefore having the putative genotype, ant-r [EYR-S CAP-S OLI-5S RHG-1R]. Tables 3 and 4 show the tetrad data obtained from these two series of backcrosses. It

TABLE 3

MEIOTIC SEGREGATION OF DRUG RESISTANCE IN CROSSES OF S3B-CR by Z1EK27-D

	NUMBER OF TETRADS ANALYZED	TETRAD CLASSES	SEGREGATION OF RESISTANCE: SENSITIVITY FOR EACH OF THE DRUGS TESTED						NUMBER OF TETRADS IN EACH CLASS
			ERY	CAP	OLI	RHG	TCN	CHX	
Cross 2A	48	I	0:4	2:2	0:4	2:2	2:2	2:2	26
		II	4:0	4:0	2:2	0:4	2:2	2:2	10
		III	4:0	2:2	2:2	2:2	2:2	2:2	3
		IV	0:4	2:2	2:2	2:2	2:2	2:2	7
		V	4:0	4:0	0:4	0:4	2:2	2:2	1
		VI	4:0	2:2	0:4	2:2	2:2	2:2	1
Cross 2B	16	II	4:0	4:0	2:2	0:4	2:2	2:2	16
Cross 2C	13	I	0:4	2:2	0:4	2:2	2:2	2:2	16

TABLE 4

MEIOTIC SEGREGATION OF DRUG RESISTANCE IN CROSSES OF DRS8-CR by Z1EK27-D

	NUMBER OF TETRADS ANALYZED	TETRAD CLASSES	SEGREGATION OF RESISTANCE: SENSITIVITY FOR EACH OF THE DRUGS TESTED						NUMBER OF TETRADS IN EACH CLASS
			ERY	CAP	OLI	RHG	TCN	CHX	
Cross 3A	16	I	0:4	2:2	0:4	2:2	2:2	2:2	16
Cross 3B	8	I	0:4	2:2	0:4	2:2	2:2	2:2	8
Cross 3C	9	I	0:4	2:2	0:4	2:2	2:2	2:2	9

is clear that these results are in full agreement with the expectations of the model.

In this chapter, we have presented tetrad data from forward and backcrosses involving the multiple drug cross resistant mutant, RD35-CR. A model concerning the genetic basis of the cross resistance mutation was proposed which is consistent with all the tetrad data obtained. This model postulates the involvement of a nuclear gene mutation, ant-r, as well as, at least in the cases of resistance to OLI and RHG, the additional involvement of two cytoplasmic mutations, OLI-5R and RHG-1R.

Although the nuclear nature of the ant-r mutation is well demonstrated by the tetrad data, additional evidence is necessary to demonstrate the cytoplasmic nature of the postulated OLI-5R and RHG-1R mutations. Such evidence will be presented in the next chapter.

CHAPTER IV

DEMONSTRATION OF THE CYTOPLASMIC NATURE OF LOCI OLI-5, RHG-1 and TCN-1:

MITOTIC SEGREGATION OF PHENOTYPES AMONG VEGETATIVE DIPLOID CLONES: ant-r X + CROSSES:

A characteristic of mitochondrial genes, and of the phenotypes they control, is their segregation among vegetatively growing diploid clones. Mitotic segregation, as this phenomenon is called, can reasonably be expected also from cytoplasmic genes not necessarily associated with mitochondrial DNA. Thus, for example, Lacroute, (1971) has demonstrated that it occurs in the case of the [URE3] gene, a cytoplasmic, although non-mitochondrial factor found in yeast. We have sought to determine whether or not such a phenomenon occurs in our system. It is possible, however, that in our mutant, mitotic segregation, if indeed it occurs at the genotypic level, may be masked at the phenotypic level by the partial dependence of the phenotype on the nuclear ant-r mutation. Indeed, in the first part of the study described below, all the diploids used for the analysis are heterozygous for the nuclear locus, i.e. ant-r/+.

Therefore, the dominance/recessiveness relationship between the ant-r and + alleles, whatever it is, may interfere with our ability to detect phenotypically the phenomenon of mitotic segregation. Bearing in mind this possible complication, individual diploid clones from each of the nine crosses described in the preceding chapter were tested for their resistance or sensitivity to each of the drugs involved in the study. This was done by the patch-replica plating technique described in the section on materials and methods. If the drug plates are scored after 4 to 5 days of incubation, mitotic segregation is not observed, except in the case of ERY.

The diploids from all nine crosses appear all sensitive to OLI, RHG, TCN and CHX, all resistant to CAP and either resistant or sensitive to ERY. Upon further incubation of the plates, (6 to 10 days depending on the particular drug tested) however, it becomes possible to distinguish two different phenotypes for all the drugs, except CHX. Table 5 lists the phenotypes observed, as well as their frequencies.

Although the phenotypes segregating among the diploid clones are not, except for ERY, the usual resistance or sensitivity one would have expected to see, they do illustrate mitotic segregation, and can be interpreted in the following manner, consistent with our model:

ERYTHROMYCIN: The segregation of resistance and sensitivity among diploids derived from crosses 1A and 2A, and the lack of segregation in all other crosses are typical of what is expected from a bona fide mitochondrial mutation.

TABLE 5

SEGREGATION OF PHENOTYPES AMONG VEGETATIVE DIPLOID CLONES: (ant-r X + type crosses)

	PHENOTYPIC CLASSES OBSERVED FOR EACH DRUG TESTED AND NUMBER OF CLONES IN EACH PHENOTYPIC CLASS											NUMBER OF CLONES SCORED
	ERY		CAP		OLI		RHG		TCN		CHX	
	S*:R*		SR*:R		S:SP*		SP:S		SR:SP		SP	
CROSS 1A	45	5	48	2	25	25	48	2	45	5	50	50
CROSS 1B	--	50	--	50	--	50	--	50	--	50	50	50
CROSS 1C	50	--	50	--	50	--	50	--	50	--	50	50
CROSS 2A	35	15	31	19	30	20	32	18	29	21	50	50
CROSS 2B	--	200	--	200	--	200	--	200	--	200	200	200
CROSS 2C	200	--	200	--	200	--	200	--	200	--	200	200
CROSS 3A	50	--	50	--	50	--	50	--	50	--	50	50

TABLE 5 (cont'd)

CROSS 3B	50	--	50	--	50	--	50	--	50	--	50		50
CROSS 3C	50	--	50	--	50	--	50	--	50	--	50		50

*Phenotype symbols: S = sensitive, R = resistant, confluent growth

SP = sensitive with numerous resistant papillae

SR = semi-resistant, confluent growth as judged qualitatively by the extent of growth of the patches, compared to the fully sensitive or fully resistant ones.

CHLORAMPHENICOL: Diploids which have inherited the CAP-R mitochondrial mutation (genotype ant-r/+ [CAP-R] are fully resistant, while those having inherited the CAP-S mitochondrial allele (genotype ant-r/+ [CAP-S]) are semi-resistant to the drug because of the presence of the nuclear gene in the heterozygous condition. This interpretation, derived from the phenotypes obtained in the nine crosses listed in table 5, is also in full agreement with the tetrad data obtained upon dissection of resistant and semi-resistant diploids: the former segregate 4:0 while the latter give 2:2 segregations for CAP resistance:sensitivity. (See tables 1, 3, and 4 in relation to data in table 5.)

OLIGOMYCIN AND RHODAMINE 6G: The individual diploid patches either acquire an extensive number of resistant papillae, or they do not, even after a prolonged period of incubation (12 to 15 days). We will give evidence in a forthcoming chapter, that these papillae are homozygous for the ant-r allele, i.e. ant-r/ant-r, most likely resulting from either mitotic recombination or mitotic gene conversion. Whether or not these ant-r/ant-r diploids, which must occur randomly in all diploid clones (this will be demonstrated in Chapter 6) will express resistance or sensitivity to OLI and/or to RHG depends upon which cytoplasmic loci were inherited by the individual diploid from which they arise. The necessary assumption here is that in the heterozygous condition ant-r/+ the wild type + allele (sensitive) is dominant over the ant-r allele with regard to OLI and RHG resistance, and that therefore, regardless of the cytoplasmic genotype of these diploids, they will all be sensitive to both drugs. However, upon appearance of the homozygous ant-r/ant-r state in any individual diploid within a patch, the determining factor, as to whether this diploid will be resistant or sensitive to OLI or RHG, is its cytoplasmic genotype, CLI-5R or OLI-5S and RHG-1R or RHG-1S.

This interpretation is consistent with the model and with the cytoplasmic genotypes which we have ascribed to the parental strains (table 2). The results obtained for the diploid progeny of crosses 1B, 1C, 2B, 2C, 3A, 3B and 3C where all the patches are either heavily or not at all papillated, argues against the possibility that the segregation of papillated and non-papillated patches seen among the diploid progeny of crosses 1A and 2A are merely the result of random fluctuations in the number of mitotic recombinants, and/or mitotic gene convertants occuring in any given patch. Figure 1 illustrates the lack of ambiguity in scoring the phenotypes. Although some resistant papillae generally occur on the oligomycin plate in the progeny of cross 2C (and likewise in crosses 1C, 3A, 3B and 3C not shown in figure 1) their number is usually small (0 to 3 on any given patch) and far less than the number of resistant papillae occuring on all the patches of diploids derived from cross 2B (and

