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Target Analysis of Mitochondrial Genetic Units in Yeast[1]

N. E. ALLEN AND ANTHONY M. MACQUILLAN
Department of Microbiology, University of Maryland, College Park, Maryland 20742

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Target analyses of the induction of ρ^- mutants indicated that the cytoplasm of an aerobic nonrepressed yeast contains approximately 20 mutable units, whereas repressed cells exhibit approximately 3. Aerobic adaptation of fully repressed cells brings about an increase in these cytoplasmic units which continues after maximal respiration has been reached. This increase can be correlated with the increase in numbers of stained mitochondria. The data are interpreted with reference to mitochondrial deoxyribonucleic acid.

Cytoplasmic mutants of *Saccharomyces cerevisiae* designated ρ^- or vegetative petite are characterized as specifically lacking cytochromes *a*, a_3, and *b* (5, 14) and as having a respiratory-deficient metabolism (5). Since these mutants have abnormal mitochondria (27) that frequently contain deoxyribonucleic acid (DNA) differing in buoyant density from that found in the wild-type organelles (7), it is currently believed that the mutation arises from an alteration of mitochondrial DNA.

The fact that these nonchromosomal mutants are readily induced in unusually high frequencies by a variety of mutagenic agents (8) provides an experimental means of calculating the number of genetic units responsible for the ρ^- mutation. Assuming that the ρ factors are associated with the mitochondria, an analysis of this kind might provide an estimate of the average number of mitochondria per cell. Several investigators, by use of acriflavine (19), heat (13), ethidium bromide (17), and ultraviolet (UV; 6) as mutagens, have estimated the number of cytoplasmic genetic units in an aerobic yeast to range between 2 and 8. These figures are substantially lower than the assumed number of mitochondria in this type of cell (2).

Yeast grown anaerobically have been reported to be devoid of mitochondria or to contain only promitochondria-like particles (10, 23, 24). The number of ρ factors in such cells is therefore a question of considerable interest. Maroudas and Wilkie (6) and Wilkie (25) have suggested, on the basis of their UV irradiation experiments, that anaerobically grown yeast retain a single ρ factor.

This paper reports on our use of target analysis, as described by Atwood and Norman (1), to study in respiration-repressed and nonrepressed yeast and during the course of derepression, the disposition of the cytoplasmic genetic determinants now commonly believed to be equivalent to mitochondrial DNA.

MATERIALS AND METHODS

Yeast strain. All experiments were performed with a diploid strain of *S. cerevisiae*, designated ×2180, which was obtained from R. K. Mortimer.

Growth of cells. Both aerobic and anaerobic cells were grown in a biphasic malt extract medium. The biphasic method eliminates the accumulation, during growth, of an undesirable dark precipitate in the medium. One-liter flasks contained 400 ml of 2% malt extract made up with 3% agar. For aerobic nonrepressed cells, 50 ml of sterile distilled water containing the inoculum was added to this basal agar medium. Fully repressed cells were grown anaerobically in the basal medium supplemented with 5% glucose in both agar and liquid phases. Anaerobic growth without added glucose and aerobic growth in the presence of 5% glucose represent intermediate states of repression.

Anaerobic cultures were prepared as follows. After autoclaving, the hot agar medium was flushed with Ultra High Purity Nitrogen (<0.0015% oxygen; Air Products, Inc.) for 5 min at 100 C. The gas was passed through two hot-copper columns to remove traces of oxygen before entrance into the flasks. The flasks were then stoppered; after cooling, they were opened under a nitrogen atmosphere, inoculated with the water or glucose overlay (preflushed with nitrogen), and flushed for an additional 10 min before airtight sealing. Under these conditions, yeast will not grow

[1] This work was performed by N. E. Allen in partial fulfillment of the requirements for the Ph.D. degree at the University of Maryland.

on an acetate or lactate synthetic medium but will grow on a glucose synthetic medium.

All cultures were incubated at 30 C with constant shaking for 72 hr, at which time cultures without glucose supplement contained less than 1% budding cells. Approximately 7% budding cells were found in cultures supplemented with glucose. No clumping was observed.

