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THE PETITE MUTATION IN *SACCHAROMYCES CEREVISIAE*: INTERRELATIONS BETWEEN THE LOSS OF THE ρ^+ FACTOR AND THE LOSS OF THE DRUG RESISTANCE MITOCHONDRIAL GENETIC MARKERS

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ABSTRACT

The survival of the $\rho+$ factor and of *Drug*^{R} mitochondrial genetic markers after exposure to ethidium bromide has been studied. A technique allowing the determination of *Drug*^{R} genetic markers among a great number of both grande and petite colonies has been developed. The results have been analyzed by the target theory. The survival of the $\rho+$ factor is always less than the survival of any *Drug*^{R} genetic marker. The survivals of C^R and E^R are similar to each other, while that of O^R is greater than that of the other two *Drug*^{R} markers. All possible combinations of *Drug*^{R} markers have been found among the ρ^- petite cells induced, while the only type found among the grande colonies is the preexisting one. The loss of the C^R and E^R genetic markers was found to be the most frequently concomitant, while the correlation between the loss of the O^R marker and the other two *Drug*^{R} markers is less strong. Similar results have been obtained after U.V. irradiation. Interpretations concerning the structure of the yeast mitochondrial genome are given and hypotheses on the mechanism of petite mutation discussed.

TWO classes of cytoplasmically inherited mitochondrial mutants are known to occur in baker's yeast: those leading to respiratory deficiency (ρ^- cytoplasmic petite mutants) and those conferring resistance to a variety of drugs (chloramphenicol, erythromycin, spiramycin, oligomycin, etc.). It has been established that mutants of the first class result from massive changes in the nucleotide sequence of the mit—DNA molecule. In some petite mutants no mit—DNA can be detected by extraction. The nature of the changes in mutants of the second class is less well known but thought to result from discrete point mutations. Recent reviews summarize the present knowledge in this field (WILKIE 1969; LINNANE and HASLAM 1970; PREER 1971; SAGER 1972; FAYE *et al.* 1973).

A great number of agents are known to induce petite mutants, acriflavine being the first one studied (EPHRUSSI, HOTTINGUER and CHIMENES 1949). Genetic relations between the ρ^- mutation and the *Drug*^{R} genes have already been considered (THOMAS and WILKIE 1968; LINNANE *et al.* 1968; GINGOLD *et al.* 1969;

SAUNDERS *et al.* 1971; NAGLEY and LINNANE 1972; COEN *et al.* 1969; BOLOTIN *et al.* 1971; MICHAELIS, PETROCHILO and SLONIMSKI 1973; AVNER *et al.* 1973; FAYE *et al.* 1973; WAKABAYASHI and GUNGE 1970; RANK 1970). The relations are not completely clear since petite mutants were first reported to obligatorily lose the $Drug^R$ markers (THOMAS and WILKIE 1968; LINNANE *et al.* 1968), while an independent reassortment between ρ^+ and $Drug^R$ markers was later suggested (GINGOLD *et al.* 1969).

The present work was aimed at studying the interrelations between the ρ^+ factor and the mitochondrial genes conferring resistance to chloramphenicol, erythromycin and oligomycin. The experiments have used the following experimental approach:

$Drug^R$ ρ^+ cells are first treated with a mutagen (ethidium bromide or U.V.) in the strict absence of cell multiplication. They are then allowed to multiply in the absence of the mutagen to form individual clones. For each hereditary trait two types of clones are found: those still containing *non-mutated cells* (i.e. ρ^+ clones and/or $Drug^R$ clones) and those composed *exclusively of mutated cells* (i.e. ρ^- clones and/or $Drug^0$ clones). Logically for two traits there are four types of clones possible, for three traits, eight possible types, etc. The interrelations between the genetic determinants are deduced from the frequency of different clonal types during a kinetic study of the action of the mutagens. The results allow new insights into the nature of the mitochondrial genome and the mechanism of petite mutation.

MATERIALS AND METHODS

I. *STRAINS*

The kinetic experiments presented here were carried out on the two following strains:

IL8–8C $\rho+$ $\omega+$ C^R_{321} E^R_{514} O^S α *his try*

IL828–4B $\rho+$ $\omega+$ C^R_{321} E^R_{221} O^R_1 α *his*

The loss of the O^R_1 genetic marker was also tested in the strains:

IL779–3C $\rho+$ $\omega+$ E^R_{221} O^R_1 α *his*

IL781–6C $\rho+$ $\omega+$ C^R_{321} O^R_1 α *his*

These strains were built by recombination in order to carry many different $Drug^R$ mitochondrial genes. Their construction and genetical properties are described elsewhere (COEN *et al.* 1969; COEN *et al.* 1974; AVNER *et al.* 1973).

The symbol $\rho-$ is used to specify the cytoplasmic petite mutation, $\rho+$ being the wild-type grande. After U.V. mutagenesis a few colonies appeared that could not grow on glycerol but did complement the $\rho-$ neutral tester strain used in the replica-cross technique (see also below RESULTS § 1.2) They were interpreted as nuclear petite mutants (CHEN, EPHRUSSI and HOTTINGUER 1950) and they were scored as $\rho+$. After ethidium induction, no nuclear petite mutants were detected.

Following COEN *et al.* (1969) the following symbols are used for mitochondrial antibiotic resistance markers: C^R, C^S for chloramphenicol resistance and sensitivity, E^R, E^S for erythromycin, O^R, O^S for oligomycin. Particular alleles are specified as such: C^R_{321}, O^R_1 for example. The C^R genetic marker (C^R_{321}) used in the experiments presented here belongs to locus R_I, the E^R ones (E^R_{514} and E^R_{221}) to locus R_{III}, the O^R one (O^R_I) to locus O_I of the mitochondrial genetic map (AVNER *et al.* 1973); GRIVELL *et al.* 1973; NETTER *et al.* 1974). When the locus is not specified the Drug symbol is used, meaning either *C*, *E* or *O*. The symbols C^0, E^0, O^0, $Drug^0$ are used to specify cells which give a negative response on the replica-cross test and are interpreted as deletions for the genetic markers (see below RESULTS § I and BOLOTIN *et al.* 1971). The symbol ω

($\omega+$ or ω^-) specifies the "mitochondrial sex", i.e. a mitochondrial locus governing genetic recombination (see BOLOTIN *et al.* 1971; COEN *et al.* 1974).

For testing the occurrence of $\rho+$ $Drug^0$ (see RESULTS § III) many other strains were analyzed. These strains, carrying one or more mitochondrial genes, were isolated in our laboratory; their geentic properties are described in COEN *et al.* (1969; COEN *et al.* (1974); and AVNER *et al.* (1973).

The tester strains (see Replica-cross technique) used mainly were strain 55R5-3C $\rho+$ ω^- $C^SE^SO^S$ *a ura*, and its derived neutral petite strain 55R5-3C/1035 ρ^- $C^oE^oO^o$ *a ura*. Occasionally, the strain D243-2B/R1 $\rho+$ $\omega+$ $C^SE^SO^S$ *a ade*, and its derived petite strain D243-2B/R1/6 were used as testers. No difference in the results was observed on varying the tester strains.

II. *MEDIA*

The media were prepared according to COEN *et al.* (1969).

N0 : Yeast Extract Difco 1%, Bacto Peptone Difco 1%, Sörensen phosphate buffer pH 6.2 0.05 M, glucose 2% as carbon source.

N2 : Same as N0; but 0.1% glucose and 2% glycerol as carbon source. This medium is also called "differential medium" as it allows the discrimination between petite and grande colonies.

N3 : Same as N0, but 2% glycerol as carbon source.

N5 : Same as N0, but 2% galactose as carbon source.

Antibiotics were added to N3 media at the following concentrations: Erythromycin 5 mg/ml, chloramphenicol 4 mg/ml, oligomycin 3 μg/ml.

W0 : Minimal medium: Yeast nitrogen base free of amino acids (DIFCO) added with 2% glucose.

For plating 3% DIFCO agar was added to all the media.

III. *ETHIDIUM BROMIDE MUTAGENESIS*

The cells were cultured overnight in complete medium and harvested at the end of log phase. Either N0 (glucose), N3 (glycerol), or N5 (galactose) media were used for culturing prior to mutagenesis without any marked effect on E.B.induction.