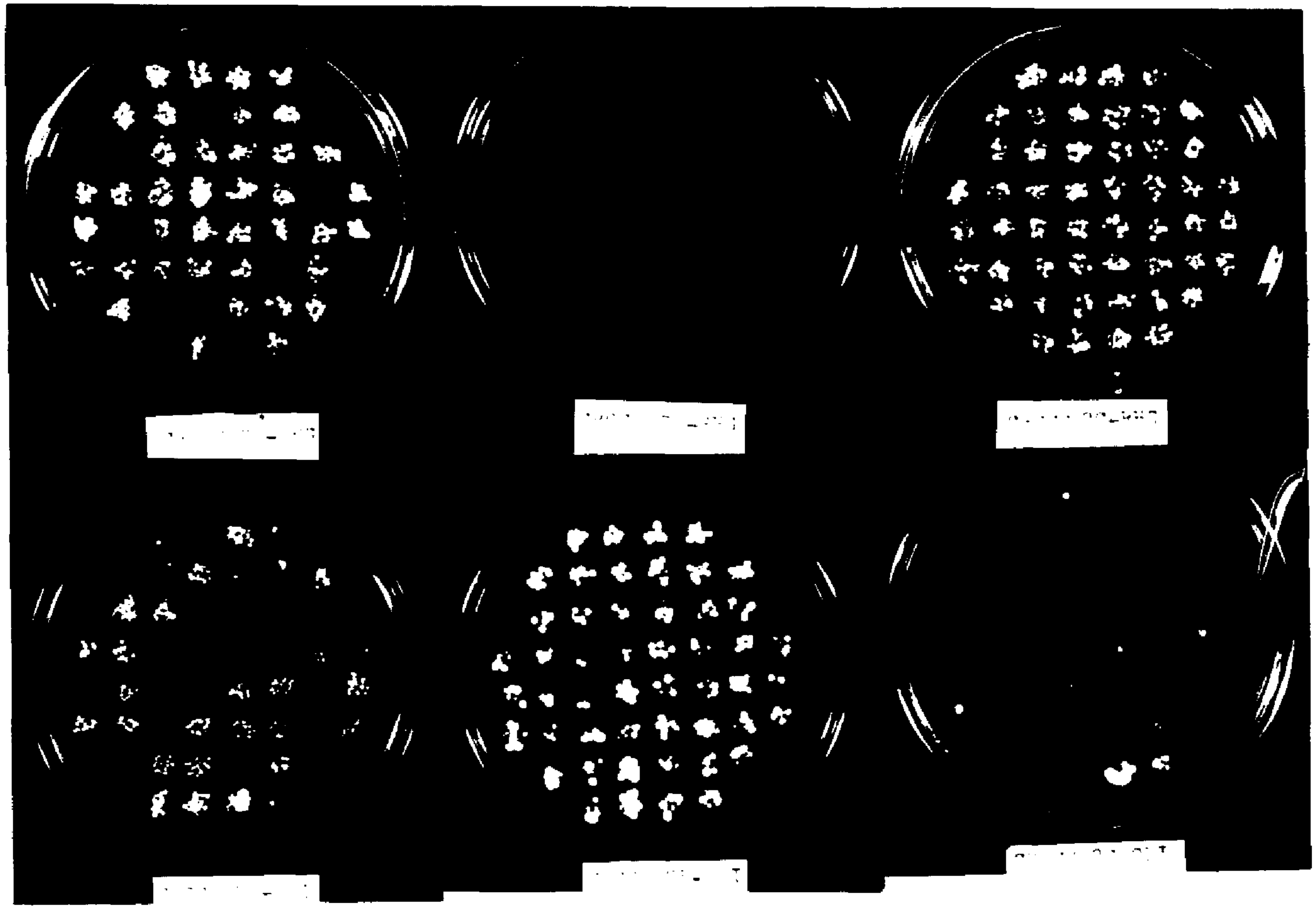


Figure 1. Segregation of phenotypes, for RHG and OLI, among vegetative diploid clones derived from crosses 2A, 2B and 2C.

from crosses 1B, 3A, B and C; not shown in figure 1). They, therefore, do not constitute a major problem in scoring the phenotype of diploids derived from crosses 1A and 2A, where mitotic segregation does occur. These few and scattered resistant papillae occuring in patches where, according to the model, they should not occur, most probably represent spontaneous mutations to OLI resistance.

TETRACYCLINE: Sensitive patches with numerous resistant papillae or semi-resistant patches occur among the diploid progeny of crosses 1A and 2A, while all other crosses produce diploid populations which are homogeneous for one or the other of these phenotypes. This result indicates that the resistance to tetracycline also depends on a mitotically segregating, and therefore cytoplasmic factor, designated TCN-1. However, in this case it is necessary to assume that both original parental strains, as well as the meiotic segregants S3B-CR and DRS8-CR, must carry resistant alleles of this factor. These may be viewed as heteroalleles of a TCN-1R gene. The heteroallele in strain RD35-CR (and in strain S3B-CR), TCN-1R-1, when present in a heterozygous diploid ant-r/+ produces a sensitive phenotype, with resistant papillae occuring upon establishment of the ant-r/ant-r condition (crosses 1B and 2B, table 5). The heteroallele in strain Z1EK27-D (and in strain DRS8-CR, TCN-1R-2, allows an intermediate level of resistance to be expressed in heterozygous ant-r/+ diploids (crosses 1C, 2C, 3A, 3B and

3C, table 5). This interpretation is consistent with the fact that diploids from all crosses, regardless of their phenotype (SR or SP), always produce 2:2 segregations for TCN resistance:sensitivity (tables 1, 3 and 4).

CYCLOHEXIMIDE: Diploids derived from all nine crosses express the same phenotype: all are sensitive with numerous resistant papillae occuring on every patch. Therefore, we do not detect the involvement of a cytoplasmic factor in the case of resistance to CHX. This observation, however, does not necessarily constitute definitive evidence against nuclear-cytoplasmic interaction. It is possible to argue that the uniformity of the phenotype of the diploids derived from all nine crosses may be due to the identity of the alleles of a possible cytoplasmic factor, CHX-1, in the two original parental strains, RD35-CR and Z1EK27-D.

MITOTIC SEGREGATION OF PHENOTYPES AMONG VEGETATIVE DIPLOID CLONES: ant-r X ant-r crosses:

The results of the preceding analysis strongly suggest that mitotic segregation does occur, at least in the cases of OLI, RHG and TCN. However, the reliance on mitotic recombination or gene conversion to enable us to distinguish between the segregating phenotypes, as well as the unorthodox nature of these phenotypes, could lend itself to some criticism. In an attempt to overcome this problem, and definitively demonstrate mitotic segregation, we repeated the above analysis using as parents two strains which both carried the ant-r mutation.

Strain S3B-CR, described previously, was crossed to strain I1C-CR, a meiotic segregant from the cross RD35-CR [RHO^{o}] X Z1EK27-D [RHO^{+}]. The following three crosses were performed:

CROSS 4A: S3B-CR [RHO^{+}] X I1C-CR [RHO^{+}]
CROSS 4B: S3B-CR [RHO^{+}] X I1C-CR [RHO^{o}]
CROSS 4C: S3B-CR [RHO^{o}] X I1C-CR [RHO^{+}]

Clearly, from the point of view of the cytoplasmic genotypes of the two strains, these crosses are analogous to crosses 1A, 1B and 1C and to crosses 2A, 2B and 2C. The phenotypes of individual random diploid clones derived from these three crosses were analyzed as previously described and the results appear in table 6. Mitotic segregation of clearly resistant and clearly sensitive diploids is now obtained for the drugs OLI and RHG, in the RHO^{+} X RHO^{+} cross (cross 4A), whereas the diploid populations from crosses 4B and 4C are homogeneously either all resistant or all sensitive to OLI and/or RHG.

This result confirms the correctness of our interpretation of the phenotypes obtained in the ant-r X + type crosses and clearly demonstrates mitotic segregation and consequently the involvement of cytoplasmic factors in the determination of resistance and sensitivity to OLI and RHG.

Unfortunately, but expectedly, no confirmation can be obtained from these crosses concerning mitotic segregation

TABLE 6

SEGREGATION OF PHENOTYPES AMONG VEGETATIVE DIPLOID CLONES: (ant-r X ant-r type crosses)

	PHENOTYPIC CLASSES OBSERVED FOR EACH DRUG TESTED AND NUMBER OF CLONES IN EACH PHENOTYPIC CLASS												NUMBER OF CLONES SCORED
	ERY		CAP		OLI		RHG		TCN		CHX		
	S*:R*		S:R		S:R		R:S		S:R		S:R		
CROSS 4A	37	63	--	100	39	61	39	61	--	100	--	100	100
CROSS 4B	--	100	--	100	--	100	--	100	--	100	--	100	100
CROSS 4C	100	--	--	100	100	--	100	--	--	100	--	100	100

*PHENOTYPES SYMBOLS: S = sensitive

R = resistant, confluent growth

for TCN. However, in so far as the interpretation of the diploid phenotypes for OLI, RHG and TCN were all based on the same sort of premises, and in so far as this interpretation is confirmed for OLI and RHG, we feel that it is very likely also correct for TCN.

The data presented in the preceding chapter clearly demonstrates that mitotic segregation of different phenotypes occur among the vegetative diploid progeny of [RHO^+] X [RHO^+] type crosses of the resistant mutant by a sensitive wild type strain, in the cases of the drug OLI and RHG and most likely in the case of TCN also. This in turn is evidence for the involvement of cytoplasmic genetic factors in the determination of the phenotypes for these three drugs.

The results of the [RHO^+] X [RHO^o] and [RHO^o] X [RHO^+] crosses further demonstrate that the cytoplasmic factors involved are eliminated or inactivated following treatment with ethidium bromide, concomitantly with the elimination of the mitochondrial genetic information. This, therefore, suggests the localization of these cytoplasmic factors on the mitochondrial genome. However, an alternative explanation, placing the cytoplasmic loci OLI-5, RHG-1 and TCN-1 on a cytoplasmic DNA species independent from mt.DNA but similarly sensitive to EB, cannot be ruled out at this stage.

In the following chapter, we will present evidence in support of the mitochondrial nature of loci OLI-5, RHG-1 and TCN-1.

CHAPTER V

DEMONSTRATION OF THE LOCALIZATION OF CYTOPLASMIC LOCI OLI-5, RHG-1, AND TCN-1 ON THE MITOCHONDRIAL GENOME

Recombination of Phenotypes among Vegetative Diploid Clones in ant-r [RHO^+] X + [RHO^+] crosses:

The question of the localization of the cytoplasmic loci OLI-5, RHG-1 and TCN-1 on the mt.DNA or on a different DNA species is amenable to genetic analysis. Indeed, the demonstration of genetic linkage or independence between, on the one hand, loci OLI-5, RHG-1 and TCN-1 and, on the other hand, well characterized mitochondrial loci would constitute good evidence for or against the localization on mt.DNA of the former three loci. We have performed such a genetic analysis by determining the recombination frequencies between loci OLI-5, RHG-1 and TCN-1 and the two mitochondrial loci ERY and CAP present in the strains we have been using.