Irradiation. After growth, cells were first cooled to 4 C, then harvested aerobically and suspended in sterile distilled water to a concentration of 10^6 CFU (colony-forming units) per ml. Samples of 5 ml were irradiated in glass petri dishes with a Westinghouse UV sterilamp, delivering an intensity of 4.5 ergs per mm^2 per sec at an exposure distance of 80 cm. Dose was determined with a UV intensity meter (Ultraviolet Products, Inc.) measuring in the 253.7-nm region.

After irradiation, samples were immediately diluted and plated on the following medium: 0.3% yeast extract, 0.3% malt extract, 0.5% peptone (Difco), 1% glucose, and 2% agar. Between 15 and 20 plates were spread for each irradiated sample, and the average number of colonies counted per dose was 1,785. All operations were performed under a yellow safelight to avoid uncontrolled photoreactivation.

Spread plates were incubated at 30 C for 4 to 6 days, and mutants were scored with the triphenyltetrazolium chloride (TTC)-agar overlay technique (9).

Aerobic adaptation. After cooling to 4 C anaerobically, fully repressed cells were harvested and exposed to oxygen in a medium consisting of 3.0% sodium acetate, 0.5% yeast extract, and 0.4% KH_2PO_4. Cells were suspended to an initial concentration of 2 mg (dry weight) per ml and were adapted in cotton-stoppered flasks in a shaker water bath at 30 C. Under these conditions, the dry weight doubled over a 72-hr period, with a maximum of only 30% budding cells observed at any given time.

Respiration. Oxygen uptake was measured on a Gilson respirometer at 30 C. Cells were washed once and suspended in 0.066 M KH_2PO_4. Q_{O_2} values were calculated as microliters of O_2 uptake per hour per milligram (dry weight) with 0.1 M ethyl alcohol as substrate.

Janus Green B staining. Mitochondria were stained by the method of Avers et al. (3), extending the time of aeration to 1 hr. Particles staining dark blue were counted on a Zeiss photomicroscope at a magnification of 1,560×.

RESULTS

ρ^-Induction in repressed and nonrepressed yeast. Patterns of mutant induction with nonrepressed and fully repressed cells by use of low doses of UV irradiation are shown in Fig. 1. When scoring ρ^- mutants by the TTC-agar overlay technique, a distinction between white and variegated colony phenotypes must be made (5). The latter designation is used to indicate a wide variety of colonial phenotypes and is generally characterized as

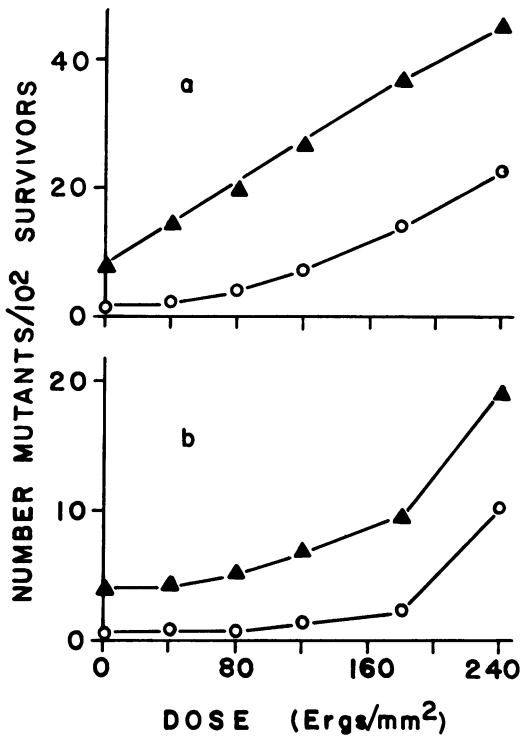

FIG. 1. *UV induction of ρ^- yeast. (a) Induction of white plus variegated colony phenotypes. (b) Induction of white colony phenotypes. Symbols: ▲, fully repressed cells; ○, aerobic nonrepressed cells.*

colonies having both wild-type (red) and mutant (white) sectors.

When scoring both white and variegated colonies as mutants, fully repressed cells exhibit a linear dose response, whereas nonrepressed cells follow a nonlinear relationship (Fig. 1a). Wilkie (25) obtained the same response differences for anaerobically and aerobically cultured yeast. His interpretation was that induction in an aerobically grown yeast exhibited a lag effect due to the presence of several mitochondria, each containing a mutable unit that must be "hit" to produce a ρ^- cell. On the other hand, an anaerobically grown yeast, apparently devoid of mitochondria, retained only a single mutable unit which when inactivated resulted in a ρ^- cell; thus the linear or single-hit response.