The cells were washed, and resuspended in Sörensen phosphate buffer pH 6.5 0.1 M. The final titer of the suspension was 10^6 cells/ml. Cycloheximide was added at a final concentration of 2 μg/ml. Exposure to ethidium bromide was made in the dark, at 28°. Ethidium concentrations and exposure time are given in the text for each experiment. They varied from 1 to 5 μg/ml and from 10 min to 7 hours. After the treatment, the cells were washed twice, diluted and plated on N0 and/or N2 media. Several times, two types of controls were made: zero time plating, and plating of the cells after shaking under the same conditions as treated samples in buffer plus cycloheximide, omitting ethidium bromide. No difference was ever found between the two controls. By comparing the number of cells and the number of colonies one finds that, on the average, two cells (the mother cell and its bud) form a colony. The plating efficiency does not vary during treatment.

IV. *FORMAL ANALYSIS OF THE KINETICS OF ETHIDIUM BROMIDE MUTAGENESIS*

We have analyzed the results of ethidium bromide mutagenesis by a formal treatment based on the target theory. The target theory (single-hit, multiple-target model) describes the inactivation of cells or viruses as follows (cf. DRAKE 1970).

$$S = 1 - (1 - e^{-h})^n$$

S is the frequency of surviving cells or viruses, h the number of hits and n the number of genetic targets. In our case S is the survival of a given genetic marker, as measured by the frequency of non-mutated colonies among the total population. Other mathematical treatments could have been applied (e.g., multiple hits, single-target model). However, as will be shown later, the one we have used has permitted us to single out a quantitative parameter, the relative target size, which is invariant of experimental conditions and more amenable, therefore, to interpretations than the survival curves alone.

In our formal approach the number of hits h and the number of targets n as calculated do not necessarily represent the true number of physical hits or targets. We assume that among the total number of real hits only a number h will be effective. We also assume that among the total

number of real targets, the inactivation of a number n will result in a mutated colony. Defined as such, it is clear that the parameters do not only describe the characteristics of the mutagen, but also depend on the properties of the cells and of the genetic markers. They therefore include the enzymatic process(es) of mutation and repair and may vary according to physiological and/or genetic variations in these processes.

In order to analyze the kinetic experiments, we have assumed that the number of effective hits h varies as a linear function of time.

$$h = K\,(t - t_0)$$

where t is the exposure time,

t_0 is a delay in the action of the mutagen

and K is the target size of the genetic marker.

In a similar way to h and n, we do not take K to be a measure of the physical size of genetic marker, but rather as a function of the probability that an effective hit may reach the genetic marker.

Graphic determination of the parameters is based most often on the approximation of the survival curve to a linear trace when the number of hits is high. It does not take into account sampling errors. In our case, the sampling error in measuring a low frequency of surviving markers is quite important. We have therefore submitted our data to a computer program in order to find the best fit between the experimental results and the theoretical equation. A computer fitting has already been applied to the kinetics of petite induction by ethidium (Mahler, Perlman and Mehrotra 1971). The basic principle of our program is slightly different from the one used by Mahler, Perlman and Mehrotra. It is based on a χ^2 test. Each point is thus weighted for the number of colonies in the sample. When the calculated figure lies between 10 and 5 colonies the Yates' correction is applied. When the calculated figure is less than 5, the point is not taken into consideration. Within a given experiment, the survival curves for the different markers (i.e. $\rho+$, C^R, E^R, O^R) were at first independently fitted, a confidence level of 95% being chosen. This had led to several pairs of parameters (K, n) being calculated for each genetic marker. As the values of n calculated for all the genetic markers have been found to overlap, the minimum χ^2 leading to the same n value for all the genetic markers has been looked for, in order to increase the resolution of the test. The parameter t_0 describes a delay in the action of the mutagen. When the induction is slow, and cells are not sampled within the first minutes of incubation, Kt_0 becomes negligible with respect to Kt; whether t_0 is taken into consideration or not, the determination of the K and n parameters is therefore not affected. This is the case with strain IL8–8C. When the induction is fast and cells are sampled within the first minutes of incubation, taking t_0 into consideration is necessary. In the case of strain IL828–4B, indeed no mutagenic effect was detected until after 10 min. Furthermore, assuming $t_0 = 0$ led, in the determination of K and n for each survival curve, to χ^2 values greater than 10^3 for 5 degrees of freedom, which is, of course, unacceptable. On the contrary, when t_0 is taken into consideration, the survival curves for all the genetic markers ($\rho+$, C^R, E^R, O^R) fitted the same t_0 ($t_0 = 17$ min $\pm$ 1 min determined with a χ^2 probability greater than 5% for $\rho+$, C^R, E^R and greater than 2% for O^R).

U.V. MUTAGENESIS

Cells cultured and harvested the same way as for ethidium mutagenesis were resuspended at 10^6 cells per ml in Sörensen phosphate buffer in a glass petri dish. They were exposed to a dose of 20 ergs·mm^{-2}.s^{-1}. A germicidal UV lamp (General Electric model G 15 T8, 15 watts) was used. At various times, as indicated in Table 8, samples were harvested, diluted and immediately plated on N0 medium.

RESULTS

I. DESCRIPTION OF THE EXPERIMENTS

The aim of the present study is to obtain information about the mitochondrial genome of yeast, by examining the survival of mitochondrial genetic markers

when submitted to mutagenesis. In order to obtain interpretable results, the following requirements must be fulfilled: (1) One must be able to score independent mutagenic events. A mutagen capable of action in the strict absence of cell multiplication is needed, and the cells must be plated immediately after treatment to form individual colonies. (2) An appropriate technique, allowing the analysis of a large number of colonies, is needed. (3) The scoring of surviving copies of mitochondrial genetic markers must not be obscured by the presence of mutant copies in the same colony. A positive screening method is therefore necessary.

We shall now describe in detail how these conditions are fulfilled.

I.1 *Conditions of Mutagenesis*

Two of the mutagens known to induce the petite mutation and fulfilling the above requirements are ethidium bromide (SLONIMSKI, PERRODIN and CROFT 1968) and U.V. light (RAUT and SIMPSON 1955). In most of the experiments ethidium bromide has been used. This choice was due to its greater efficiency and to the fact that cell lethality does not interfere with petite induction. In order to insure a complete lack of cell multiplication, and to ascertain therefore that the petite colonies derive from independent mutagenic events, 2 μg/ml cycloheximide was added to the reaction mixture. Controls with cycloheximide alone did not show any increase in the frequency of petites. There was neither any loss of markers in these controls. We have verified that under our conditions the essential features (see below) of petite induction are not influenced by the presence of cycloheximide.

I.2 *Scoring of the cells; The replica-cross technique*

A technique has been developed which allows the determination of the absence or presence of *Drug^{R}* gene in a large number of colonies. The phenotypic determination of the presence of a *Drug^{R}* gene in a petite colony is not possible since petite cells are unable to grow under the conditions required for the effective inhibition of a sensitive strain by the drugs—that is, on respirable substrates. The *Drug^{R}* genes are thus detected in petite cells through a cross by a grande tester strain carrying the *Drug^{S}* alleles. If grande *Drug^{R}* cells are found among the diploid progeny of a cross between a petite haploid cell and a drug-sensitive grande haploid cell the drug resistance must be derived from the petite cell, which therefore will be scored as *Drug^{R}*. If not, it will be scored as *Drug^{o}*, meaning that it has lost the *Drug^{R}* gene.

The cross-replica technique is shown in Figure 1. The cells are plated, immediately after mutagenesis, on YP Glucose medium, a non-selective medium which allows the growth of grande and petite cells, both sensitive and resistant. This master plate is then incubated 2–3 days at 28° and replicated using velveteen (LEDERBERG and LEDERBERG 1952) onto a series of plates in the following order:

YP Glycerol plus Drug(s). Only the drug-resistant grande colonies can grow on this plate.

YP Glycerol. On this plate both drug-sensitive and *Drug^{R}* grande colonies can grow.

Minimal-Glucose. This plate, on which no colony should grow, is a control for the presence of the auxotrophic markers.

