# YEAST EPISOME : OLIGOMYCIN RESISTANCE ASSOCIATED WITH A SMALL COVALENTLY CLOSED NON-MITOCHONDRIAL CIRCULAR DNA.

Michel Guerineau[†], Piotr P. Slonimski[*¶] and Philip R. Avner[*]

Centre de Génétique Moléculaire du CNRS[*], Gif-sur-Yvette, France and Laboratoire de Pharmacologie Moléculaire associé au CNRS N° 147, Institut Gustave Roussy[†], Villejuif, France.

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Summary : We have isolated a single step spontaneous mutant of *S.cerevisiae* resistant simultaneously to oligomycin, venturicidin, chloramphenicol, cycloheximide and triethyltin. This multiple drug resistance results from the interaction of two genetic factors showing both chromosomal location and episomal characteristics. One factor ($\pi$) confers oligomycin resistance, the other ($\tau$) confers the other resistances. $\pi$ can be lost spontaneously while $\tau$ can be completely eliminated with ethidium bromide. All $\pi^+$ strains, whether grande or petite, $\tau^+$ or $\tau^-$, carry a covalently closed circular DNA while $\pi^-$ strains are devoid of it. We hypothesise that this circular DNA may play an informational role in the biogenesis and/or function of membranes.

Recent advances in our understanding of mitochondrial genetics have greatly depended on the study of mutants resistant to drugs interfering with a variety of metabolic processes (cf.1). During the course of an attempt to isolate a new class of spontaneous drug resistance mutants in *Saccharomyces cerevisiae* we have found a mutant which displayed pleiotropic resistance and unusual genetic features. The resistance is not coded by the mitochondrial DNA and results from the interaction of two genetic factors which show a complex chromosomal location and episomal characteristics. A search was undertaken to correlate the genetic properties with a molecular species of nucleic acids. This paper demonstrates that a one-to-one correlation exists between the presence of the oligomycin resistance conferring genetic factor carried by the mutant and the presence of a small covalently closed circular DNA. This DNA belongs to the class of molecules of nuclear buoyant density which have been previously isolated and purified from yeast (2,3,4,5,6,7,8) but the function of which has remained until now completely unknown.

## Isolation on a non-fermentable substrate containing a mixture of chloramphenicol and cycloheximide of a single step mutant displaying a variety of drug resistances.

A haploid strain IL125-6B ($\alpha his_1\ \rho^+\ \omega^-$), sensitive to all the antibiotics used in this study, was plated onto glycerol media containing 4 mg/ml

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[¶]Requests for reprints should be sent to P.P. Slonimski

chloramphenicol + 1 µg/ml cycloheximide at a density of ~ $10^8$ cells/dish and incubated at 28°C. Resistant colonies arising spontaneously appeared only slowly being absent after five days, but reached a frequency of ~ $10^{-7}$ per cell plated by the fifteenth day. This resembles, in both frequency and rate of appearance of resistant colonies, the behaviour of IL125-6B when plated onto glycerol media containing 1 µg/ml CYC* alone, but is clearly different from that observed on glycerol media containing 4 mg/ml CAP* alone when resistant colonies started to appear on the third day of incubation and reached a frequency of ~ 5 X $10^{-5}$ by the $15^{th}$ day.

The nine resistant colonies so isolated were subcloned once on glycerol containing chloramphenicol media, the seven clones which had maintained their resistance crossed to a sensitive tester strain IL125-10C (a $ura_1$ $\rho^+$ $\omega^-$) and the diploids tested for their resistance to chloramphenicol. The mutant DRI9 alone showed clear mitotic segregation for $CAP^R/CAP^S$ and $CYC^R/CYC^S$ and was therefore retained for further study. By replica plate testing on glycerol media containing either OLI* (3 µg/ml), or TET* (20 and 30 µg/ml), or VEN* (2 µg/ml), DRI9 was shown to be resistant to all these drugs but was sensitive to erythromycin and spiramycin (5 mg/ml) and showed increased sensitivity to paromomycin and ethidium bromide. It was also confirmed to be resistant to both CAP (4 mg/ml) and CYC (1 µg/ml) alone as well as on the double media plates and was equally resistant to CYC alone whether glucose or glycerol was the carbon source.