The frequencies of the various phenotypic classes occurring among the diploid progeny of cross S3B-CR [RHO^+] X ZIEK27-D [RHO^+] were determined and the frequencies of recombination between the various cytoplasmic loci were derived from this data. The results of this analysis are presented in tables 7 and 8. As there are 32 theoretically possible different phenotypes in the cross: [ERY-R CAP-R OLI-5R RHG-1S TCN-1R-1] x [ERY-S CAP-S OLI-5S RHG-1R TCN-1R-2], the data in table 7 have been presented, for reasons of clarity, as a

4 point cross involving ERY, CAP, RHG and TCN (table 7a), and two 3 point crosses involving ERY, CAP and OLI;and RHG, TCN and OLI, respectively (tables 7b, c). The observed recombination frequency for each gene pair, shown in table 8, corresponds to the frequency of the recombinant phenotypes for the particular gene pair, as derived from table 7a, b, and c. Table 8 also lists for comparaison the recombination frequencies expected on the basis of random assortment. The latter frequencies were derived from the frequency with which each allele occurs among the diploid progeny of the cross.

Although the observed recombination frequencies cannot be extrapolated into actual map distances, they can, however, be used as a qualitative indication of genetic linkage by comparing them to the recombination frequencies expected on the basis of random assortment. The data in table 8 therefore clearly demonstrates that the cytoplasmic loci RHG-1 and TCN-1 are genetically linked to the mitochondrial loci ERY and CAP and thus are themselves mitochondrial loci.

The recombination frequencies between OLI-5 and the four other markers are, in all cases, approximately 20%. This value is close to the upper limit of recombination observed for mitochondrial genes, and is generally interpreted as indicative of unlinked genetic markers which are, nevertheless, carried by the same mt.DNA molecule (Dujon _et al_., 1974). Therefore, although a doubt may persist as to the localization of the OLI-5 locus, it is likely that it is also on the mitochondrial genome.

TABLE 7

RECOMBINATION OF PHENOTYPES AMONG VEGETATIVE DIPLOID CLONES DERIVED FROM CROSS 2A (ant-r [RHO$^+$] X + [RHO$^+$])

7a. Expected phenotypic classes and their observed frequencies, for the four drugs: ERY, CAP, RHG and TCN, listed in this order.

Phenotypes								Number of clones scored
RRSS	RRSR	RRRS	RRRR	RSSS	RSSR	RSRS	RSSR	
72	1	8	4	1	0	6	13	
SRSS	SRSR	SRRS	SRRR	SSSS	SSSR	SSRS	SSRR	
6	4	3	4	0	0	24	245	391

7b. Expected phenotype classes and their observed frequencies, for the three drugs: ERY, CAP and OLI, listed in this order.

Phenotypes								Number of clones scored
RRR	RRS	RSR	RSS	SRR	SRS	SSR	SSS	
71	14	7	13	9	8	47	222	391

TABLE 7 (cont'd.)

7c. Expected phenotypic classes and their observed frequencies, for the three drugs: RHG, TCN and OLI, listed in this order.

Phenotypes								Number of clones scored
RRS	RRR	RSR	RSS	SRR	SRS	SSS	SSR	
218	48	18	23	2	3	13	66	391

The cross analyzed in this table is essentially a 5 point genetic cross, with the two parents having the following cytoplasmic genotypes: S3B-CR [ERY-R CAP-R RHG-1S TCN-1R-1 OLI-5R RHO$^+$] X Z1EK27-D [ERY-S CAP-S RHG-1R TCN-1R-2 OLI-5S RHO$^+$]. For clarity, we have used the following symbols in reference to the phenotypes described in Table 5: Erythromycin resistant = R; Erythromycin sensitive = S; Chloramphenicol resistant = R; Chloramphenicol semi-resistant = S; Rhodamine 6G sensitive with resistant papillae = R; Rhodamine sensitive = S; Oligomycin sensitive with resistant papillae = R; Oligomycin sensitive = S; Tetracycline semi-resistant = R; Tetracycline sensitive with resistant papillae = S. Therefore, the parental phenotype classes in Table 7a, b and c are respectively: RRSS and SSRR, RRR and SSS, and RRS and SSR. All other classes represent all the possible recombinant phenotypes.

TABLE 8

% RECOMBINATION BETWEEN THE CYTOPLASMIC MARKERS ERY, CAP, RHG-1, TCN-1 and OLI-5, TAKEN PAIRWISE

(as derived from the data in Table 7)

GENE PAIR	% RECOMBINATION OBSERVED	% RECOMBINATION EXPECTED*
ERY - CAP	9.5 (37)**	38.9 (152)
ERY - RHG-1	10.5 (41)	36.7 (143)
CAP - RHG-1	5.1 (20)	36.1 (141)
ERY - TCN-1	13.0 (51)	41.3 (161)
CAP - TCN-1	11.2 (44)	40.9 (160)
RHG-1 - TCN-1	11.8 (46)	39.0 (152)
ERY - OLI-5	21.2 (83)	42.6 (166)
CAP - OLI-5	19.4 (76)	42.3 (165)
RHG-1 - OLI-5	21.0 (82)	40.7 (159)
TCN-1 - OLI-5	22.0 (86)	43.9 (172)

*The data appearing in this column were derived from the frequency with which each allele occurs among the diploid progeny.

**Numbers in parentheses represent the observed or expected number of recombinant clones for a particular gene pair.

RECOMBINATION OF PHENOTYPES AMONG VEGETATIVE DIPLOID CLONES IN ant-r [RHO$^+$] X ant-r [RHO$^+$] CROSS:

A recombination analysis formally identical (with respect to cytoplasmic genotypes of parents) to the preceding one was performed on the diploid progeny of cross: S3B-CR [RHO$^+$] X I1C-CR [RHO$^+$], in which both parents carry the ant-r allele. Although this cross has advantages described in the preceding chapter, it eliminates loci TCN-1 and CAP from this analysis, since ant-r/ant-r homozygous diploids are all fully resistant to both TCN and CAP (see table 6). Nevertheless, it still allows the determination of the recombination frequencies between loci ERY, RHG-1 and OLI-5.

The results of this analysis are shown in tables 9 and 10. It is clear that the observed recombination frequencies obtained here for the gene pairs ERY-RHG-1, ERY-OLI-5 and RHG-1 — OLI-5 are very similar to those obtained from the ant-r [RHO$^+$] X +[RHO$^+$] cross. These results therefore confirm our conclusions regarding the mitochondrial location of RHG-1 and the liklihood of a similar location for locus OLI-5.

LOSS OF OLI-5R IN PETITES OF THE RHO$^-$ TYPE:

It is important to attempt to provide further evidence for the localization of the OLI-5 locus on the mitochondrial genome. This obligation stems from the fact that most of the claims concerning the non-mitochondrial and/or episomal nature of mutations similar to ours have centered mainly on the

TABLE 9

RECOMBINATION OF PHENOTYPES AMONG VEGETATIVE DIPLOID CLONES DERIVED FROM CROSS 4A

(ant-r [RHO^+] X ant-r [RHO^+])

EXPECTED PHENOTYPIC CLASSES AND THEIR OBSERVED FREQUENCIES, FOR THE THREE DRUGS ERY, RHG AND OLI, LISTED IN THIS ORDER.

PHENOTYPES								NUMBER OF CLONES SCORED
RSR	RSS	RRR	RRS	SSR	SSS	SRR	SRS	
294	34	28	16	18	16	34	156	596

The cross analyzed in this table is a 3 point cross with the two parental strains having the following cytoplasmic genotypes: S3B-CR [ERY-R RHG-1S OLI-5R] X I1C-CR [ERY-S RHG-1R OLI-5S]. Therefore, the parental phenotypic classes are: RSR and SRS. All other classes represent all the possible recombinant phenotypes.

TABLE 10

% RECOMBINATION BETWEEN THE CYTOPLASMIC MARKERS ERY, RHG-1, and OLI-5, TAKEN PAIRWISE (as derived from data in table 9).

GENE PAIR	% RECOMBINATION OBSERVED	% RECOMBINATION EXPECTED*
E - R	13.1 (78)**	47.6 (284)
E - O	17.1 (102)	47.2 (282)
R - O	18.8 (112)	47.6 (284)

*The data appearing in this column were derived from the frequency with which each allele occurs among the diploid progeny.

**Numbers in parentheses represent the observed or expected number of recombinant clones for a particular gene pair.

genetic and other characteristics of the resistance to OLI in such cross resistant mutants (Avner *et al.*, 1973b; Guerrineau *et al*;, 1974; 1976a and 1976b).

Such supporting evidence can be provided through a careful genetic analysis of the fate of the OLI-5 locus in petites of the [RHO$^-$] type. These petites, in contrast to the [RHO^o] type, which lack detectable mt.DNA, have, by definition, retained some mt.DNA.

Although the [RHO$^+$]$\longrightarrow$[RHO$^-$] mutational event is not yet fully understood in molecular terms, it is clearly demonstrated now that in established [RHO$^-$] mutants large segments of the wild type mitochondrial genome have been deleted and that the remaining DNA sequences are reiterated (Faye *et al.*, 1973; Locker *et al.*, 1974a, b). That the retained sequences have conserved meaningful genetic information is evidenced by the possibility of rescuing mitochondrial genetic markers from [RHO$^-$] strains through [RHO$^-$] X [RHO$^+$] crosses (Deutsch *et al.*, 1974).

This situation has often been exploited in locating cytoplasmic loci on the mt.DNA, by inducing the [RHO$^-$] mutation and demonstrating, in rescue experiments, the concomitant loss of the genetic locus under study in some [RHO$^-$] petites and its retention in others (Coen *et al.*, 1970). The results we have reported in the preceding chapters already suggested the concomitant loss of the OLI-5R locus and mt.DNA upon treatment with ethidium bromide. But it could be argued that both the extent of the EB treatment and its possible lack of specificity does not preclude the localization of the OLI-5R locus on a cytoplasmic DNA species other than mt.DNA, but which is also sensitive to the EB treatment. Therefore it would be of greater value to analyze the fate of the OLI-5R gene in petites induced by a possibly more specific technique. We have attempted to do this by taking advantage of the presence in mutant strain RD35-CR of a nuclear temperature sensitive mutation, pet-ts. Upon growth at 37°C, cells carrying the pet-ts mutation are irreversibly converted to the petite state. The kinetics of this conversion are shown in figure 2. Although the mechanism of this conversion has not been investigated in great detail, it appears that growth at 37°C leads to the loss of mitochondrial genetic information, possibly through physical alterations or loss of mt.DNA sequences. This is suggested by the loss of a series of mit^- mitochondrial markers (Slonimsky _et_ _al_., 1976) as well as the loss of ERY-R and CAP-R in a proportion of petites induced by growth at 37°C, as shown by the data in table 11.