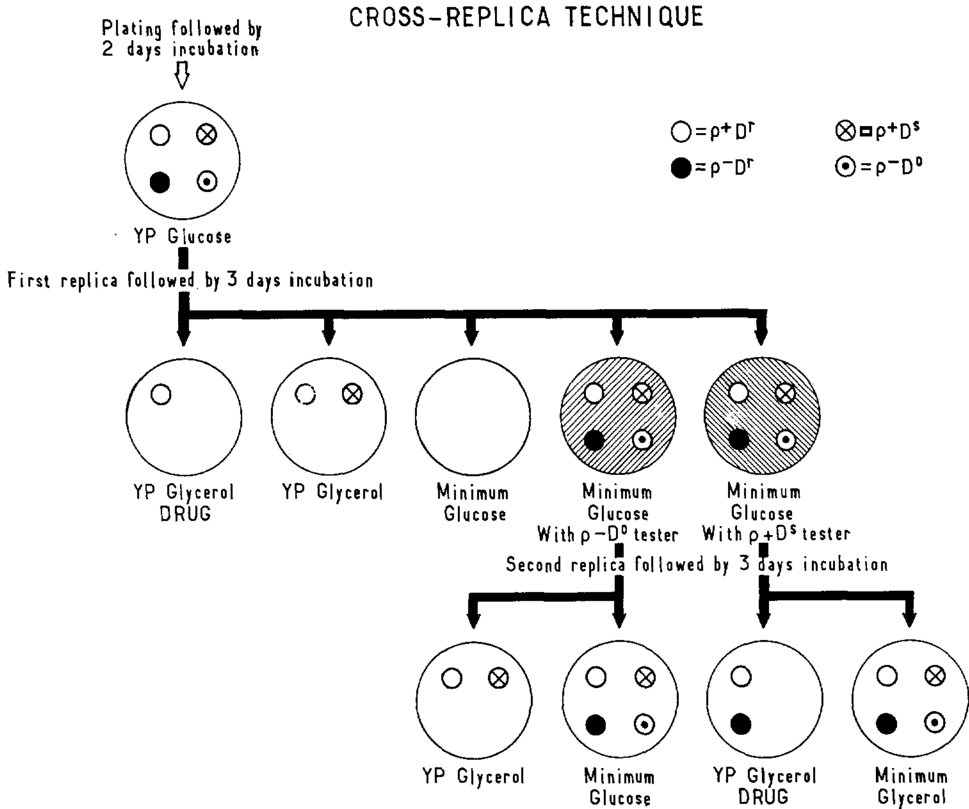

FIGURE 1.—The replica-cross technique.

Minimal-Glucose plated with a lawn of neutral petite ρ^- $C^oE^oO^o$ tester strain of the opposite mating type. Minimal medium is used to select prototrophic diploids. This is the first cross plate.

Change of velveteen.

Minimal-Glucose plated with a lawn of drug-sensitive ρ^+ $C^SE^SO^S$ tester strain of the opposite mating type. This is the second cross plate.

The YP Glycerol plus Drug(s) plates are incubated for 3–6 days, depending on the drug, while the YP Glycerol and the cross plates are incubated for 3 days. After 3 days, diploid patches have grown on the cross plates, each patch corresponding to a colony on the first YP Glucose master plate. Each diploid patch is made up of more than a hundred colonies. After 3 days, the cross-plates are used as master plates to make new replicas. The first cross plate (cross by the ρ^- tester) is replicated on YP Glycerol and Minimal-Glucose media. On YP Glycerol the diploid colonies arising from matings involving grande haploid cells or from petite haploid cells which complement the ρ^- petite tester (e.g. from nuclear petite cells), will grow. The Minimal-Glucose plate is a control plate on which all the colonies should grow. The second cross plate (cross by the drug-sensitive grande tester) is replicated onto glycerol media with and without drug(s). On drug con-

taining media only the diploid colonies arising from matings involving *Drug*^{R} haploid cells: both ρ^+ *Drug*^{R} cells as well as ρ^- *Drug*^{R} ones, will grow. The plate without drug is again a control plate on which all colonies should grow. This technique allows the scoring of all possible combinations of the various Drug resistance genes and the ρ factor. The media are buffered (see above) because the discrimination between resistant and sensitive colonies is facilitated by keeping the pH constant.

The replica cross technique is a positive screening method. It means that the haploid colonies scored as positive (ρ^+, *Drug*^{R}) may be, and in fact often are, mixed, being composed of both positive and negative types of cells. Examples of the composition of colonies scored as ρ^+ are given later on. The limit of detection of positive cells among mixed colonies may be estimated as $1/10^6$ for the ρ^+ factor and $1/10^4$ for the *Drug*^{R} genetic markers. Negative colonies (ρ^-, *Drug*^{0}) are pure, being composed of the negative type only.

II. THE SURVIVAL OF THE ρ^+ FACTOR AND DRUG^R MITOCHONDRIAL GENES AFTER ETHIDIUM BROMIDE TREATMENT

The same experiment gives information about the survival of the various genetic markers and about their correlations. In this section we discuss the survival of each individual genetic marker.

Figures 2 and 3 show, respectively, the survival of the ρ^+ factor, the C^R and E^R genetic markers in strain IL8-8C and of the ρ^+ factor, C^R, E^R and O^R genetic markers in strain IL828-4B.

The points shown are experimental while the curves are drawn from the theoretical equation based on target theory as described in MATERIAL AND METHODS. Table 1 gives the results of the computer determinations of the parameters, target size K and the number of targets n.

The results are in satisfactory agreement wtih the theoretical equation.

The survival of the *Drug*^{R} markers is always greater than the survival of the ρ^+

TABLE 1

The parameters of the survival curves of the genetic markers

Strain	Number	Size (Targets)				Relative size			χ^2	Degrees of freedom
		$K\rho$ h^{-1}	KC h^{-1}	KE h^{-1}	KO h^{-1}	$KC/K\rho$ %	$KE/K\rho$ %	$KO/K\rho$ %		
IL8–8C $\rho^+\omega^+C^R_{321}\ E^R_{514}$	2.0	0.65	0.47	0.47	—	72	72	—	11.0	13
IL828–4B $\rho^+\omega^+C^R_{321}\ E^R_{221}\ O^R_{1}$	1.8	6.3	4.1	4.1	3.3	65	65	52	41.6	20

The parameters of the survival curves (Figures 2 and 3) were calculated to fit the theoretical equation:

$$S = 1 - (1 - e^{-K(t-t_0)})^n$$

Where K is the target size,
n is the number of targets,
t is the exposure time, and t_0 a delay.

The calculation of the parameters is made by a computer program based on a χ^2 test (see text).

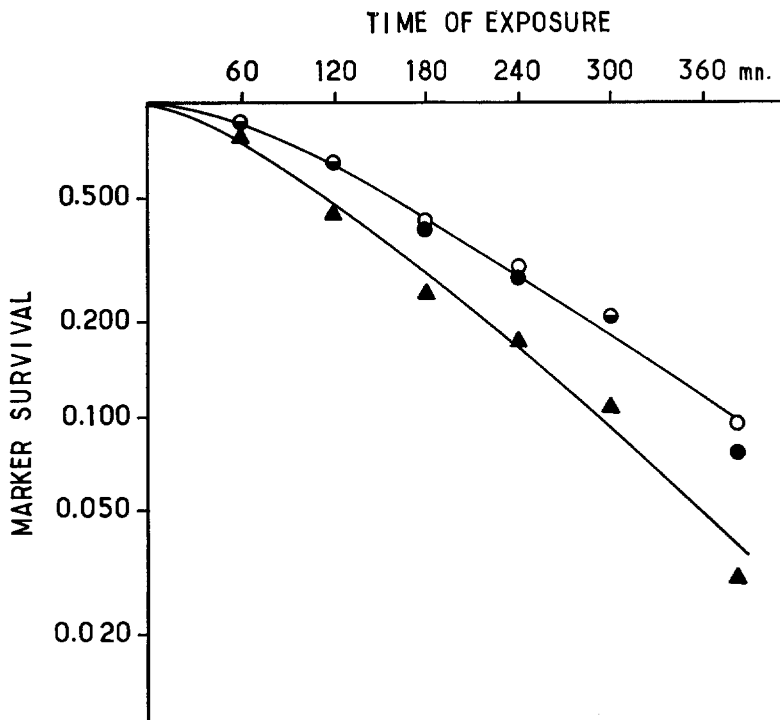

FIGURE 2.—Kinetics of the survival of $\rho+$, C^R_{321} and E^R_{514} genetic markers after ethidium bromide treatment.