Genetics of the multiple drug resistance

Crosses, back-crosses and test-crosses of the mutant DRI9 have been performed using various grande or $\rho^-$ petite strains carrying multiple nuclear and mitochondrial genetic markers. The complete results will be published elsewhere (Slonimski, in prep.). The salient features of this study are : a) The mutant DRI9 carries two genetic factors which behave in meiosis like bona fide non-allelic mendelian genes. b) The genetic factor designated $\pi$ confers resistance to OLI and VEN and is closely linked to the centromere. c) The genetic factor designated $\tau$ confers resistance to CAP, CYC and TET when $\pi$ is present and is not centromere linked. d) $\pi$ can be lost during vegetative multiplication with the corresponding loss of OLI resistance and mitotic segregation for this character while maintaining the resistance to CAP, CYC and TET ; this loss is accompanied by the appearance of a novel non-allelic mendelian gene conferring low resistance to VEN only and mapping

*CAP : chloramphenicol, CYC : cycloheximide, OLI : oligomycin, VEN : venturicidin, TET : triethyltin. R : resistant clone, S : sensitive clone.

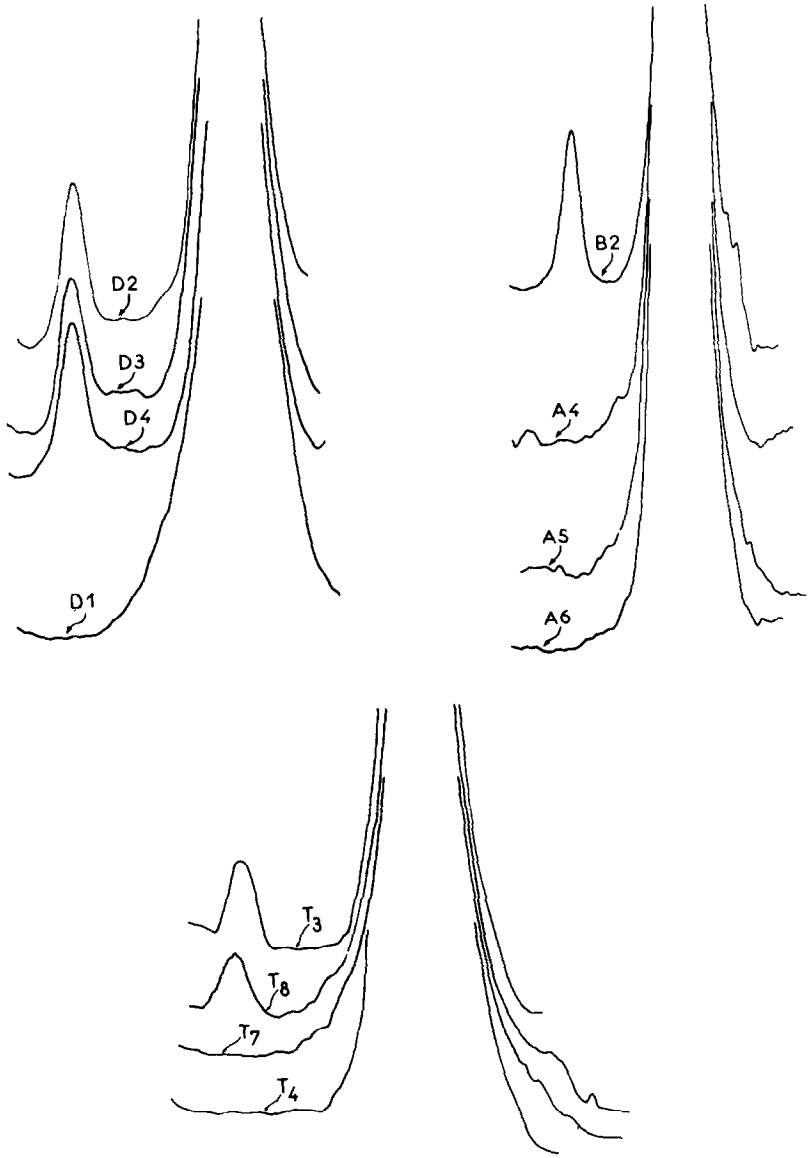


Figure 1

close to the centromere but on a different chromosome from the one where $\pi$ is located. e) $\tau$ can be completely eliminated by growing cells in ethidium bromide ; this loss occurs more slowly and requires higher concentrations of the dye from those required to produce deletions and repetitions in the mitochondrial DNA (cf. 9,10). f) $\pi$ is not located on the mitochondrial DNA as $\rho^-$ clones completely devoid of mitochondrial genetic information can be obtained still carrying $\pi$; the resistance is not expressed in such clones, but the presence

of the genetic factor can be demonstrated by test-crosses and subsequent analysis of the mitotic and meiotic progeny by techniques similar to those used in demonstrating the retention of mitochondrial genes in $\rho^-$ (cf. 10). g) The loss of $\pi$ does not lead to $\rho^-$. h) Various combinations of drug resistance can be realized by appropriate associations of $\pi$ and $\tau$ ($VEN^R$; $OLI^R$-$VEN^R$; $VEN^R$-$CAP^R$-$CYC^R$-$TET^R$; $OLI^R$-$VEN^R$-$CAP^R$-$CYC^R$-$TET^R$) ; all of them segregate independently from all the presently known (cf.1) mitochondrial genes.