Table 11 also demonstrates that the fate of OLI-5R parallels that of the mitochondrial markers in that some of these petites have also lost the OLI-5R marker. This result argues in favor of the OLI-5R gene being a mitochondrial marker.

Admittedly, the absolute validity of this conclusion depends on whether the effect of the pet-ts mutation is specific to mit.DNA or whether it can also lead to the loss

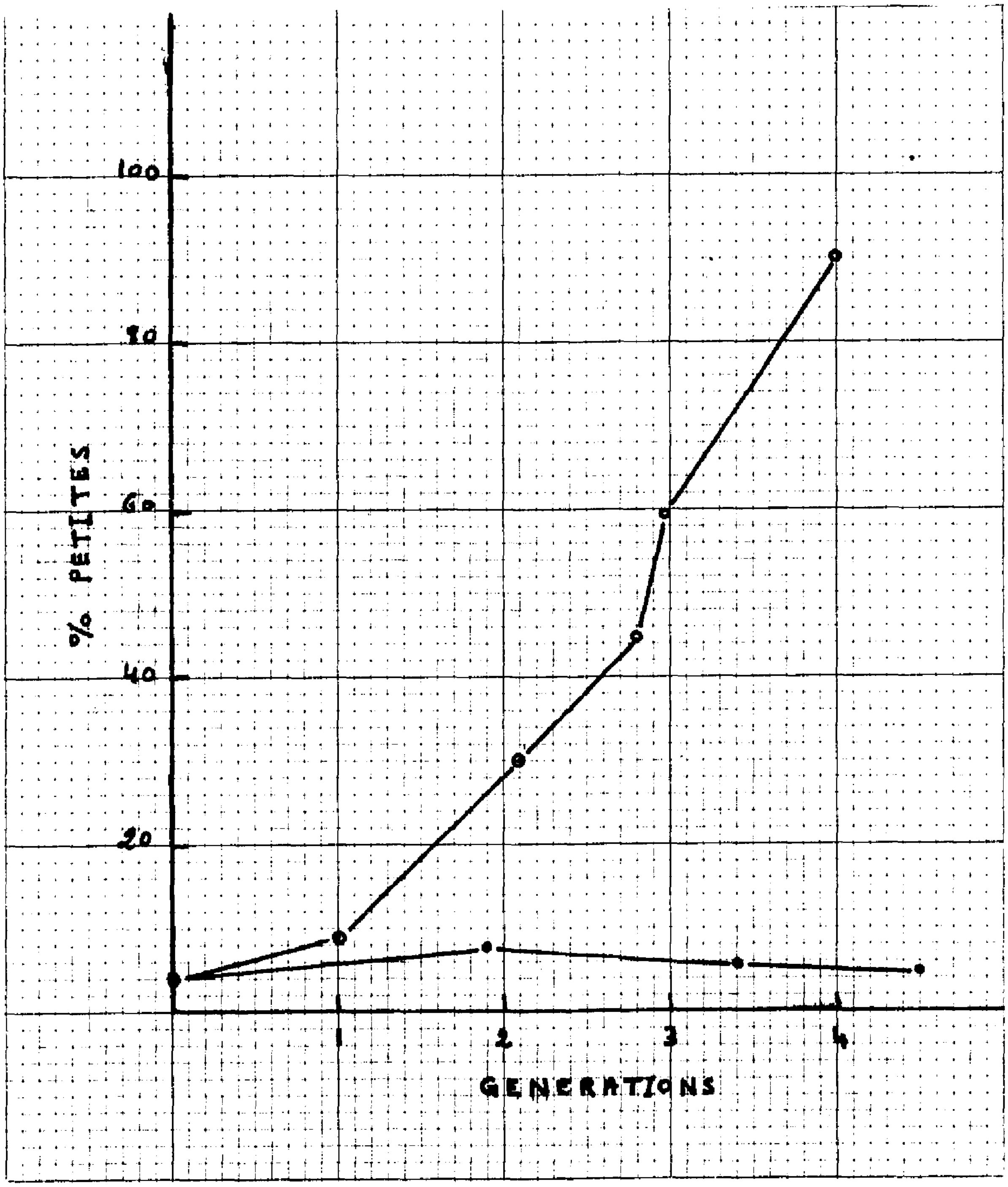


Fig. 2 - Kinetics of the induction of petites in strain RD35-CR, grown in YEPD medium, at 25°C (•—•) and at 37°C (o—o).

FIGURE 2 (Legend)

RD35-CR cells were inoculated into 10 ml of liquid YEPD medium (approximately 2 X 10^6 cells/ml) incubated at 25° with shaking until the increase in OD590 mm indicate 1 one doubling. The culture was then quickly equilibrated to 37°C and shifted to growth at 37°, with shaking. At various times after the temperature shift .1 ml aliquots were collected, diluted, plated on YEPD plates and the plates incubated at 25°C. Calories growing on these plates were then replica plated onto YEPGE plates to determine the % of petites.

The number of generations at each time point was derived from the formula $G_x = 3.3 \text{ X } \log \frac{N_x}{N_o}$ when G_x is the number of generations at time x, N_x the concentration of cells at time x and N_o the initial cell concentration (at the shift to 37°C).

TABLE 11

LOSS AND RETENTION OF MITOCHONDRIAL MARKERS AND OF MARKER OLI-5R among [RHO$^-$] PETITES INDUCED AT 37°C IN STRAIN RD35-CR

MARKER	NUMBER OF PETITES HAVING LOST MARKER	NUMBER OF PETITES HAVING RETAINED MARKER
M9-3	32	68
M5-121	84	16
M5-60	80	20
M6-239	52	48
M6-200	35	65
M7-40	40	60
CAP-R	61	39
ERY-R	43	57
OLI-5R	74	26

100 petites were obtained by growing strain RD35-CR at 37°C for approximately 1.5 generations (20% petites). Loss or retention of the mit$^-$ markers was determined by performing a complementation test between the petites and each of 6 mit$^-$ mutants kindly supplied by Dr. R.B. Needleman. This was done by crossing the petites to the mit$^-$ mutants and determining whether or not grande (complementing) diploids can be obtained from these crosses: Loss or retention of CAP-R, ERY-R and OLI-5R was determined by crossing each petite to strain Z1EK-27D ([CAP-S ERY-S OLI-5S]) and determining whether or not the resulting grande diploids are sensitive or resistant to CAP, ERY and OLI.

of other cytoplasmic DNA species as well. Nevertheless the result obtained here must be interpreted in conjunction with the body of evidence presented previously and pointing toward a mitochondrial location for the OLI-5R locus (loss upon EB treatment and linkage to known mitochondrial loci).

In summary, the data presented in this chapter demonstrate the localization of the cytoplasmic loci RHG-1 and TNC-1 on the mt.DNA and strongly suggest a similar location for locus OLI-5.

CHAPTER VI

MAPPING OF THE ant-r LOCUS AND ANALYSIS OF CROSSES BETWEEN OUR MUTANT AND TWO SIMILAR MUTANTS REPORTED BY OTHERS

MAPPING OF THE ant-r LOCUS:

The segregation of the ant-r and + alleles of the nuclear gene during meiosis can be followed by scoring the ascospore clones for resistance or sensitivity to the drugs CHX and TCN, since the phenotypes for these two drugs always segregate in a 2:2 fashion in all tetrads. It is therefore possible to map the ant-r locus relative to the other nuclear markers present in the crosses, by determining the relative frequencies of the various types of tetrads obtained for any given pairwise combination of markers. The results of such an analysis, for the two markers ant-r and leu-1 are reported in table 12.

The obvious inequality, PD $\gg$ NPD, indicates linkage between these two genes. The distance between them can be derived from the equation:

$$x = \frac{100 \text{ X } (TT + 6NPD)}{2 \text{ X } (TT + PD + NPD)}$$

Where X is the distance, in centimorgans, (cM), between the two genes (Perkins, 1949). In this instance, $x = 3.3$cM.

In addition, we have attempted to determine the position of the ant-r locus relative to the centromere-linked

TABLE 12

MAPPING OF THE ant-r LOCUS

Segregation of leu-1/+ and ant-r/+ in complete and true tetrads*	Type of tetrads	Number of tetrads of each type
2 leu-1 ant-r : 2 + +	PD	223
2 leu-1 + : 2 + ant-r	NPD	0
1 leu-1 ant-r : 1 leu-1 + : 1 + ant-r : 1 + +	TT	16

*The data in this table represents the pooled results of tetrad analyses of random diploids derived from several different crosses. However, in all these crosses the two markers involved here were in a cis configuration, i.e. ant-r leu-1 X + +.

leu-1 gene as well as to the centromere itself. This was done by determining the fate of the leu-1 locus among mitotic recombinants for the ant-r locus (ant-r/ant-r). Individual resistant diploid papillae, putative mitotic recombinants or gene convertants, were picked from the selective (drug) media on which they occured and were purified on the same selective media. A total of thirty independently occuring resistant papillae were selected from various drug plates and from various crosses. They were then tested for their resistance or sensitivity to all the other drugs and for their leucine requirement. In addition, twelve representative clones among these thirty were sporulated, dissected and subjected to tetrad analysis. The results of this work appear in tables 13 and 14. The salient features of these results are:

a. All thirty papillae, regardless of the drug plate or cross from which they arose, are fully resistant to CHX, TCN and CAP.

b. A proportion of these papillae (13/30) are anxotrophic for leucine (they grow on YNBD + leucine but not on YNBD), although the diploids from which they arose were all heterozygous leu-1/+ and, therefore, had no requirement for leucine.

c. Tetrad analysis of the 12 selected papillae produce 4:0 segregations for resistance:sensitivity to CHX, CAP and TCN, and either 4:0 or 0:4 segregations for OLI and RHG.