The cells (strain IL8-8C, $\rho+$ $\omega+$ C^R_{321} E^R_{514}) are suspended in buffer plus cycloheximide (2 μg/ml) and added with ethidium bromide (2 μg/ml) at zero time. Samples are taken at various times, washed, diluted and immediately plated. The colonies are scored by the replica-cross technique (see Figure 1). The frequency of the $\rho+$ grande colonies was tested both by the replica cross technique and by plating on N2 differential medium. The average value of the two determinations is taken as the $\rho+$ survival.

—▲— Survival of the $\rho+$ factor
—●— Survival of the C^R genetic marker
—○— Survival of the E^R genetic marker

factor. The different *Drug*^{R} genetic markers display different relative target sizes. While the survival curves of the C^R and E^R genetic markers are very similar to one another in both strains studied, the survival of the O^R genetic marker is greater than the survival of the other two *Drug*^{R} markers. There is therefore a differential effect of ethidium bromide on the different *Drug*^{R} markers. In order to know if this differential effect was a peculiarity of the strain IL828-4B, two other strains bearing either the C^R and O^R, or the E^R and O^R genetic markers were treated with ethidium. Although a complete kinetic study has not been made, the results, presented in Table 2, show that the survival of O^R is always greater than that of C^R and E^R.

There also seems to be (Tables 3 and 4) a slight difference in the loss of the C^R

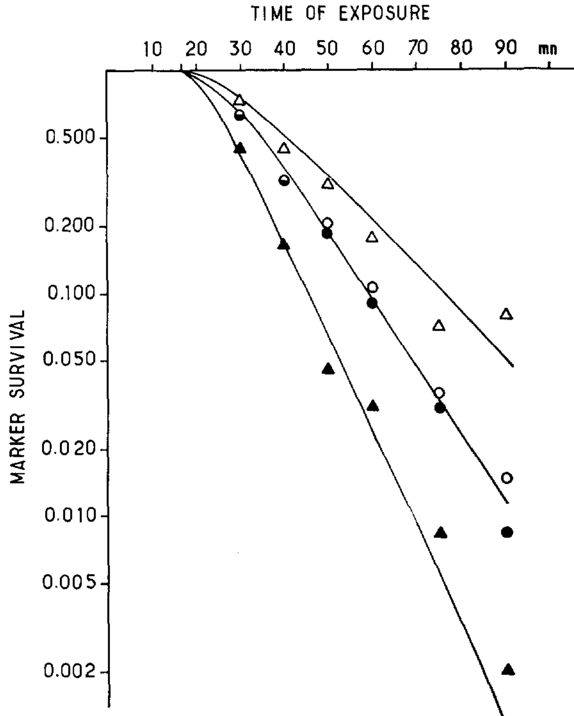

FIGURE 3.—Kinetics of the survival of $\rho+$, C^R_{321}, E^R_{221} and O^R_1 genetic markers after ethidium bromide treatment.

The same experimental procedure as for Figure 2 except that the strain IL828–4B ($\rho+\omega+C^R_{321}\ E^R_{221}\ O^R_1$) is used.

—▲— Survival of the $\rho+$ factor
—●— Survival of the C^R genetic marker
—○— Survival of the E^R genetic marker
—△— Survival of the O^R genetic marker

TABLE 2

The difference between the survival of the O^R *and either* C^R *or* E^R *genetic markers*

Strain	Number of colonies scored	ρ^+ C^RO^R or E^RO^R %	ρ^- C^RO^R or E^RO^R %	ρ^- C^0O^R or E^0O^R %	ρ^- C^RO^0 or E^RO^0 %	ρ^- C^0O^0 or E^0O^0 %	C^R or E^R survival %	O^R survival %
IL779–3C $\rho+\omega+E^R_{221}\ O^R_1$	206	34	17	18	5	26	56	69
IL781–6C $\rho+\omega+C^R_{321}\ O^R_1$	221	12	4	14	2	68	18	30

Similar experimental procedure as described in the legend of Figure 2 was used. The table gives the results of the replica-cross scoring of the colonies.

TABLE 3

Kinetics of the survival of the ρ^+, C^R *and* E^R *genetic markers: distribution of the different types of colony*

Time hours	Number of colonies scored	ρ^+ C^RE^R %	ρ^- C^RE^R %	ρ^- C^RE^0 %	ρ^- C^0E^R %	ρ^- C^0E^0 %
1	237	71.5	16.2	0.0	0.0	12.3
2	288	39.0	23.0	0.4	0.4	37.2
3	237	10.9	28.5	0.5	1.0	59.1
4	440	6.9	20.0	0.7	1.7	70.7
5	273	6.0	13.2	0.4	0.4	80.0
6.3	154	1.5	5.2	0.8	2.3	90.3

The same experiment as presented in Figure 2. Colony types are scored by the replica-cross technique. Spontaneous background of ρ^- and $Drug^0$ colonies was substracted from the results. Spontaneous ρ^- colonies represented 11% of the total, composed of 6% ρ^- C^RE^R and 5% ρ^- C^0E^0 counted on 319 colonies.

and E^R genetic markers. Since these two markers were found to be lost for the most part together (see below § III), the difference depends on the two rare classes, C^0E^R and C^RE^0, the C^0E^R type being more frequent than the C^RE^0 one. In individual experiments, the difference between the frequency of the C^0E^R and C^RE^0 types is not statistically significant. We have therefore summed all the experiments with the strain IL8-8C (see Table 5). Among 9266 colonies tested, there appeared to be a significant excess of C^0E^R colonies as compared to C^RE^0 ones. The standard error test gives an ε value of 7.4 corresponding to a probability less than 10^{-9}.

When the experiment was repeated on the same strain the absolute rate of petite induction varied by a maximum of twofold. Since the cells were grown either in glucose or glycerol medium, such variations may be accounted for by variations in the physiological state of the cells.

TABLE 4

Kinetics of the survival of the ρ^+, C^R, E^R *and* O^R *genetic markers: distribution of the different types of colony*

Time min	Number of colonies scored	ρ^+ $C^RE^RO^R$ %	ρ^- $C^RE^RO^R$ %	ρ^- $C^RE^RO^0$ %	ρ^- $C^RE^0O^R$ %	ρ^- $C^RE^0O^0$ %	ρ^- $C^0E^RO^R$ %	ρ^- $C^0E^RO^0$ %	ρ^- $C^0E^0O^R$ %	ρ^- $C^0E^0O^0$ %
20	394	89.2	5.4	1.8	0.0	0.0	0.4	0.0	2.4	0.7
30	498	44.3	14.6	2.2	0.0	0.2	1.1	0.0	11.5	26.1
40	482	16.2	9.9	5.5	0.4	0.2	0.5	0.0	16.3	51.0
50	572	4.6	10.0	4.4	0.0	0.2	1.6	0.4	14.3	64.5
60	326	3.1	3.2	2.5	0.0	0.3	0.5	0.9	10.8	78.7
75	481	0.8	1.6	0.2	0.0	0.4	0.0	0.8	4.5	91.6
90	491	0.2	0.0	0.2	0.0	0.4	0.0	1.0	7.7	90.4

The same experiment as presented in Figure 3. Colony types are scored by the replica-cross technique. Spontaneous background of ρ^- and $Drug^0$ colonies was substracted from the results. Spontaneous ρ^- colonies represented 1.5% of the total, composed of 0.3% of ρ^- $C^RE^RO^R$, 0.1% ρ^- $C^0E^RO^R$, 0.1% ρ^- $C^0E^0O^R$, 1.0% ρ^- $C^0E^0O^0$, counted on 764 colonies.