It is clear therefore, that the genetic factors responsible for drug resistance are not located in the mitochondrial DNA and constitute a novel class of heredity elements in yeast. By some criteria (2:2 meiotic segregation, centromere linkage) they behave like typical nuclear genes mutations, while by other criteria (loss during vegetative multiplication, apparent migration from one chromosome to another, complete elimination by growth in ethidium) they are reminiscent of bacterial plasmids and episomes.

Covalently closed circular DNA molecules associated with the genetic factor $\pi$.

We have studied 14 clones derived by vegetative multiplication from the original mutant DRI9. Each clone was of independant origin and the presence or absence of the genetic factors $\pi$ and $\tau$ was ascertained by appropriate test crosses. The search for covalently closed circular DNA was undertaken by a method similar to the one we have used previously to purify this molecular species from yeast (3). Exactly the same procedure was used for all clones.

Cells were grown for 24 hours in a Yeast Extract 1%; Bacto-Peptone 1% and glucose 2% medium and harvested one generation before the stationary phase. After mechanical breakage, the membrane rich 16 X kg fraction was iso-

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Figure 1. Densitometer traces of CsCl - propidium - buoyant - density gradients from $\pi$ positive and $\pi$ negative clones.

The membrane enriched fraction (16 X kg) was isolated and the total DNA extracted from it as described in the text. Density was adjusted to 1.56 g/ml with a propidium diiodide concentration of 500 µg/ml and with a sodium lauryl sarcosinate concentration of 1 %. Centrifugations were performed in a Spinco fixed angle rotor 40 at 35,000 rpm, 15°C for 60 hours. Photographs were taken through a Kodak Wratten filter N°23 by illuminating with ultraviolet light at 365 µm. Negatives were scanned by a Joyce Loebl densitometer. Heavy density is to the left of the diagram.

The results of three centrifugation runs, each comprising four gradients are given. Each gradient corresponds to a different clone.