We found that if only white-colony phenotypes are scored as mutant, both repressed and nonrepressed cells exhibit multihit induction curves (Fig. 1b). In an attempt to explain these data, we made the following working assumptions (5): (i) a white-colony phenotype will result only when all mutable units within an irradiated cell have been inactivated and (ii) a variegated colony results

when fewer than the total number of units have been inactivated so that a ρ^- cell may arise in some future generation.

Inherent in these assumptions is the idea that cell duplication involves a dilution as well as duplication of mutable units present in the irradiated cell. If all units are inactivated, all progeny must be ρ^- and the colony will be white. However, if only some units are damaged, a cell having only inactivated or defective cytoplasmic units could arise in a future generation. The progeny of this cell will be ρ^-, thus producing a sectored or variegated colony. The degree of sectoring would depend on the number of mutable units originally inactivated in the irradiated cell.

Interpretation of the data in Fig. 1 in terms of these assumptions suggests that, in a fully repressed cell, an average of one unit must be inactivated for expression of the ρ^- phenotype. However, since the induction of white-colony mutants follows a nonlinear dose response (Fig. 1b), a fully repressed cell could conceivably contain several of these cytoplasmic units, albeit a reduced number.

It is possible to estimate the number of genetic determinants by subjecting these data to a method of curve fitting (6), but, because of the low dose responses, it is difficult to obtain a realistic value for the sensitivity of the determinant assumed to be exponentially inactivated in the two classes of cells. After exposure to 240 ergs/mm^2, approximately 90% of the irradiated population is still viable and it is possible to observe the mutation kinetics over a much wider dose range.

Target analysis with repressed and nonrepressed yeast. In an attempt to determine the actual numbers of mutable units, a series of irradiation experiments was conducted employing a wide dose range. Semilog survival curves were plotted. Survival is here defined as the fraction of wild-type (ρ^+) yeast among those cells that have survived UV irradiation. This approach makes possible an interpretation by conventional target theory (1).

If a cell contains a single mutable unit or target, a semilog survival plot should exhibit first-order inactivation according to the expression $N/N_0 = e^{-kD}$, where N_0 is the fraction of wild-type colonies at zero dose and N equals the fraction of wild-type colonies after irradiation with dose D; k is the inactivation constant.

The presence of more than one mutable unit will result in a multitarget survival curve exhibiting a shoulder before exponential inactivation begins. A curve of this type can be expressed by: $N/N_0 = 1 - (1 - e^{-kD})_n$. Extrapolation of the straight line portion to zero dose gives the target number (n), the number of mutable units within a cell.

Wild-type survival (N/N_0) of aerobic nonrepressed yeast is shown in Fig. 2. Two curves are shown. One represents survival of red plus variegated colonies; i.e., only white colonies are scored as ρ^-. This curve has an extrapolation number of approximately 20 (two separate additional experiments gave target numbers of 18 and 25) and, in