TABLE 5

Summary of the pattern of the types of colony obtained after ethidium bromide treatment

		Total	ρ^+ C^RE^R	ρ^- C^RE^R	ρ^- C^RE^0	ρ^- C^0E^R	ρ^- C^0E^0
Without E.B.	colonies	1265	1180	30	0	0	55
	Percent	*100.0*	*93.3*	*2.4*	*0.0*	*0.0*	*4.3*
Plus E.B.	colonies	9266	3024	1579	38	99	4526
	Percent	*100.0*	*32.6*	*17.0*	*0.4*	*1.1*	*48.9*

The results of six independent experiments similar to the one presented in Table 3 and Figure 2 are pooled together, whatever the incubation time and whatever the dye concentration are.

The absolute rates of induction are quite different in strains IL828-4B and IL8-8C, the latter being about *ten times less sensitive* than the former. In spite of these variations, the target size of the *Drug^R* genetic markers relative to the ρ^+ target size does not differ from one strain to another, the difference between the two strains not being significant (Table 1). *The relative target size is thus an intrinsic property of the genetic marker.*

The relative target size is very large. The target size of the C^R and E^R genetic marker is about ⅔ of that of the ρ^+ target size, while that of O^R is about ½ that of the ρ^+ target size.

The same number of targets n is found for all the genetic markers, when the different survival curves are independently fitted. The number of targets is also the same in the two strains studied. This number is very low, around 2.

III. CORRELATION BETWEEN THE LOSS OF THE DIFFERENT GENETIC MARKERS

The results presented and discussed above represent for each survival curve the pooled data of various types of colonies.

The detailed data of the different types of colonies are presented in Tables 3 and 4.

III. 1. *The absence of ρ^+ Drug^o cells*

Each genetic marker can occur in two forms: ρ^+/ρ^-, C^R/C^o, E^R/E^o, O^R/O^o. It is obvious in Tables 3 and 4 that all possible combinations of the drug resistance markers were found among the petite colonies. On the contrary, the only type of grande colonies found was the preexisting $\rho^+C^RE^R(O^R)$, the ρ^+ *Drug^o* type being missing. The ρ^+ *Drug^o* type was not found for any of the Drug markers in either of the experiments presented here. It was also absent from every repetition of these experiments. For example, in strain IL8-8C, about 3000 colonies of the total 9000 remained grande after ethidium treatment (see Table 5), and were all found to be C^RE^R. In order to strengthen this evidence, many strains carrying *Drug^R* genetic markers were submitted to ethidium and/or U.V. light. These strains were different from the previous ones, in both their mitochondrial and chromosomal background. In all the experiments, not a single *Drug^o* grande colony was found.

In order to ensure that *Drug^o* cells were not escaping our screening procedure

TABLE 6

The analysis of the composition of some colonies remaining $\rho+$ *after ethidium treatment*

Colony no.	Number of subclones scored	ρ^+ $C^RE^RO^R$ %	ρ^- $C^RE^RO^R$ %	ρ^- $C^RE^RO^0$ %	ρ^- $C^RE^0O^R$ %	ρ^- $C^RE^0O^0$ %	ρ^- $C^0E^RO^R$ %	ρ^- $C^0E^RO^0$ %	ρ^- $C^0E^0O^R$ %	ρ^- $C^0E^0O^0$ %
3	196	81	0.5	1.5	0	0	0	0	2	15
2	221	39	0	15	0	0	0	0	4	42
5	140	21	1	6	0	0	0	0	2	69
1	169	1	0	0	0	0	0	0	4	95
4	174	0.5	0.5	3	0	0	0	0	0	96
6	233	0.5	1	1	0	0	0	0	13	85

Six colonies remaining grande after ethidium treatment of strain IL828-4B were picked up, dissociated in sterile water and the cells plated. The resulting subclones (second column) were scored by the replica-cross technique.

by being masked in mixed clones containing both $\rho+$ *Drug*^{R} and $\rho+$ *Drug*^{0} cells, some colonies which remained grande after ethidium bromide treatment, were picked up and individually dissociated in sterile water. Samples were plated on complete glucose medium and analyzed by the replica cross technique. These grande colonies were found to be mixed, containing both $\rho+$ and ρ^- cells of different types, including ρ^- *Drug*^{0}, but all the $\rho+$ cells had retained all their *Drug*^{R} genetic markers. (Some examples of this analysis of the composition of the grande colonies are given in Table 6.)

It can be asked what phenotype a cell of putative $\rho+$ *Drug*^{0} genotype would have. Such a cell could have a petite phenotype, i.e., would not grow on glycerol plates. However, because of its $\rho+$ genotype, it should be able to complement a ρ^- petite tester, giving rise to $\rho+$ grande diploid progeny. This is not the case; all the *Drug*^{0} colonies are *both phenotypically and genotypically petite.*

Our conclusion is that $\rho+$ *Drug*^{0} cells do not exist. This statement is valid for all the mitochondrial genes conferring drug resistance studied until now—that is, those concerned with mitochondrial ribosome functions (R_I, R_{II}, $_{III}$, P) conferring resistance to chloramphenicol, erythromycin, spiramycin, paromomycin as well as those concerning the mitochondrial ATPase (O_I, O_{II}) (see AVNER *et al.* 1973; WOLFF, DUJON and SLONIMSKI 1973; NETTER *et al.* 1974; GRIVELL *et al.* 1973; SOMLO *et al.* 1973).

III.2. *The correlations between the loss of the different* Drug^R *markers*

A very strong correlation between the loss of the C^R and E^R genetic makers can be seen from the results given in Tables 3 and 4. The single C^0E^R and C^RE^0 mutants are seldom found: the two markers are usually either lost or maintained together. This means that a single mutagenic event induces the simultaneous loss of both the markers.

On the contrary, the C^R and E^R on one hand, and the O^R on the other hand, behave as two different groups: petite cells having lost one of these groups (ρ^- $C^RE^RO^0$ and ρ^- $C^0E^0O^R$) are numerous. However, there still exists a correlation between the loss of these two groups. Should the two groups be lost independently the frequency of the double mutants C^0O^0 and E^0O^0 would be equal to the product

of the frequency of the single events leading to C^o and O^o mutations. But the frequencies of C^oO^o and E^oO^o types (see Tables 2 and 4) are in excess of the expected values. This means that, whatever the mutagenic mechanism, a single event may hit the two *Drug*^{R} genetic markers at once. Such events are however, more frequent for the C^R and E^R genes than for the C^R and O^R or the E^R and O^R ones.

IV. THE EFFECT OF ETHIDIUM BROMIDE CONCENTRATION

Samples of a suspension of the strain IL8-8C were incubated for a constant time in ethidium bromide at concentrations ranging from 2 to 4 μg/ml. In Figure 4 the results of such an experiment where the incubation time was 5 hours are presented for both petite induction and the loss of *Drug*^{R} markers, the mutagenic process is highly dependent on ethidium concentration, saturation being reached with 3.6 to 4.0 μg/ml (about 10^{-5} M).

Table 7 gives examples of the pattern of the types of colonies obtained at saturating and limiting concentrations. The main relations between the markers remain unchanged on variation of the ethidium concentration: no ρ^+ *Drug*^{o} colony is found, the C^oE^R and C^RE^o types are very rare, and the linkage between the loss of the two *Drug*^{R} markers is unaffected by the changes in the concentration.

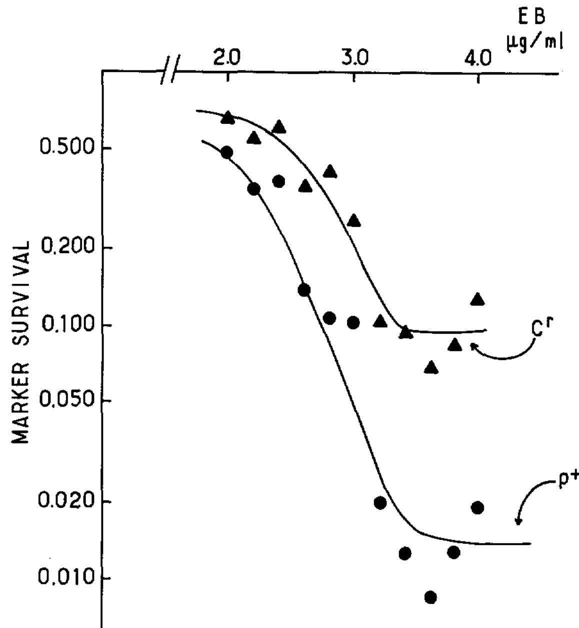

FIGURE 4.—Effect of ethidium bromide concentration on the survival of ρ+ and C^R_{321} genetic markers.