TABLE 13

GENETIC ANALYSIS OF RESISTANT PAPILLAE: CROSS RESISTANCE AND LEUCINE REQUIREMENT

	ORIGINS OF PAPILLAE:		PHENOTYPE OF DIPLOID PATCH FROM WHICH PAPILLAE ORIGINATE	PHENOTYPE OF PAPILLAE:						
PAPILLAE#	CROSS	DRUG PLATE		YNBD	YNBD + leu	CHX	CAP	TCN	THG	OLI
1	1A	RHG	CHX^{SP} CAP^{SR} TCN^{SR} RHG^{SP} OLI^{S}	-	+	R	R	R	R	S
2	"	"	"	-	+	R	R	R	R	S
3	"	"	"	+	+	R	R	R	R	S
4	"	"	"	+	+	R	R	R	R	S
5	"	"	"	+	+	R	R	R	R	S
6	"	CHX	"	-	+	R	R	R	R	S
7	"	"	"	+	+	R	R	R	R	S
8	"	"	"	+	+	R	R	R	R	S
9	"	OLI	CHX^{SP} CAP^{R} TCN^{SP} RHG^{S} OLI^{SP}	-	+	R	R	R	S	R
10	"	"	"	+	+	R	R	R	S	R
11	"	"	"	-	+	R	R	R	S	R
12	"	CHX	"	-	+	R	R	R	S	R

TABLE 13 (cont'd.)

	ORIGINS OF PAPILLAE:		PHENOTYPE OF DIPLOID PATCH FROM WHICH	PHENOTYPE OF PAPILLAE:						
PAPILLAE#	CROSS	DRUG PLATE	PAPILLAE ORIGINATE:	YNBD	YNBD + leu	CHX	CAP	TCN	RHG	OLI
13	"	"	"	+	+	R	R	R	S	R
14	"	"	"	+	+	R	R	R	S	R
15	"	"	"	+	+	R	R	R	S	R
16	"	"	"	+	+	R	R	R	S	R
17	1B	CHX	CHX^{SP} CAP^{R} TCN^{SP} RHG^{S} OLI^{SP}	–	+	R	R	R	S	R
18	"	"	"	+	+	R	R	R	S	R
19	"	"	"	+	+	R	R	R	S	R
20	"	OLI	"	+	+	R	R	R	S	R
21	"	"	"	+	+	R	R	R	S	R
22	"	"	"	–	+	R	R	R	S	R
23	"	"	"	+	+	R	R	R	S	R
24	1C	CHX	CHX^{SP} CAP^{SR} TCN^{SR} RHG^{SP} OLI^{S}	+	+	R	R	R	R	S
25	"	"	"	–	+	R	R	R	R	S
26	"	"	"	–	+	R	R	R	R	S

TABLE 13 (cont'd.)

	ORIGINS OF PAPILLAE:		PHENOTYPE OF DIPLOID PATCH FROM WHICH PAPILLAE ORIGINATE:	PHENOTYPE OF PAPILLAE:						
PAPILLAE#	CROSS	DRUG PLATE		YNBD	YNBD + leu	CHX	CAP	TCN	RHG	OLI
27	"	"	"	–	+	R	R	R	R	S
28	"	RHG	"	+	+	R	R	R	R	S
29	"	"	"	–	+	R	R	R	R	S
30	"	"	"	–	+	R	R	R	R	S

TABLE 14

GENETIC ANALYSIS OF RESISTANT PAPILLAE: TETRAD ANALYSIS OF SELECTED RESISTANT PAPILLAE:

Papillae#	Segregation of drug resistance:sensitivity (R:S) and of leucine prototrophy:auxotrophy (+:-)						#Tetrad analysed
	LEU	CAP	CHX	TCN	RHG	OLI	
	+:-	R:S	R:S	R:S	R:S	R:S	
1	0:4(10)*	4:0(10)	4:0(10)	4:0(10)	4:0(10)	0:4(10)	10
10	2:2(8)	4:0(8)	4:0(8)	4:0(8)	4:0(8)	4:0(8)	8
11	0:4(10)	4:0(10)	4:0(10)	4:0(10)	0:4(10)	4:0(10)	10
12	0:4(10)	4:0(10)	4:0(10)	4:0(10)	0:4(10)	4:0(10)	10
13	2:2(9)	4:0(9)	4:0(9)	4:0(9)	0:4(9)	4:0(9)	9
17	0:4(10)	4:0(10)	4:0(10)	4:0(10)	0:4(10)	4:0(10)	10
18	2:2(9)	4:0(9)	4:0(9)	4:0(9)	0:4(9)	4:0(9)	9
20	2:2(8)	4:0(8)	4:0(8)	4:0(8)	0:4(8)	4:0(8)	8
21	2:2(9)	4:0(9)	4:0(9)	4:0(9)	0:4(9)	4:0(9)	9
22	0:4(8)	4:0(8)	4:0(8)	4:0(8)	0:4(8)	4:0(8)	8
25	0:4(6)	4:0(6)	4:0(6)	4:0(6)	4:0(6)	0:4(6)	6
29	0:4(8)	4:0(8)	4:0(8)	4:0(8)	4:0(8)	0:4(8)	8

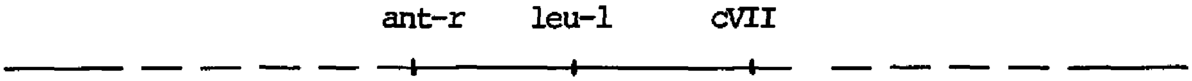
TABLE 14 (cont'd.)

*Numbers in parentheses represent the number of tetrads displaying the segregation ratio which they follow.

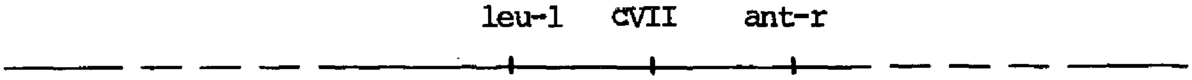
d. All 7 leucine requiring papillae dissected segregate 4:0 for leu-1:+. The remaining 5 papillae, all prototrophic for leucine, segregate 2:2 for leu-1:+.

Taken together, these results confirm the assumption we made earlier in this work (Chapter IV) that the resistant papillae are homozygous ant-r/ant-r. The fact that, among these ant-r/ant-r homozygotes, several also involved the conversion of the leu-1 locus to a homozygous state, leu-1/leu-1, argues strongly in favor of mitotic recombination or gene conversion being the event responsible for their production. The data also demonstrate that this event (the conversion of ant-r/+ to ant-r/ant-r) does indeed occur randomly among the diploid progeny of <u>all</u> crosses. Therefore, the absence of resistant papillae in certain crosses and for certain drugs (RHG and OLI) is not due to the failure of mitotic recombination, or gene conversion to occur in these particular crosses and for these particular drugs.

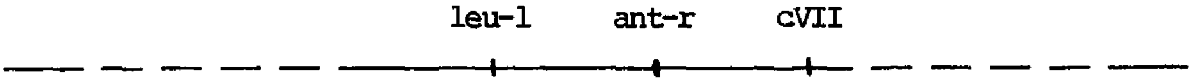
If it is accepted that the event leading to the occurence of resistant papillae is in fact the production of ant-r/ant-r homozygotes by mitotic recombination or gene conversion, the significant observation relevant to the mapping of the ant-r locus is the simultaneous conversion, in approximately half the cases, of the closely-linked leu-1 locus to a homozygous leu-1/leu-1 state. Indeed, this result is consistent only with the following order for the three genetic markers ant-r, leu-1 and cVIII (centromere of chromosome VII):



The other two possible orders:



or:



would have produced, among ant-r/ant-r mitotic recombinants or gene convertants, predominantly leu-1/+ or predominantly leu-1/leu-1 diploids, respectively.

CROSSES BETWEEN OUR MUTANT AND TWO SIMILAR MUTANTS REPORTED BY OTHERS:

Our interpretation of the genetic basis of the cross-resistant phenotype in our mutant is clearly in contradiction with some of the claims made in the literature concerning the genetic basis of mutants similar to ours, particularly the claim made by Guerineau et al. (1974, 1976a and b) concerning the episomal nature of their mutation. This discrepancy may of course be the result of a basic genetic difference between their mutant and ours. On the other hand, the many similarities (phenotypic as well as genetic) between these mutants seem to argue in favor of a common genetic basis for all of them. It was therefore of interest to try to determine whether or not our mutant indeed shares some common basic genetic feature with any of the mutants reported by others, and for which a different genetic interpretation had been given.

Two such mutants, the one reported by Rank _et_ _al_. (1973) and that isolated by Guerineau _et_ _al_. (1974) were kindly made available to us by Dr. Julius Marmur. These are strain 332-7c, carrying the oli^{PR1} mutation (Rank _et_ _al_., 1973) and strain KL9D which carries the drug resistance mutation described by Guerineau _et_ _al_. (1974). Both strains were crossed to our strain 28D-CR, a meiotic segregant from the cross S3B-CR [RHO$^+$] X Z1EK27-D [RHO$^+$], which carries the ant-r mutation and has the drug resistance phenotype shown in table 15. The diploids obtained from these crosses were analyzed with respect to their drug resistance phenotype and were further subjected to tetrad analysis, in an attempt to detect any genetic similarity between our mutation and those present in strains 332-7C and KL9D. The results of this analysis appear in tables 15 and 16. Clearly the diploids phenotypes and the tetrad ratios for CHX, CAP and TCN indicate that both strains 332-7C and KL9D carry a nuclear mutation allelic (or heteroallelic) to our ant-r mutation. Therefore all three mutant strains share at least this

TABLE 15

CROSSES BETWEEN OUR MUTANT AND MUTANT STRAINS 332-7c and KL9D: Phenotypes of the haploid parental strains and of the diploids

PARENTAL STRAIN OR DIPLOID	DRUG RESISTANCE PHENOTYPE				
	CHX	CAP	TCN	RHG	OLI
28D-CR	R	R	R	R	R
332-7c	R	R	R	R	R
KL9D	R	R	R	R	S
28D-CR X 332-7c(50)*	R	R	R	R	R
28D-CR X KL9D(50)*	R	R	R	R	R

*Number of individual diploid colonies tested

TABLE 16

CROSSES BETWEEN OUR MUTANT AND MUTANT STRAINS 332-7c and KL9D :

Tetrad analysis of random diploids:

CROSS	#TETRADS* ANALYSED	SEGREGATION OF RESISTANCE:SENSITIVITY (R:S) IN TETRADS				
		CHX	CAP	TCN	RHG	OLI
		R:S	R:S	R:S	R:S	R:S
28D-CR X 332-7c	15	4:0(15)**	4:0(15)	4:0(15)	4:0(15)	4:0(10) 2:2(5)
28D-CR X KL9D	16	4:0 (16)	4:0(16)	4:0)16)	4:0(5) 2:2(2) 1:3(9)	2:2(13) 1:3(1) 0:4(2)

*Only true and complete tetrads are reported here.