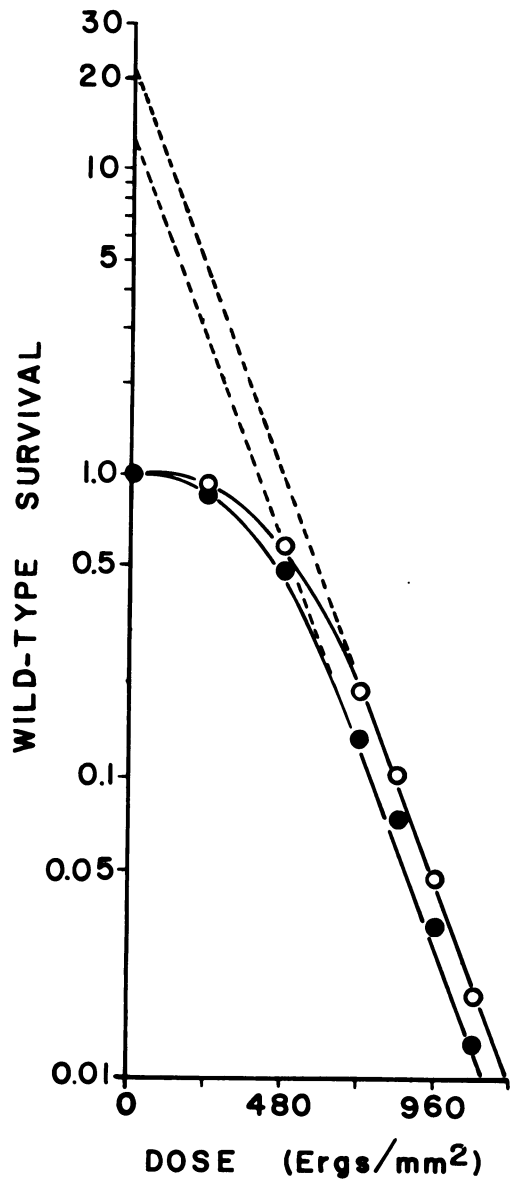

FIG. 2. *Semilog wild-type survival curves for aerobic nonrepressed yeast. Symbols:* ○, *red plus variegated colony survival;* ●, *red colony survival.*

terms of the assumptions made, represents the total number of mutable units in this type of cell. The other curve in Fig. 2 illustrates the survival of red colonies; i.e., both white and variegated are scored as ρ^-. The extrapolation number of 12 is explained as indicating the average number of units that must be inactivated for phenotypic expression.

Cells grown under three different physiological conditions were used to determine the number of mutable units in a repressed cell (Fig. 3). All three states of repression show similarly low extrapolation numbers suggesting that a repressed cell does indeed contain fewer mutable units than the nonrepressed cell. Cells grown under these conditions exhibit differences in respiratory ability (*unpublished data*), yet there seems to be little difference among the three cell types either in extrapolation numbers or in slopes.

The survival data for repressed yeast can be plotted as mutation induction curves (*see* Fig. 1); when this is done, the induction of white plus variegated colonies is always linear at low doses. On the other hand, the analogous target data (red colony survival curves) always exhibit a slight multiplicity (n = 1.4 to 1.8).

Although there appears to be a significant difference in extrapolation numbers between nonrepressed and repressed yeast (Fig. 2 and 3), there is also an apparent difference in slopes. Specifically, the cytoplasmic units of an aerobic nonrepressed cell seem to be more susceptible (k = 0.0066) to the mutagenic action of UV than do the units of a repressed cell (k = 0.0032).

Target analysis during aerobic adaptation. The difference in target numbers between respiration-repressed and nonrepressed cells would lead one to expect that target analysis with cells sampled during derepression would result in a series of curves with increasing values of n. Several investigators have regarded respiratory adaptation as a period of mitochondrial biogenesis (15, 22, 24). If we assume that the target responsible for the ρ^- state is associated with the mitochondrion, then determination of target increases during respiratory adaptation might provide a measure of mitochondrial DNA formation. Therefore, we established conditions whereby fully repressed cells could develop respiratory ability on exposure to oxygen in the absence of extensive growth.

Figure 4a depicts the changes in Q_{O_2} over a 24-hr adaptation period. A maximum rate of 112 μliters of O_2 per hr per mg (dry weight) was reached in 12 hr. Wild-type survival data during aerobic adaptation resulted in a series of curves which were the same as those predicted by target theory (Fig. 4b, c). There is a progressive increase in targets without any noticeable change in sensitivity. This applies to both red plus variegated colony survival (Fig. 4b) and red colony survival (Fig. 4c) curves.