The cells (strain IL8-8C) are suspended in buffer plus cycloheximide (2 μg/ml) and added with ethidium bromide at concentrations ranging from 2 to 4 μg/ml. After 5 hours incubation, the cells are washed, diluted and immediately plated. The colonies are scored by the replica-cross technique (see Figure 1).

— ● — Survival of the ρ+ factor
— ▲ — Survival of the C^R genetic marker

TABLE 7

The types of colony obtained with saturating and limiting concentrations of ethidium

EB concentration μg/ml	Time hours	Number of colonies scored	Types of colonies $\rho^+ C^R E^R$ %	$\rho^- C^R E^R$ %	$\rho^- C^R E^0$ %	$\rho^- C^0 E^R$ %	$\rho^- C^0 E^0$ %
2.0	2	266	75	9	0	1	15
	5	194	48	16	0	0.5	36
4.0	2	172	33	23	0	4	40
	5	375	2	9	2	2	85

The table gives examples of the results of the replica-cross scoring of the colonies in the experiments described in Figures 4 and 5.

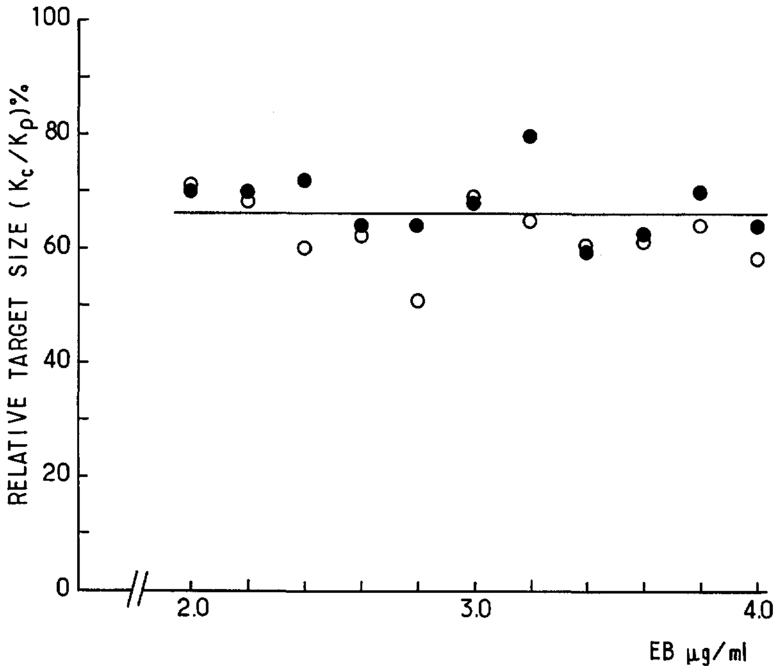

FIGURE 5.—Effect of ethidium bromide concentration on the target size of the C^R_{321} marker relative to the target size of $\rho+$.

From the experiment shown in Figure 4 and from a similar experiment where the incubation time was only 2 hours, the target size of the $\rho+(K_\rho)$ and C^R (K_C) genetic markers were calculated (see text for more details). The relative target size (K_C/K_ρ) is plotted against the ethidium concentration.

—O— 2 hours incubation
—●— 5 hours incubation

Assuming that the number of targets does not vary with the dye concentration, and considering a number of targets of 2.0 (see Table 1), the target sizes of the ρ^+ and of the $Drug^R$ markers were calculated. Figure 5 shows that the relative target size (K_0/K_ρ) remains constant when the dye concentration is varied.

Summarizing, the variation in ethidium concentration strongly affects the frequency of mutants induced without changing the intrinsic parameters of the mutagenic process: the target size of mitochondrial $Drug^R$ genetic markers relative to the ρ factor remains constant.

V. THE PETITE MUTATION AND THE LOSS OF THE C^R AND E^R MARKERS INDUCED BY UV LIGHT

Since ethidium bromide has been described as provoking large modifications and degradation of the mit-DNA of yeast (GOLDRING *et al.* 1970; GOLDRING, GROSSMAN and MARMUR 1971; PERLMAN and MAHLER 1971; NAGLEY and LINNANE 1972; MAHLER and PERLMAN 1972), the question arose whether the results just presented were due to a pecularity of the mutagen or are a more general feature of the petite mutation. We have therefore applied the same techniques on the same strain (IL8-8C, ρ^+ C^RE^R) to the induction of the petite mutation and to the loss of C^R and E^R markers by U.V. light. The mutagenic effect of ethidium is likely due to intercalation into DNA, while the petite induction by UV light is mainly due to the formation of pyrimidine dimers (PITTMAN, RANGANATHAN and WILSON 1959).

The results, presented in Table 8, show striking similarities to the results obtained on ethidium bromide treatment:

The survival of the $Drug^R$ markers is greater than the survival of the ρ^+ factor. The survivals of the C^R and E^R markers are very similar to each other. Again, no ρ^+ $Drug^o$ colony is found and neither ρ^- C^oE^R nor ρ^- C^RE^o colonies were found. Taking into account the small size of the sample tested, it is probable that these types occur just as rarely as after ethidium bromide induction. The most interesting feature is that U.V. irradiation like ethidium bromide treatment, induces a simultaneous loss of the C^R and E^R genes together.

VI. SPONTANEOUS ρ^- PETITE MUTANTS

It is well known that ρ^- petite mutants can arise spontaneously with high fre-

TABLE 8

The survival of ρ^+, C^R and E^R genetic markers after U.V. irradiation

Time (sec)	Number of colonies scored	Cell survival %	ρ^+ C^RE^R %	ρ^- C^RE^R %	ρ^- C^RE^o %	ρ^- C^oE^R %	ρ^- C^oE^o %
30	149	91	77.2	7.4	0	0	15.4
60	167	59	76.0	12.0	0	0	12.0
90	208	40	58.2	32.2	0	0	9.6
120	205	32	41.5	29.8	0	0	28.8

The cells (strain IL8–8C), were irradiated by U.V. (20 ergs.s^{-1}.mm^{-2}) and plated immediately after irradiation. The colonies were scored by the replica-cross technique (see Text and Figure 1).

quencies. GINGOLD *et al.* (1969) (see also SAUNDERS *et al.* (1971)) have claimed that the euflavine-induced petite mutants rarely retain the E^R determinant, while the spontaneously arising petite mutants frequently retain it. As shown in the present work, the frequency of $Drug^R$ petite colonies depends on the dose of mutagen received. The frequency of E^R petite colonies is quite high on induction by short exposure to ethidium (see above) or euflavine (data not shown). On the contrary, spontaneously arising petite mutants (for which of course the causal agent is unknown), may lose the E^R marker frequently (see Table 6). Therefore, in this respect, the distinction between spontaneous and induced petite mutants suggested by GINGOLD *et al.* (1969) does not seem to apply. Furthermore, it is important to stress that both induced and spontaneous petite mutants share at least one basic feature of the mutagenic process, i.e. the simultaneous loss of the C^R and E^R markers (see Table 6).

DISCUSSION AND CONCLUSIONS

We shall now discuss to what extent the results give information on two topics: the mitochondrial genome and the petite mutation mechanism.