**Numbers in parentheses represent the number of tetrads showing the R:S ratio indicated.

genetic element with regard to their drug resistance phenotype.

However, no simple explanation can account for the tetrad ratios obtained for RHG and OLI. In the cross 28D-CR X 332-7c, in all 5 tetrads showing 2:2 segregations for OLI resistance:sensitivity the 2 sensitive spores are also and always leucine negative. This result could be explained in terms of nuclear-mitochondrial interaction if the following assumptions are made, (keeping in mind the fact that, given the tight linkage between the ant-r locus and the leu-1 locus, the leu-1 spores most probably carry our ant-r mutation):

a. Strain 332-7c carries a nuclear mutation, ant-rx heteroallelic to our ant-r mutation.

b. Strain 332-7c carries a mitochondrial mutation OLI-5Rx, possibly heteroallelic to our OLI-5R mutation.

c. In the haploid condition, genotypes ant-r [OLI-5R]; ant-rx [OLI-5Rx] and ant-rx [OLI-5R] produce an oligomycin resistant phenotype, whereas genotype ant-r [OLI-5Rx] expresses an oligomycin sensitive phenotype.

d. The five tetrads giving 2:2 segregation result from diploids having inherited the mitochondrial OLI-5Rx genotype of strain 332-7c and would therefore have the following genotypes and phenotypes:

leu-1 ant-r [OLI-5Rx] (sensitive to OLI)
leu-1 ant-r [OLI-5Rx] (sensitive to OLI)

+ ant-rx [OLI-5Rx] (resistant to OLI)

+ ant-rx [OLI-5Rx] (resistant to OLI)

As for the segregations for RHG and OLI obtained in the case of the cross involving strain KL9D, the situation seems to be even more complex. It must be noted here that strain KL9D is not the original mutant described by Guerineau *et*. *al*. (1974), but a derivative which has lost resistance to OLI. We have not been able to establish its origin (meiotic segregant, partial revertant obtained, spontaneously or after mutagenic treatment, etc...). It is possible that the tetrad ratios obtained in this case are the result of interactions between the nuclear ant-r type mutations, other nuclear gene mutations affecting the phenotypic expression of these primary mutations (see following chapter for a description of two such mutations) and mitochondrial genes.

The data presented above do not provide a definitive answer as to the identity between the genetic phenomena underlying the drug resistant mutation in our strain and that underlying the mutation in strains 332-7c̄ and KL9D. However, they demonstrate that all three mutants share in common, and at the very least, a mutation of a nuclear nature at the locus we have designated ant-r. In addition we feel that nothing in these results is irreconcilable with a nuclear-mitochondrial interpretation of the drug resistant mutations in all three strains.

CHAPTER VII

ISOLATION AND GENETIC CHARACTERIZATION OF PARTIAL REVERTANTS OF THE CROSS RESISTANT MUTANT

The genetic nature of revertants of the cross resistance phenotype could have some bearing on the question of the genetic nature of the forward resistance mutation itself. Whether revertants could be demonstrated to be of a nuclear, mitochondrial or cytoplasmic nature, or related to the absence or physical alteration of 2μDNA, this would lend support, however circumstantial it may be, to one or the other of the several models proposed to explain the genetic basis of the forward resistance mutation. It is not surprising, therefore, that several workers in this field have reported the isolation of complete or partial revertants of their cross resistant mutants.

Guerineau _et_ _al_. (1974) report the isolation of revertants of their cross resistant mutant, DR19, (resistant to OLI, CAP, CHX, VEN* and TET**), having lost either OLI resistance only or CAP, CHX, and TET resistance, or OLI, CHX, CAP and TET resistance. Although it is stated that these revertants were derived either spontaneously or following EB treatment, it is not clear how each particular revertant

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*VEN = VENTURICIDIN

**TET = TRIETHYLTIN

was obtained. These authors make the claim that the revertants were due to the loss of either one or both of two *episomal* genetic elements, which they have designated π (pi) and τ (tau). Pi, it is claimed, confers resistance to OLI and VEN while τ confers resistance to all the other inhibitors. In addition, π is claimed to be identical or related to the 2μ circular DNA species. In support of these claims, the authors presented biochemical data purportedly demonstrating the physical absence of 2μ DNA in OLI sensitive revertants. However, no genetic analysis of any of the revertants is reported in either the publication cited above or in a subsequent paper dealing with the same subject (Guerineau *et al*., 1976b).

On the other hand, Rank *et al*., (1975) have obtained two spontaneous revertants of their cross resistant mutant. One showed a complete loss of the pleiotropic phenotype (sensitive to all 13 drugs to which the original mutant was resistant). Genetic analysis of this revertant demonstrated that it resulted from a back mutation of the nuclear $oli^{PR}1$ locus responsible for the resistance phenotype.

The other is a partial revertant, retaining resistance to OLI, but sensitive to all other drugs. Genetic analysis of this revertant indicated that it was most likely due to a mutation at a secondary locus, segregating independently of the $oli^{PR}1$ locus and suppressing resistance to CAP, CHX etc..., without altering resistance to OLI.

Finally, Griffiths et al. (1975) reported revertants of their VEN^R TET^R RHG^R mutant (Lancashire et al., 1975) having lost resistance to all three inhibitors. But an attempt by them to show a concomitant loss of 2μ DNA in these revertants failed, as 2μ DNA was found in all sensitive revertants analyzed. No further genetic analysis of these revertants was reported.

In an effort to extend further the genetic analysis of our drug resistant mutant, and possibly to gain more insight into its genetic basis, sensitive revertants were selected and subjected to genetic analysis in order to ascertain the genetic basis of the reversion. Two strains, RD35-CR and DRS8-CR, were utilized as starting points for the selection of revertants. No mutagenesis or special selection technique was employed. Revertants were selected simply by testing several thousand individual isolates for loss of resistance to one or more of the drugs. The two strains differ in their mitochondrial genotype (see table 2) and consequently in their phenotype. Strain RD35-CR has the genotype ant-r [ERY-R CAP-R OLI-5R RHG-1S TCN-1R1] and, therefore, is resistant to ERY, CAP, OLI, TCN, CHX but sensitive to RHG. Strain DRS8-CR, on the other hand, originates from the cross RD35-CR [RHO^o] X Z1EK27-D[RHO^+] and has the genotype ant-r [ERY-S CAP-S OLI-5S RHG-1R TCN-1R2] and is therefore resistant to CHX, CAP, TCN and RHG but sensitive to the other drugs. One revertant from each strain was analyzed.

PHENOTYPES OF THE REVERTANTS:

Revertant RD35-S1, derived from strain RD35-CR, has lost resistance to CHX but retains resistance to all other inhibitors (OLI, TCN, CAP and ERY).

Revertant DRS8-S2, derived from strain DRS8-CR, has lost resistance to CHX, CAP and RHG, but has retained resistance to TCN. In addition, this revertant has acquired resistance to OLI, which the parental strain DRS8-CR did not exhibit.

GENETIC ANALYSIS OF THE REVERTANTS

Both revertants were crossed to the sensitive wild type strain Z1EK27-D and random diploids were sporulated and subjected to tetrad analysis. In both cases, [RHO$^+$] X [RHO^o] and [RHO^o] X [RHO$^+$] crosses were performed in addition to the [RHO$^+$] X [RHO$^+$] cross, in order to detect any possible involvement of mitochondrial genes in the mechanism of the reversions.

The results of these tetrad analyses are presented in tables 17 and 18.

DISCUSSION OF THE TETRAD DATA:

a. Revertant RD35-S1: Although at first glance the tetrad data appear confusing, they are by no means uninterpretable. Consider the segregation of CHX resistance:sensitivity, in which case mitochondrial genes are not involved in determining the original resistant phenotype: the ratio

TABLE 17

GENETIC ANALYSIS OF REVERTANT RD35-S1*:

CROSS	# TETRADS** ANALYZED	TETRAD CLASSES	SEGREGATION OF RESISTANCE:SENSITIVITY FOR EACH OF DRUGS TESTED***					# TETRADS IN EACH CLASS
			TCN	OLI	CAP	RHG	CHX	
RD35-S1[RHO$^+$]	16	I	2:2	0:4	1:3	1:3	1:3	10
X		II	2:2	2:2	1:3	1:3	1:3	1
Z1EK27-D[RHO$^+$]		III	2:2	2:2	4:0	0:4	1:3	2
		IV	2:2	0:4	2:2	2:2	2:2	2
		V	2:2	2:2	0:4	0:4	0:4	1
RD35-S1[RHO$^+$]	8	III	2:2	2:2	4:0	0:4	1:3	7
X								
Z1EK27-D[RHO^o]		VI	2:2	2:2	4:0	0:4	2:2	1

TABLE 17 (cont'd.)

CROSS	# TETRADS** ANALYZED	TETRAD CLASSES	SEGREGATION OF RESISTANCE:SENSITIVITY FOR EACH OF DRUGS TESTED					# TETRADS IN EACH CLASS
			TCN	OLI	CAP	RHG	CHX	
RD35-S1[RHO^{o}]	25	I	2:2	0:4	1:3	1:3	1:3	20
X		IV	2:2	0:4	2:2	2:2	2:2	1
Z1EK27-D[RHO^{+}]		VII	2:2	0:4	0:4	0:4	0:4	4

*The phenotype of revertant RD35-S1 is TCN^{R} OLI^{R} CAP^{R} RHG^{S} CHX^{S}

**Only complete and true tetrads were recorded.

***Segregation of ERY resistance:sensitivity is not reported here, as it should not be and is not affected by either the ant-r mutation or reversion.

PD : TT : NPD tetrads (2:2, 1:3 and 0:4 respectively) is not significantly different from a 1:4:1 ratio ($\chi^2 = 4.9$, $P \sim .10$) and is therefore most simply explained by postulating that revertant RD35-S1 contains a single nuclear gene mutation, designated scr-1, suppressing CHX resistance and segregating independently of the ant-r mutation. The effect of scr-1 on the expression of resistance to the other drugs can be readily deduced from the examination of the types of segregation obtained in the [RHO$^+$] X [RHO^o] and [RHO^o] X [RHO$^+$] crosses:

Cross RD35-S1 [RHO$^+$] X Z1EK27-D [RHO^o] indicates that scr-1 does not suppress resistance to OLI as 2:2 segregation is the rule, regardless of what type of segregation is obtained for CHX. It is also clear that there is no suppression of TCN resistance either. However, nothing can be deduced from this cross regarding the effect of scr-1 on resistance to CAP and RHG.