A surprising observation was the time course of

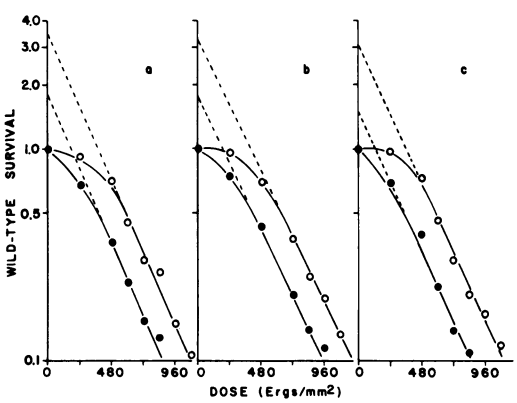

FIG. 3. *Semilog wild-type survival curves for repressed yeast. (a) Cells grown anaerobically with 5% glucose (fully repressed). (b) Cells grown anaerobically without added glucose. (c) Cells grown aerobically with 5% glucose. Symbols* ○, *red plus variegated colony survival;* ●, *red colony survival.*

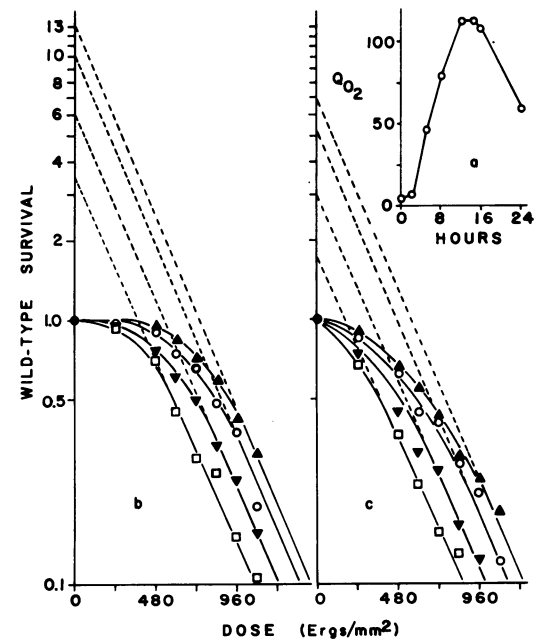

FIG. 4. *Aerobic adaptation of fully repressed yeast. (a) Development of respiratory ability (Q_{O_2}) during aerobic adaptation. (b) Wild-type survival curves: red plus variegated colonies. (c) Wild-type survival curves: red colonies. Symbols:* □, *unadapted cells;* ▼, *12-hr adapted cells;* ○, *24-hr adapted cells;* ▲, *72-hr adapted cells.*

target development. Increases were observed over a 72-hr period. Although respiration reached a peak in 12 hr, the target number less than doubled and continued to increase, whereas respiratory ability dropped.

Although desirable, wild-type survival data at higher doses is impossible to obtain with this strain of yeast because of the lethal effects of UV. Nevertheless, the data appear to fit the curves as they have been drawn. Data for 48-hr adapted cells give an extrapolation number of 12, and the points fall between the 24- and 72-hr curves. These data have not been included in Fig. 4 because of spatial limitations.

Janus Green B staining of particles. Mitochondrial counts with the Janus Green B staining technique were performed on an adapting culture. There is an obvious correlation between increases in stained particle counts (Fig. 5a) and target numbers (Fig. 5b). A considerable range in particle counts per cell was noticed, and values given represent counts averaged from at least 120 single cells.

DISCUSSION

The first question relevant to this study concerns the probable nature of the UV susceptible unit. The high frequency of the mutation induced by UV suggests that the target is the cytoplasmic factor, ρ, which most investigators now associate with the mitochondrion (5, 21, 26). Earlier investigators (11, 25) showed that the action spectrum of UV inducton of the ρ^- state in *S. cerevisiae* points to a nucleic acid, presumably DNA, as the susceptible target. The lower target number observed for repressed yeast, when compared with nonrepressed yeast in the present work, is consistent with recent data which show that the total mitochondrial DNA content of a cell is diminished under conditions of glucose repression (18, 20). The reappearance of targets, increasing in number, during conditions of respiratory adaptation, as well as the high degree of correlation between increased numbers of targets and Janus Green-staining particles, constitute, together with the other observations, presumptive evidence of the identity of targets and mitochondrial DNA.