I. THE MITOCHONDRIAL GENOME

The ρ factor was defined a long time before the discovery of mit-DNA as the genetic determinant responsible for respiratory competence in yeast. The ρ factor has been shown to be cytoplasmically inherited (EPHRUSSI, HOTTINGUER and CHIMENES 1949) and the ρ^- mutants to bear large defects in mitochondrial function (SLONIMSKI and EPHRUSSI 1949; SCHATZ 1968). Later work has shown ρ^- mutants to possess either modified mit-DNA (MOUNOLOU, JAKOB and SLONIMSKI 1966; MEHROTRA and MAHLER 1968; BRENARDI *et al.* 1968; HOLLENBERG, BORST and VAN BRUGGEN 1972) or no detectible mit-DNA (NAGLEY and LINNANE 1970; MICHAELIS *et al.* 1971) and allowed the ρ factor to be identified with the mit-DNA. This identity is now commonly admitted. The question remains, however, whether *the ρ factor is the whole mit-DNA or only that part of it, (a single gene or a cluster of genes)* responsible for the mitochondrial functions which are always lacking in ρ^- petite strains. A similar situation occurs in relation to a second problem: the location of the so-called mitochondrial $Drug^R$ markers. Although these are all concerned with mitochondrial functions and clearly exhibit a cytoplasmic pattern of inheritance (LINNANE *et al.* 1968; COEN *et al.* 1969; WAKABAYASHI and GUNGE 1970; KLEESE, GROTBECK and SNYDER, 1972; AVNER *et al.* 1973; THOMAS and WILKIE 1968) evidence concerning their localization within the mit-DNA comes from studies of their relations with the ρ factor among grande and petite colonies. Unfortunately, contradictory interpretations have been advanced. As some petite strains have lost $Drug^R$ markers, a clone or compulsory linkage between ρ^+ and $Drug^R$ markers has been claimed (THOMAS and WILKIE 1968; LINNANE *et al.* 1968). As some petite strains have kept $Drug^R$ markers, an independent reassortment between ρ^+ and $Drug^R$ markers has been considered (GINGOLD *et al.* 1969).

We are thus faced with three hypotheses:

a) *the ρ^+ factor is the whole mit-DNA, and* Drug^R *markers an independent set of cytoplasmic genetic elements.* The ρ^+ factor and the *Drug^R* markers are two independent sets of targets. Two independent mutagenic events are necessary to produce a ρ^- *Drug^o* mutant. The frequency of the double event ρ^- *Drug^o* should be the product of the frequency of the two single events, ρ^- and *Drug^o*. Results given in Tables 3, 4 and 8 show that this is not true.

b) *the ρ^+ factor is localized in one part of the mit-DNA, the* Drug^R *markers are localized in another part, the two parts being more or less linked.* The linkage between the two targets ρ^+ and *Drug^R* should lead to an excess of ρ^- *Drug^o* cells, in a similar manner as the linkage between C^R and E^R leads to an excess of C^oE^o cells.

In all the cases studied one finds an excess of ρ^- *Drug^o* cells over the calculated frequency from the product of two single events, ρ^- and *Drug^o*. This is shown in the present work for the genetic markers C^R_{321}, E^R_{514} and O^R_I and is also true for the genetic marker E^R_{354} (locus R_{II}) and P^R_{454} (data not shown). The ρ factor appears therefore to be linked to every known *Drug^R* marker and it is impossible to identify a precise location for the ρ^+ mit-DNA sequence. This hypothesis is therefore not satisfactory. The main reason to reject this hypothesis is that it predicts that ρ^+ *Drug^o* cells should exist in an analogous way to the reciprocal ρ^- *Drug^R* cells. As we have seen (RESULTS III) ρ^+ *Drug^o* cells are never found.

c) *The ρ^+ factor is the whole mit-DNA, the* Drug^R *markers being included in it.* This hypothesis takes into account all the results, and explains obviously why a single event hitting any *Drug^R* target, hits the ρ^+ target at the same time. The *Drug^o* mutation is by itself a ρ^- mutation. The converse is not necessary: the mutation in the ρ^+ factor may be limited to a part of the genome not including the *Drug^R* genetic marker, producing a ρ^- *Drug^R* cell.

We thus conclude that:

1) The ρ^+ factor is the entire mitochondrial DNA.

2) The *Drug^R* markers are located within it.

A question immediately arises: we know that we can preserve any known mitochondrial genetic marker in a ρ^- petite mutant. How can a mutation, located anywhere along the mit-DNA sequence, always confer the petite phenotype, that is, mainly, lack of cytochromes a and b leading to respiratory deficiency?

Two types of hypotheses may well explain this extreme pleiotropy:

a) The expression of the mitochondrial genes is achieved through such a highly coordinated regulatory system that the genetic inactivation of any single gene leads to the inhibition of the expression of the whole genome. In this respect it is of interest to note that the whole mit-DNA of HeLa cells seems to be a single transcription unit (ALONI and ATTARDI 1971).

b) The genes coding for the different components of the mitochondrial protein synthesis system (ribosomal RNA's and/or proteins, transfer RNA's) are distributed all along the mit-DNA sequence. The mutagenic lesions are large enough that the probability that the whole synthetic machinery will be preserved is virtually nil.

II. THE PETITE MUTATION MECHANISM

The main characteristics of the petite mutation process are: (a) the lesions are irreversible, of a very large size and cover simultaneously different genes; (b) some pairs of genes are mutationally more linked than others; (c) some individual gene regions appear more susceptible to loss than others.

All these conclusions could have been reached without the help of the target analysis of the survival curves. The absolute parameters (K, n) that can be determined by such an analysis have to be handled with the greatest caution, since they vary considerably from strain to strain and from one set of experimental conditions to another. However, the formal analysis allowed us to single out a *relative* parameter, the target size of a $Drug^R$ genetic marker relative to that of the ρ^+. We have shown that the *relative target size is an intrinsic property of a genetic marker, being independent of the strain used, mutagen concentration and time of exposure*. This relative parameter has therefore a biological significance, whatever that of the absolute parameters. The relative target size permits the description of the results by a single estimate. Even if this were its only advantage, it would have justified, in our opinion, the application of the target theory.

The size of the mutagenic event

The $Drug^R$ mutations do not obliterate the functions of the mitochondrial genes: for example, the C^R, S^R, E^R mutations have been shown to be correlated with changes in the mitochondrial ribosomes (GRIVELL *et al.* 1973), though these still remain active in protein synthesis. The $Drug^R$ mutations must therefore be discrete mutations, involving probably one or at the most a small number of base pairs. It is obvious that the relative *Drug* target size observed in the present work does not represent the nucleotide (or few nucleotides) which differentiates the resistant allele from the sensitive one. The relative target size is the probability that one hit on the mitochondrial genome hits the genetic marker. The relative *Drug* target sizes are quite large, representing about 50–70% of the total mitochondrial genome. One way to explain such high target size values is to assume that the hits are not discrete but large. If so, the probability for a hit to reach a given marker will increase with the size of the hit. This hypothesis is strongly supported by the fact that the *Drug* targets overlap. A single mutagenic event yields the concomitant loss of several $Drug^R$ markers far enough apart along the mitochondrial DNA sequence to be unlinked by recombination (eg C^R and O^R). This means that most often the mutagenic event leading to the petite mutation is very large, overlapping a significant part of the entire genome. This rules out any hypothesis that the petite mutation can occur through a multiplicity of small events (cf. BORST and KROON 1969 and HOLLENBERG, BORST and VAN BRUGGEN 1972).

We wish to stress that the main features of petite induction by U.V. (and possibly even, the spontaneous process) are strongly similar to petite induction by ethidium. This seems rather surprising in view of the differences of the two mutagens. The similarity between the action of ethidium and U.V. light indicates

that the intrinsic mechanism leading to the petite mutation is the same regardless of the nature of the inducing agent. It is well known that a great number of chemically unrelated agents are able to induce the petite mutation: chemical mutagens, intercalating dyes, non-intercalating dyes, base analogs, ions, antibiotics (cf. NAGAI, YANAGASHIMA and NAGAI 1961; SAGER 1972; SCHWAIER, NASHED and ZIMMERMAN 1968; NORDSTROM 1967; MAHLER and PERLMAN 1973; WILLIAMSON, MAROUDAS and WILKIE 1971). We think that all these agents simply trigger the same mechanism and would like to predict that they should yield the same pattern of relations between the loss of the ρ^+ factor and the *Drug*^{R} markers as do U.V. light and ethidium bromide. In addition, since it has been reported that ethidium bromide treatment is rapidly followed by degradation of the preexisting mit-DNA, we would predict that the same events are likely to happen after U.V. exposure.