Four $\rho^+$ grande clones are shown in the bottom of the figure : clones T3 and T8 are $\pi^+$ $\tau^+$ and $OLI^R$, $VEN^R$, $CAP^R$, $CYC^R$, $TET^R$ while T4 and T7 are $\pi^-$ $\tau^+$ and $OLI^S$, $VEN^R$, $CAP^R$, $CYC^R$, $TET^R$. Eight $\rho^-$ petite clones are shown in the top : clones B2, D2, D3 are $\pi^+$ $\tau^+$ and carry the genetic factors conferring the resistance to OLI, VEN, CAP, CYC and TET ; clone D4 is $\pi^+$ $\tau^-$ and carries the genetic factor conferring the resistance to OLI and VEN but not those conferring resistance to CAP, CYC and TET ; clones A4, A5, A6, D1 are $\pi^-$ $\tau^-$ and carry the genetic factor conferring resistance to VEN only.

lated as before (3). To 35 g wet weight of cells correspond 10 ml of the 16 X kg fraction in standard SSC, which was extracted by adding 0.4 volume of SSC saturated phenol pH 9. The mixture was gently stirred for 15 min. and centrifuged at 15 X kg for 20 min. at 0°C. The supernatant was mixed with 0.2 vol of SSC saturated phenol pH 9 and gently stirred for 10 min. The clear supernatant (8 ml) was dialysed against SSC to remove phenol and the DNA fractionated into closed circular and linear molecules by equilibrium centrifugation in a CsCl propidium diiodide (11) gradient. Fig. 1 shows some of the results obtained.

Two characteristically distinct patterns were obtained : the heavy band being either present or absent. All clones displayed a lighter band containing the bulk of DNA, both mitochondrial and nuclear and composed of linear and nicked circular molecules. We have checked by electron microscopy that the heavy band contained closed circular supercoiled DNA molecules of ca 2 μm contour length similar if not identical to those previously described (3). Fig. 1 and table 1 summarize the data. It is clear that the circular DNA is present only in those clones which carry the genetic factor π conferring resistance to oligomycin. This correlation occurs without exception and is independent of whether the mitochondrial DNA is $\rho^+$ or $\rho^-$ and also of whether the genetic factor τ conferring resistance to other drugs is present or absent.

To check this correlation further two control experiments were performed. It could have been argued that in $\pi^-$ clones a fluorescent background due to an excess of non-circular nucleic acids (RNA or DNA) could possibly mask the band of the circular DNA. To test this hypothesis the corresponding regions of the gradients were collected and recentrifuged again in the absence of the bulk of nucleic acids. Fig. 2 shows that the circular band could not be detected in this purified preparation of a $\pi^-$ strain. Electron microscopy examinations have confirmed the absence of supertwisted circular molecules which were present in the $\pi^+$ strain. In the second experiment we have extracted the circular DNA with phenol directly from the whole cell homogenate and not from the membrane enriched 16 X kg fraction. CsCl (1.56 g/ml) and sarkosyl (1%) were added to the DNA extract and kept at 0° overnight. After first centrifugation (90 min. at 36 X Kg in the Spinco rotor 40) propidium diiodide was added to the clear supernatant at a concentration of 800 μg/ml, the density readjusted to 1.56 g/ml and a second centrifugation performed under standard conditions. A heavy band of closed circular DNA was present in a $\pi^+$ clone and absent in a $\pi^-$ one.

## DISCUSSION

In spite of its characterisation as a distinct molecular species (2,3,4, 5,6,7,8) the biological significance of the small closed circular DNA of yeast

| PRESENCE OF GENETIC FACTORS | | | | | | NUMBER OF INDEPENDENTLY ISOLATED CLONES | CIRCULAR DNA |
|---|---|---|---|---|---|---|---|
| RHO | $OLI^R$ | $VEN^R$ | $CAP^R$ | $CYC^R$ | $TET^R$ | | |
| + | + | + | + | + | + | 2 | + |
| + | - | + | + | + | + | 2 | - |
| - | + | + | + | + | + | 3 | + |
| - | - | + | + | + | + | 1 | - |
| - | + | + | - | - | - | 2 | + |
| - | - | + | - | - | - | 4 | - |

Table 1. Correlation between the presence of the genetic factor $\pi$ conferring resistance to oligomycin and the presence of covalently closed circular DNA.