In the former case, this is due to the presence of the mitochondrial CAP-R mutation resulting in 4:0 segregation in all tetrads of this cross. In the latter case, the absence of the RHG-1R gene, necessary in the first place for the expression of resistance to RHG, results in 0:4 tetrads and therefore precludes any analysis of the effect of scr-1 on RHG resistance.

On the other hand, cross RD35-S1 [RHO^o] X Z1EK27-D [RHO$^+$] reveals that scr-1 also suppresses the ant-r-dependent resistance to both CAP and RHG in addition to CHX. Indeed 2:2, 1:3, and 0:4 segregations are obtained for these two drugs, and the spore clones are always simultaneously resistant or simultaneously sensitive to the three drugs CHX, CAP and RHG.

In summary, the reversion in strain RD35-S1 is most likely the result of a secondary nuclear gene mutation, designated scr-1, segregating independently of ant-r. Mutation scr-1 suppresses the ant-r dependent resistance to CHX, CAP and RHG, but has no effect on resistance to TCN and OLI.

b. <u>Revertant DRS8-S2</u>: As pointed out earlier, this revertant has simultaneously lost resistance to CHX, CAP and RHG, and has acquired resistance to OLI. Here again, the tetrad data shown in table 18 are consistent with the presence in this revertant of a secondary nuclear gene mutation, designated scr-2, which suppresses resistance to CHX, CAP and RHG, has no effect on TCN resistance and segregates independently of ant-r ($\chi^2 = .46$; $P \sim .80$ for a PD : TT : NPD ratio of 1:4:1).

In addition, the analysis of the pattern of segregation of OLI resistance:sensitivity in spore clones, relative to the segregation of CHX, CAP and RHG resistance:sensitivity (table 19), is readily explained by postulating that the same scr-2 mutation is also responsible for the expression of ant-r specified OLI resistance in strain DRS8-S2 and in spore clones carrying the ant-r gene, although these strains and spore clones carry the OLI-5S mitochondrial allele.

TABLE 18

GENETIC ANALYSIS OF REVERTANT DRS8-S2*:

CROSS**	# TETRADS ANALYZED	TETRAD CLASSES	SEGREGATION OF RESISTANCE:SENSITIVITY FOR EACH OF THE DRUGS TESTED					# TETRADS IN EACH CLASS
			TCN	OLI	CAP	RHG	CHX	
DRS8-S2 X Z1EK27-D	44	I	2:2	1:3	1:3	1:3	1:3	28
		II	2:2	0:4	2:2	2:2	2:2	7
		III	2:2	2:2	0:4	0:4	0:4	9

*The phenotype of revertant DRS8-S2 is TCN^R OLI^R CAP^S RHG^S CHX^S

**In so far as the three crosses: [RHO$^+$] X [RHO$^+$], [RHO$^+$] X [RHO^o] and [RHO^o] X [RHO$^+$] gave homogeneous segregations and tetrad ratios, tetrad data were pooled.

TABLE 19

SEGREGATION OF RESISTANCE (R) : SENSITIVITY (S) IN INDIVIDUAL SPORE CLONES, IN TETRADS FROM THE CROSS DRS8-S2 X Z1EK27-D:

TETRAD CLASS*	SPORE CLONE	PHENOTYPE OF SPORE CLONE FOR EACH DRUG TESTED				
		CHX	CAP	RHG	OLI	TCN
I	A	R	R	R	S	R
	B	S	S	S	R	R
	C	S	S	S	S	S
	D	S	S	S	S	S
II	A	R	R	R	S	R
	B	R	R	R	S	R
	C	S	S	S	S	S
	D	S	S	S	S	S

TABLE 19 (cont'd.)

TETRAD CLASS*	SPORE CLONE	PHENOTYPE OF SPORE CLONE FOR EACH DRUG TESTED				
		CHX	CAP	RHG	OLI	TCN
III	A	S	S	S	R	R
	B	S	S	S	R	R
	C	S	S	S	S	S
	D	S	S	S	S	S

*Tetrad classes correspond to those described in table 18.

MAPPING OF scr-1 AND scr-2:

In the tetrads listed in tables 17 and 18, the suppressor genotype, i.e. scr-1 or + and scr-2 or +, of some of the spore clones can be determined. In 1:3 tetrads only two of the spore clones can be genotyped; whereas all four spore clones can be genotyped in 2:2 and in 0:4 tetrads. These genotypes can be deduced from the phenotype of the spore clones for TCN and CHX. For example, a TCN^R CHX^R spore clone is necessarily + (wild type) for the suppressor gene. A TCN^R CHX^S spore clone is necessarily of genotype scr-1 (or scr-2). Although this is not sufficient to allow the mapping of scr-1 and scr-2 by tetrad analysis (1:3 tetrads are not completely genotyped), a large enough number of spore clones can be genotyped in this way to permit mapping by random spore analysis. For any pair of markers, the frequency of recombinant spore clones among the total number of spore clones scored represent the distance, in centimorgans, between the two markers. The results of such an analysis, for the gene pairs scr-1 — ade-2 and scr-2 — ade-2, are presented in table 20. For comparison, and as a confirmation of the legitimacy of this random spore technique, recombination frequencies between the two independent markers lys-1 and ade-2 is also reported, as well as the recombination frequencies between scr-1 and lys-1 and scr-2 and lys-1. These results clearly indicate that both scr-1 and scr-2 are linked, although loosely, to the ade-2 marker

TABLE 20

MAPPING OF LOCI scr-1 and scr-2:

CROSS	GENE PAIR	TOTAL # OF SPORE CLONES ANALYZED	# OF RECOMBINANT SPORE CLONES	% RECOMBINATION
RD35-S1*	scr-1 -- ade-2	116	18	15.5
X	scr-1 -- lys-1	116	56	48.3
Z1EK27-D	ade-2 -- lys-1	196	99	50.5
DRS8-S2**	scr-2 -- ade-2	120	20	16.7
X	scr-2 -- lys-1	120	53	44.2
Z1EK27-D	ade-2 -- lys-1	176	83	47.2

*Parental genotypes for the relevant genes are: scr-1 ade-2 X lys-1

**Parental genotypes for the relevant genes are: scr-2 ade-2 X lys-1

TABLE 21

ALLELE TESTING OF MUTATIONS scr-1 and scr-2: TETRAD ANALYSIS OF DIPLOIDS DERIVED FROM CROSS T18D-S1 X T32C-S2

	SEGREGATION OF CHX RESISTANCE:SENSITIVITY
4 spored Asci (31)*:	0:4 (31)
3 spored Asci (8) :	0:3 (8)

*Numbers in parentheses indicate the number of Asci of the type described.

on chromosome XV. The distance between the markers is, in both cases, approximately 16 cM.

ALLELE TESTING OF scr-1 AND scr-2:

Although not identical, the effects of both scr-1 and scr-2 on the pleiotropic phenotype are very similar. In addition, the recombination frequencies (distance) between each of these markers and the ade-2 locus are practically identical. These observations suggest that the two suppressor mutations may in fact be heteroalleles of a unique genetic locus. This possibility was investigated by allele testing scr-1 and scr-2.

The following two strains were constructed:

T18D-S1, of genotype α ade-2 leu-1 ant-r scr-1 and T32C-S2, of genotype a lys-1 leu-1 ant-r scr-2. Diploids resulting from the cross T18D-S1 X T32C-S2, selected on YNBD + leucine plates, are sensitive to CHX, i.e. scr-1 and scr-2 do not complement each other. This result strongly suggests that scr-1 and scr-2 are heteroallelic. However, it might be argued that the same result would have been obtained even if scr-1 and scr-2 were non-allelic, provided either one or both of them were dominant. Therefore, confirmation of the allelism of scr-1 and scr-2 was sought by tetrad analysis of random diploids derived from the above cross. The results of this tetrad analysis appear in table 21.

All 39 asci analyzed segregated 0:4 (or 0:3 in the case of 3 spored asci) for CHX resistance:sensitivity, demonstrating that scr-1 and scr-2 are indeed allelic mutations.

SUMMARY:

Two phenotypically similar, but not identical, partial revertants of our cross resistant mutant were isolated and genetically characterized. Each revertant is the result of a single nuclear gene mutation, scr-1 or scr-2, suppressing part of the pleiotropic phenotype and segregating independently of the ant-r locus. Both are located on chromosome XV, approximately 16 cM from the ade-2 locus and allele testing demonstrates that they are very likely heteroalleles of the same genetic locus.

Phenotypically and genetically, both are similar to one of the two revertants reported by Rank et al., (1975).

Although these results do not disprove the episomal hypothesis advanced by Guerineau et al. (1974 and 1976b), they appear to be more in line with the model of nuclear-mitochondrial gene interaction which we have presented in preceding chapters.

Possibly, the most useful purpose of the genetic analysis of revertants, described in this chapter, is to illustrate the genetic complexity inherent to this system. Several nuclear gene mutations and several mitochondrial mutations are interacting here to control and affect in different ways different facets of a pleiotropic phenotype. This complexity may very likely be the source of the conflicting interpretations and claims which have been made with regard to this type of mutant.

CHAPTER VIII

DISCUSSION

In this thesis, we have reported the results of the genetic analysis of a mutant of Saccharomyces cerevisiae, cross-resistant to several structurally and functionally unrelated drugs. The data presented show that the resistance of our mutant to the five drugs, chloramphenicol, tetracycline, oligomycin, rhodamine 6G and cycloheximide, is primarily determined by a single nuclear gene mutation designated ant-r. In addition to the ant-r mutation, several mitochondrial genes are also required for expression of resistance to oligomycin, tetracycline and rhodamine 6G.

The evidence of the involvement of a nuclear gene is indisputable and comes from the tetrad analyses reported in chapter III. Several crosses of strains carrying the cross-resistance mutation to a sensitive wild type strain always yield 2:2 meiotic segregation ratios for resistance: sensitivity to cycloheximide, chloramphenicol and tetracycline. The patterns and ratios of segregations obtained for the remaining two drugs, oligomycin and rhodamine 6G, suggested that the resistance phenotype for these two drugs resulted from the interaction of the same nuclear ant-r mutation with several cytoplasmic genes.