The large size of the petite mutagenic event is well corroborated by the fact that all the petite strains studied until now show considerable changes in their mit-DNA sequence. Moreover, recent DNA-DNA hybridization studies on petite strains especially selected for keeping *Drug*^{R} genes in a hereditary stable manner reveal that these have nevertheless lost at least 50% of the wild-type mit-DNA sequence (LAZOWSKI *et al.* 1974; FAYE *et al.* 1973).

How could such a large mutagenic event be achieved? The petite mutation seems at the first glance to be very peculiar. We wish to stress, on the contrary, that the events leading to the petite mutations may not greatly differ from those produced by the action of mutagens in other organisms. One must keep in mind that one recovers with the petite mutations all the mutations which appear lethal in other organisms. We feel then that the large extended lesions found during petite induction might, in fact, be found in many mutagenic processes if their recovery was not obliterated by lethality.

Linkage of mitochondrial genes in petite mutation

If two genes are located close to each other on the ρ^+ mit-DNA, the probability that they should be lost simultaneously due to single macrolesion should be quite high, while if they are distantly located this probability should be lower. As shown in RESULTS §III, the genetic markers of the loci R_I (genetic marker $C^R{}_{321}$) and R_{III} (genetic markers $E^R{}_{514}$, $E^R{}_{221}$) are lost simultaneously very frequently, while the correlation between the loss of the markers of the R loci (C^R, E^R) and the marker of the O_I locus (genetic marker $O^R{}_1$) is less strong. In recombination studies, the $R_I(C^R)$ and $R_{III}(E^R)$ loci have been shown to be linked, while the R and O loci segregate independently (COEN *et al.* 1969; BOLOTIN *et al.* 1971; AVNER *et al.* 1973). The agreement between the present results and recombination studies is thus quite satisfactory. Moreover, it seems of interest to be able by means of the analysis of mutational events to detect a linkage between loci which are unlinked by recombination. The mapping of mitochondrial genes has until now been carried out by the study of the frequency of recombination between variously marked ρ^+ strains (COEN *et al.* 1969; BOLOTIN *et al.* 1971; KLEESE, GROTBECK and SNYDER 1972; RANK 1973; AVNER *et al.* 1973; WOLF, DUJON and SLONIMSKI

1973). Macrolesions occurring during the petite mutation may provide an independent method for assessing the topographical relations between mitochondrial genes.

The differential loss of mitochondrial genes during petite mutation

In the RESULTS §II we have shown that the C^R and E^R genetic markers are lost more frequently than the O^R genetic marker. It is even possible that the C^R genetic marker is lost slightly more frequently than the E^R genetic marker. While the former difference is well established, the latter, although statistically significant, is much less prominent. This differential loss may be explained in two ways: it could result either from the very nature of the base sequence in different regions of the mit-DNA or it could be related to the direction of replication. As it has been shown that the distribution of G + C is very heterogeneous along the ρ^+ mit-DNA molecule (BERNARDI *et al.* 1970; PIPERNO, FONTY and BERNARDI 1972; FAYE *et al.* 1973), it is therefore conceivable that the efficiency of the mutagenic process could depend locally on the nature of the sequence. Another attractive hypothesis is that the probability of the loss of a given genetic marker could be related to its distance from the replication initiation site of the mit-DNA. Several other genetic markers will have to be studied in order to improve our understanding of this phenomenon.

The number-of-targets paradox

Several authors have previously attempted to determine the number of mitochondrial genetic units by applying the target theory to the petite induction (SHERMAN 1959; SUGIMURA, OKABE and IMAMURA 1966; MAROUDAS and WILKIE 1968; SLONIMSKI, PERRODIN and CROFT 1968; ALLEN and MCQUILLAN 1969; MAHLER, PERLMAN and MEHROTRA 1971). The number of targets calculated by these authors (between 2 and 20), as well as the one we have estimated (2), is small with respect to the number of mit-DNA molecules per cell, which is generally admitted to lie between 50 and 100 (WILLIAMSON 1969). A number of only 2 targets is even too low to be related to the number of mitochondria per cell.

Several interpretations have been proposed to explain this discrepancy, such as that the molecular target of ethidium is not mit-DNA itself, but rather a membrane compound or complex (MAHLER and PERLMAN 1972) or the number of genetically competent, replicating and/or segregating, mit-DNA molecules is much smaller than the number of physical mit-DNA molecules (cf. master copy hypothesis, MAROUDAS and WILKIE 1968; WHITTAKER, HAMMOND and LUHA 1972).

It seems to us that there is only an apparent paradox, for the target theory is not an adequate method to determine the *absolute* numbers of genetic units, since, as pointed out before, the parameters greatly depend on physiological, enzymatic and other repair processes involved. The apparent number of targets could especially be decreased by genetic recombination.

An hypothesis on the mechanism of petite mutation

We would like to propose a hypothesis which emphasizes the role played by

recombination. It is postulated that during the petite mutation process, mit-DNA molecules which are hit by the mutagen and mutated during the first step in this process recombine with those which remain unaffected; this recombination, if achieved by unequal crossing over or non-reciprocal recombination, may lead, unlike the multiplicity reactivation (marker rescue) phenomenon (LURIA and DULBECCO 1949), to the spreading of the initial errors among almost all the mit-DNA molecules of the cell. This hypothesis is indirectly supported by the following observations: (a) As pointed out before (see RESULTS §I), the mutagenic process probably continues for a long time after the mutagen is removed, and the mutagenic event observed is the final result of the whole process. (b) Recombination is known to be involved in mutation and repair process. (c) Petite mit-DNA molecules are able to recombine with grande mit-DNA molecules (COEN *et al.* 1969; BOLOTIN *et al.* 1971; GINGOLD *et al.* 1969) and even with other petite mit-DNA's (MICHAELIS, PETROCHILO and SLONIMSKI 1973). (d) After U.V. exposure, liquid holding leads to an increase in the yield of petite mutants instead of repair (MOUSTACCHI and ENTERIC 1970). (e) The final products of the process, i.e. the petite mit-DNA molecules, are both greatly deleted and highly repetitive (FAYE *et al.* 1973), which is precisely what one would expect as a result of several rounds of non-reciprocal recombination. Points (a) to (d) are compatible with both reciprocal and non-reciprocal recombination mechanisms, while points (e) favors the latter one. A general discussion of the mechanism of recombination in mitochondria will be given elsewhere (DUJON *et al.* 1974).

A similar mechanism may be involved in another phenomenon, the suppressiveness of petite mutants (EPHRUSSI, HOTTINGUER and ROMAN 1955). Hypotheses involving recombination in suppressiveness have been proposed previously (CARNEVALI, MORPURGO and TECCE 1969; COEN *et al.* 1969; SHANNON, DOUGLASS and CRIDDLE 1972; MICHAELIS, PETROCHILO and SLONIMSKI 1973). If the two processes, petite mutation and suppressiveness, are due to the same basic mechanism, i.e. spreading of errors in the mit-DNA sequence by successive rounds of recombination, the petite mutation process can be visualized as a type of "internal suppressiveness." It would suffice, therefore, to produce one modified mit-DNA molecule in the cell to "suppress" eventually all the remaining normal molecules.

The present work has dealt exclusively with primary mutated clones, only the *inter*-clonal distribution of mitochondrial genetic markers being considered. The *intra*-clonal distribution of the genetic markers, their segregation and heredity stability during mitotic division, will be presented in a separate article.

Some of hypotheses presented in the discussion are rather large extrapolations from the results. Many others, probably, could account as well for the petite mutation mechanism and the discussion is still open.

III. CONCLUSIONS

In conclusion, the results show that:

1) the ρ^+ factor is not a localized part of the mitochondrial genome and must be identified with the whole mit-DNA sequence. Any macrolesion in any part of this sequence leads to ρ^- petite mutants.

2) The $Drug^R$ markers are unambiguously proved to be included within the mitochondrial genome.

3) Petite induction proceeds through large mutagenic events. A single mutagenic event almost invariably overlaps genes linked by recombination and may even overlap genes far enough apart to be unlinked by recombination.

4) Petite induction by ethidium bromide has a differential effect on the loss of the various $Drug^R$ genes studied.

5) Petite induction by ethidium bromide and by U.V. light have many features in common.

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