14 clones derived either spontaneously or by treatment with ethidium bromide from the strain DRI9 have been analysed for the presence of drug resistance conferring genetic factors by appropriate test crosses (see text). The presence of circular DNA was determined by CsCl-propidium-buoyant density gradients like in Fig. 1.

has remained enigmatic until now. Hypotheses have been advanced (4,5,6) and given up (7,12). The interest of the present work is two-fold : a) it demonstrates, for the first time, a biological property which shows a one-to-one correlation with the presence of this molecular species, b) it discloses the existence of a novel nucleo-cytoplasmic genetic system, distinct from the mitochondrial one, displaying several unusual features in the control of cellular drug resistance and related to this DNA. The properties of this system will be discussed extensively elsewhere (Slonimski, in prep.). It is clear, however, that it is reminiscent of episomal and plasmidial systems of bacteria (cf.13) with various states of integration of the genetic information. Of particular interest would be the study of $\pi^-$ strains in order to know whether the absence of circles results from the physical loss of the corresponding DNA sequences, from their integration into chromosome or from a change in the physical (eg. nicked) conformation of the molecules. Various hypotheses could be advanced concerning the mechanisms of drug-resistance : permeability barriers, detoxyfing processes, modifications of the sensitive targets, etc. The first one seems most probable to us for following reasons : the resistance to OLI at the cellular level is not accompagnied by the resistance of the purified mit.ATPase

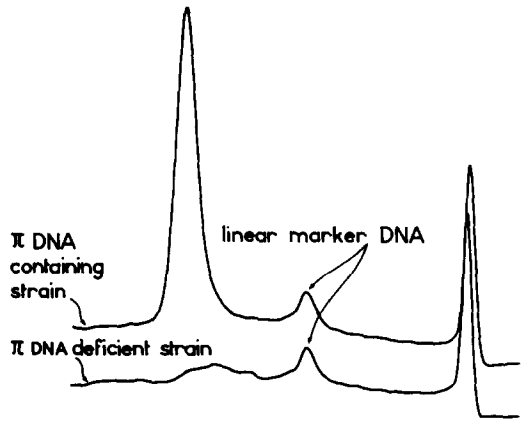


Figure 2. Densitometer traces of circular DNA purified from a $\pi^+$ clone and of the corresponding region of the gradient from a $\pi^-$ clone.

Density gradients from the clones B2 and A4 were first performed like in fig. 1. 0.7 ml was collected at the expected position of the closed circular DNA in both cases. 1 μg of linear calf thymus DNA (serving as a marker) in 4 ml of CsCl solution in SSC with a density of 1.56 g/ml containing 250 μg/ml of propidium diiodide were added to the fractions and centrifuged again in a Spinco SW 50 rotor at 36 000 rpm, 15°C, during 48 hours. The circular band is absent in the clone A4 which lacks the $OLI^R$ conferring genetic factor.

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complex (M. Somlo, personal communication) in contradistinction to the resistance specified by the mutations located in the mitochondrial DNA (14,23); the cellular resistance to CYC is not accompagnied by the resistance of purified cytoplasmic ribosomes (W. Jachimczyk, personal communication); the cells showing resistance to variety of chemically unrelated drugs display a collateral oversensitivity to other drugs. It is tempting to hypothesize that the circular DNA could play an informational role in the biogenesis and/or function of cellular membranes. Numerous (50 to 100) circular DNA molecules present in the cell would correspond to an amplification of the membrane-specific DNA segments of the chromosome. Whatever the true mechanism of drug-resistance could be, it will be of interest to reinvestigate for the presence vs absence of circular DNA and its episomal correlates a certain number of observations made in yeast concerning resistance to cycloheximide (15), tetracycline (16), oligomycin of the class I mutants (17), many drugs (18), ethidium bromide (19), mikamycin (20) and triethyltin (21). Furthermore, the search for drug-resistance correlates of the covalently closed circular DNA observed in *Euglena* (22) may be of interest.

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