Our ability to demonstrate genetic linkage between the ant-r marker and a known nuclear locus, leu-1, and the subsequent mapping of the ant-r gene on chromosome VII, leaves no doubt as to the nuclear nature of this gene.

The involvement of mitochondrial genes in determining the resistance to oligomycin, rhodamine 6G and tetracycline, is supported by several lines of evidence:

Firstly, vegetative diploids derived from crosses of the type: resistant mutant [RHO$^+$] X sensitive wild type [RHO$^+$] display mitotic segregation for oligomycin, rhodamine 6G and tetracycline resistance:sensitivity. This establishes the cytoplasmic nature of the genetic factors involved, in conjunction with the nuclear gene, in the expression of resistance to these drugs.

Secondly, the elimination of these cytoplasmic factors concurently with the elimination of mt. DNA and the establishment of the [RHO^o] state, following treatment with ethidium bromide, suggests that these factors are in effect associated with mt. DNA or alternatively are dependent for their expression, on mitochondrial functions.

Finally, conclusive evidence for the actual localization of these cytoplasmic loci on the mt. DNA was provided by the demonstration of their genetic linkage to two well characterized mitochondrial markers, ERY-R and CAP-R. The three new mitochondrial loci thus characterized were designated RHG-1, TCN-1 and OLI-5. In the case of locus OLI-5, additional supportive evidence of its mitochondrial location was obtained. Petites of the [RHO$^-$] type, induced via the pet-ts mutation, lose or retain the OLI-5R mutation, concurently with the loss or retention of other mitochondrial genetic markers.

The analysis of two revertants of the mutant phenotype, does not reveal any anomalous genetic phenomenon involved in the loss of resistance. Both revertants were unambiguously characterized as single nuclear gene mutations, segregating independently of the ant-r gene, and partially suppressing the pleiotropic resistance phenotype.

Our findings are clearly in disagreement with interpretations and claims, advanced by others, concerning the possible genetic basis of mutations expressing a cross-resistant phenotype similar to that of our mutant. In particular, the claim that such mutations are of an episomal nature (Guerineau et al., 1974; 1976b), and the interpretation that similar mutations are the result of an interaction between nuclear and non-mitochondrial cytoplasmic factors (Avner et al., 1973b; Lancashire et al., 1975; Griffiths et al., 1977), are contradicted by our work. It could, of course, be argued that these discrepancies result from basic genetic differences between our mutant and those reported by others. However, there are striking similarities between the phenotypes and genetic "peculiarities" displayed by all these mutants. This observation seems to argue in favor of the hypothesis that these mutants probably represent a genetically homogeneous class. We have reported in chapter VI the results of the analysis of crosses performed between our mutant and two similar mutants isolated by others. These results show that all three mutants share, at the very least, a mutation at the ant-r locus. We therefore feel justified in discussing the claims mentioned above in the light of our own findings.

We can first turn our attention to the reports that have originated from the group of investigators working at the University of Warwick, Coventry, England. Starting with the paper by Avner _et al._, (1973b), several publications emanating from this laboratorv have described mutants cross-resistant to a variety of drugs, in a variety of combinations: OLI^R VEN^R TET^R "1799"^{R} CAP^R MIK^R SPI^R mutants (Avner _et al._, 1973b): VEN^R, VEN^R OLI^R, VEN^R TET^R and VEN^R OLI^R TET^R mutants (Lancashire _et al._, 1975); RHG^R VEN^R TET^R "1799"^{R} CHX^R mutants (Carignani _et al._, 1977). Several of these were unambiguously characterized as nuclear or mitochondrial mutants. Others, however, failed to fall into either category. Instead, the genetic analysis of this latter class of mutants led these investigators to interpret them as being the result of an interaction between nuclear gene(s) and cytoplasmic, but non-mitochondrial. factors. Their line of reasoning leading to this interpretation can be summarized as follows:

a. Meiosis of diploids derived from crosses between the resistant mutant, and sensitive wild types yields segregation ratios of 2:2 (or 1:1 in case of random spore analysis), indicating the involvement of nuclear genetic factors.

b. These diploids also exhibit mitotic segregation, therefore indicating the involvement of cytoplasmic factors as well.

c. When the mutant strain is made petite by treatment with ethidium bromide, and then crossed to sensitive wild type [RHO$^+$] strains, the resistance factors can be "rescued", as the diploids (or some of them) can express the resistant phenotype. Therefore, the cytoplasmic factors involved are not located on the mitochondrial genome, as they were not lost concurently with mt. DNA following the treatment with ethidium bromide.

Whereas the first two conclusions are acceptable the third one, we feel, is not justified. Indeed, the retention of the cytoplasmic resistance genetic determinant in [RHO^o] petites would lead to the conclusion that it is not mitochondrially located if, and only if, it is the <u>only</u> factor involved in the control of the phenotype. But, the demonstrated and admitted involvement of nuclear genetic factors interacting with the cytoplasmic ones renders such a conclusion inappropriate. It does not eliminate the possibility that the interacting cytoplasmic factors are mitochondrially located. It is only necessary to assume that the sensitive [RHO$^+$] parent used in the cross is a "carrier" of the appropriate mitochondrial gene. The cross would therefore be of the type: ant-r [RHO^{O}] X + [A^{R} RHO^{+}] where ant-r and A^{R} represent the nuclear and mitochondrial genetic factors, respectively. The diploids derived from such a cross would have the genotype: ant-r/+ [A^{R} RHO^{+}], and could conceivably express the resistant phenotype provided the ant-r nuclear mutation is dominant over its wild type allele. This result would mimic the "rescue" of a cytoplasmic non-mitochondrial factor from the [RHO^{O}] parent although it is clear from the example given, that a nuclear-mitochondrial interaction would also produce the same result, and cannot, therefore, be excluded.

There is in addition one intriguing observation, made by these investigators, which also argues in favor of a mitochondrial location for the cytoplasmic genes involved in their mutants: In all the cross-resistant mutants they have studied, the loss of resistance to one or more of the drugs is always accompanied by the conversion of the cells to the petite state.

Clearly, therefore, the conclusions arrived at by these investigators will need to be reevaluated in the light of our results.

The other set of claims, which our work does not support, is that made by the group working at Gif sur Yvette, France. Here it is claimed that three nuclear genes and two episome-like genetic factors, designated π (pi) and τ (tau), are involved in controlling the phenotype of a cross resistant mutant (Guerineau *et al.*, 1974; Guerineau *et al.*, 1976b). Unfortunately it is difficult to evaluate this claim since no genetic data have yet been published in its support. There have been, however, data of a biochemical nature presented in support of the claim that one of the episomes, π1 , which is said to determine the resistance to oligomycin, is identical to the yeast 2μ DNA. Among 14 clones derived from the original mutant, 7 had lost resistance to oligomycin (spontaneously or following ethidium bromide treatment). All 7 OLI^S clones were reported to have also lost the 2μ DNA, whereas all 7 OLI^R clones had retained this DNA species (Guerineau *et al.*, 1974). But in a subsequent publication (Guerineau *et al.*, 1976b), this absolute correlation between the loss of resistance to oligomycin and the loss of 2μ DNA is not seen in the data or the model presented. Instead the authors seemingly suggest that various genotypic combinations of the three postulated nuclear loci, in conjunction with "mutations" and/or "translocations" of the 2μ DNA, may also lead to the loss of resistance to oligomycin.

Clearly, until such time when genetic data, crucial in this case, are published to substantiate such claims, it is impossible to evaluate them further.

A particularly interesting aspect of our mutant, as well as of mutants of this type described by others, is their bearing on the question of the interaction between the nuclear and mitochondrial (or other cytoplasmic) genetic systems in the biogenesis of mitochondria as well as in the control of other cellular characteristics. There have been several reports of nucleo-cytoplasmic genetic interaction in yeast. Wickner (1974) has shown that nuclear genes are necessary for the maintenance and expression of the cytoplasmic killer factor. Cox (1965) has described the interaction of the cytoplasmic ψ(psi) factor with nuclear suppressors and Young et al. (1975) have shown that a nuclear gene is required for the maintenance of ψ. Rotman (1975) has demonstrated that the resistance of a yeast mutant to the nucleic acids intercalating dye, primaquin, is under a dual nuclear-cytoplasmic genetic control. There are also several reports of specific nuclear-mitochondrial genetic interaction. Barrere et al. (1976) have shown that such an interaction exists in the case of a yeast mutant resistant to the dye basic fuchsin, and Howell et al. (1974) have demonstrated a similar interaction in the case of a mikamycin resistant mutant.

Our mutant, however, provides the first example of such a nuclear-mitochondrial genetic interaction operating in the control of a complex pleiotropic phenotype, affecting several different mitochondrial functions (oxydative phosphorylation, protein synthesis) as well as at least one non-mitochondrial cellular function (cytoplasmic protein synthesis). We did not attempt to investigate the physiological nature of our mutation. It is clear, however, that such an investigation could provide new insight into the problems of the integration and cooperation of the nuclear and mitochondrial genomes in the specification of mitochondrial and other cellular characteristics.

There also exists an interesting application of our findings in the field of mitochondrial genetics. One of the new mitochondrial genes we have characterized, the RHG-1 locus, is closely linked to the CAP-R (5% recombination) and ERY-R (10% recombination) mitochondrial loci. It is therefore located within the region of the mitochondrial genome known as the omega (ω) region. This region is believed to be the site of extensive gene conversion, when appropriate crosses (ω^- X ω^+ "heteropolar crosses") are made. These molecular events lead to a bias in the transmission of mitochondrial genes located near or in the omega region as well as to asymmetrical recombination between genes in this region (Netter et al., 1974). But the genetic investigation of the actual events taking place in the omega region has been hampered by the paucity of genetic markers at this location. Questions such as the length of genome involved in the conversion events, the bidirectionality or unidirectionality of the conversion, etc..., still remain unanswered. The discovery of a new genetic marker in this region may prove to be useful in solving these problems.

APPENDIX

LIST OF ABBREVIATIONS

CAP	Chloramphenicol
CHX	Cycloheximide
EB	Ethidium bromide
ERY	Erythromycin
MIK	Mikamycin
mt. DNA	Mitochondrial DNA
OLI	Oligomycin
RHG	Rhodamine 6G
SPI	Spiramycin
TCN	Tetracycline
TET	Triethyltin
VEN	Venturicidin

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