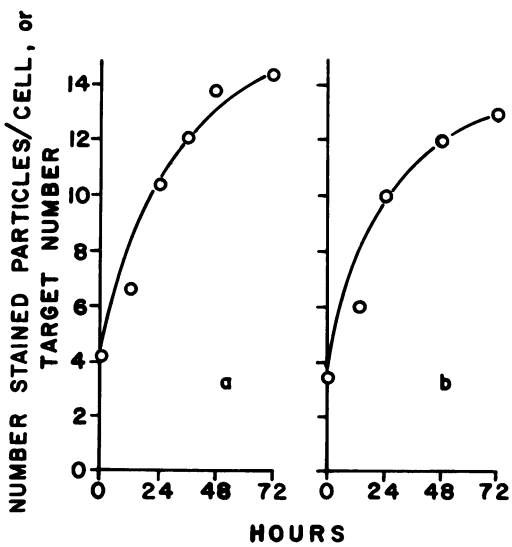

FIG. 5. *(a) Increase in average number of Janus Green B staining particles per cell during aerobic adaptation. (b) Increase in target numbers (from Fig. 4b) during aerobic adaptation.*

Sinclair et al. (16) estimated the weight of one strand of yeast mitochondrial DNA to be 10^7 daltons. If we equate this unit with our target, then 20 such strands would give rise to 3.3×10^{-16} g of mitochondrial DNA per cell, a figure which agrees fairly well with published values (20).

The second question concerns the behavior of the targets under environmental manipulation of the cells. The target analyses support the contention, derived from mutation induction data (Fig. 1), that repressed cells contain fewer cytoplasmic units than nonrepressed cells, but there appears to be more than one unit per repressed cell. It may be argued that our conditions of anaerobiosis were not perfect; however, despite considerable effort, we have not been able to reduce this target number below 3. Red colony survival data approximate single target curves (average n from Fig. 3 equals 1.6), but we assume that this figure merely reflects the average number of units that must be inactivated for phenotypic expression.

The estimate of 20 targets in an aerobically grown yeast suggests a 1:1 correlation between mitochondria and ρ factors. This concept does not agree with recent findings by Maroudas and Wilkie (6) nor with several previous reports (13, 17, 19). It is quite possible that the increased sensitivity observed with nonrepressed yeast (Fig. 2), attributable perhaps to repair phenomena or to secondary effects of UV at high doses, could have caused an overestimation of n. Although we have not yet studied repair in detail, the potential for repair in aerobic and anaerobic yeast has been reported to be quite similar (6). In addition, there was no indication of any significant change in sensitivity during aerobic adaptation (Fig. 4) and fully adapted cells still give a relatively high estimate of n.

If variegated colonies are also scored as ρ^- (red-colony survival), the target numbers for aerobic cells are substantially lowered. Non-

repressed cells (Fig. 2) and fully adapted cells (Fig. 4c) have 12 and 7 targets, respectively. The latter figure agrees with the above-mentioned reports, suggesting 2 to 8 targets in aerobically grown yeast. Nevertheless, we stress that white colony data alone (survival of red plus variegated colonies) should be used to estimate the total number of units present in a cell.

The increase in target number during respiratory adaptation provides, we believe, an indirect means of following mitochondrial DNA formation in the cell. Unfortunately, our method does not provide information concerning early events during adaptation, and other reports (6) suggest that there may be a very rapid rate of DNA synthesis at this time. The fact that the maximum target number in adapting cells was only 13 was not unexpected, since other workers (15) have shown that respiratory adaptation, without good growth, does not reach the level of completely nonrepressed cells.

Of particular interest was the observation that the appearance of targets and the development of respiratory ability occur at different rates, yet targets and Janus Green-staining particles, presumably mitochondria (3), develop concurrently. If targets and mitochondrial DNA are identical, then the acquisition of genetically competent mitochondria lags behind respiratory development. One might account for this observation by assuming that either (i) a functional electron transport system is formed, at least in part, by assembly of preexisting subunits (4, 12), or (ii) transcription and translation of respiratory information provided by mitochondrial DNA proceed at considerably faster rates during adaptation than does replication of mitochondrial DNA. The decline in the ability to respire on ethyl alcohol after 12 hr of adaptation while targets continue to increase may be attributable to some limiting feature of the system which apparently does not influence target formation.

Although target numbers have been emphasized in this report, it should be obvious that experimental limitations do not permit an exact calculation of numbers of cytoplasmic genetic units within a cell. It is also important that these experiments were performed with a diploid strain of *S. cerevisiae*. This strain was particularly suited for the target experiments since it provided mutation responses over a wide dose range; it is conceivable that other strains of yeast may not lend themselves to this type of analysis.

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