Proc. Nat. Acad. Sci. USA
Vol. 72, No. 1, pp. 372–375, January 1975

Complementation in Cytoplasmic *Petite* Mutants of Yeast to Form Respiratory Competent Cells

(mitochondrial DNA/information decay/recombination)

G. D. CLARK-WALKER AND G. L. GABOR MIKLOS

Departments of Developmental Biology and Population Biology, Research School of Biological Sciences, Australian National University, Canberra City, A.C.T. Australia

Communicated by D. G. Catcheside, November 8, 1974

ABSTRACT Complementation has been observed in cytoplasmic respiratory deficient yeast cells (*petites*) to yield respiratory competent diploids. This successful demonstration depended on the use of spontaneous *petites* of recent origin and on crosses involving all possible pairwise combinations between the many different *petite* isolates of opposite mating type. The possibility of deletion of a single unique region of yeast mitochondrial DNA as the initial lesion in *petite* formation has been eliminated by using strains isogenic for their mitochondrial DNA.

In the yeast *Saccharomyces cerevisiae* a spontaneous cytoplasmic irreversible mutation in mitochondrial DNA (the *petite* mutation) converts a cell from respiratory competence (ρ^+) to respiratory deficiency (ρ^-) (1). A feature of *petites* is the occurrence of deletions from the circular mitochondrial genome (ρDNA) (2–4). A mechanism for the generation of these deletions by excision within and insertion between ρDNA molecules has recently been proposed (5, 6). An implication of this hypothesis is that complementation between spontaneous *petites* to give respiratory competent cells should be possible, provided that deletions do not occur from one particular region in ρDNA and that initially the deletions together do not exceed 50% of the combined genomes. However, complementation between cytoplasmically inherited *petites* has never been reported (7–10). Nevertheless, it has been demonstrated in genetic experiments that recombination between ρDNA molecules of different *petites* can occur (11), though ρ^+ progeny were not observed as a consequence. Therefore, complementation could be possible in the suitable circumstances mentioned above.

In this paper complementation between cytoplasmic *petites* is reported. The successful results are due, first, to using spontaneous *petites*, second, to using those of recent origin, and third, to using crosses involving all possible pairwise combinations between the many different *petite* isolates of opposite mating type.

MATERIALS AND METHODS

Yeast Strains. The strain T of *S. cerevisiae* 4342-2B *a ade 8-18 lys2*, was obtained from Prof. S. Fogel, Department of Genetics, University of California, Berkeley. The strain F, having *α adel arg4* and ρDNA isogenic to strain T, was a segregant from a cross of strain T to ATCC 26500, which is *α adel arg4* and completely lacks ρDNA (12). Strains A, *α adel*, and B *α ade2*, closely related in their nuclear genomes, also obtained from Prof. Fogel, were derived by ethyl methanesulfonate mutagenesis of strains X2180 1Aα and X2180 1Bα, which were isogenic except at the mating type locus. Neutral *petites* (ρ^0) of all strains were generated as previously described (13); these *petites* completely lack ρDNA.

Spontaneous Petite Mutants (ρ^-). Spontaneous *petite* mutants were selected by both small colony size and pale appearance compared to the larger buff colored respiratory competent (ρ^+) colonies on GGYP plates (media are described later). To ensure that spontaneous *petites* were newly arisen, cultures stored on silica gel were revived by growth on ethanol YP liquid medium for 16 hr. Cells were plated on GGYP at a density of 100–200 per plate and after 44 hr at 30° *petite* colonies were picked and subcultured into 2 ml of GGYP liquid medium. Approximately 30 *petites* of each mating type were picked per experiment and each *petite* was taken from a separate plate.

Tests for ρ^+ Contaminants and Nuclear Petites. After 10 hr growth in GGYP at 30°, 1 ml of each *petite* isolate was inoculated onto GlyYP solid medium with a pasteur pipette. A further 0.2 ml of the culture was then used for crossing to the opposite mating type neutral *petite*, and after overnight incubation at 30° each sample was inoculated onto both GSyV and GlySyV plates. Plates were scored for ρ^+ after a further 48 hr at 30°, during which time the *petite* isolates were stored at 4°.

Tests for Complementation. In the first experiment the 36 *petite* isolates of strain T which were free of ρ^+ contaminants were pooled into an equal volume of GYP medium (pool T). Similarly the 19 *petites* of strain F were also pooled (pool F). The two pools were then used in three different ways. A quarter of pool T was used for crossing to an equal volume of pool F. After overnight incubation of this mating mixture, samples were inoculated onto GlySyV plates.

As an added precaution against ρ^+ contamination, a further 25% of each pool was crossed to the ρ^0 *petites* of opposite mating type and the mated samples were inoculated onto GlySyV plates after overnight incubation. Additionally the remaining half of each pool was then inoculated onto GlyYP plates. The numbers of unmated and diploid yeasts in the above experiments were determined by plating samples onto GYP and GSyV prototrophic selection media, respectively. All plates were incubated at 30° and scored after 3 days.

In the second and third experiments *petites* were crossed separately rather than being pooled as before. Samples of 0.03 ml of each of 30 *petites* were crossed with 30 *petites* of opposite mating type to produce a 30 × 30 matrix. In ad-

Abbreviations: ρ^+, ρ^-, ρ^0, respiratory competence, deficiency, and neutrality, respectively; ρDNA, the circular mitochondrial DNA of buoyant density 1.684 g/cm^3.

dition each individual *petite* was crossed with ρ^0 and ρ^+. The remainder of each *petite* sample (1 ml) was inoculated onto GlyYP as before.

After overnight incubation each individual cross was inoculated onto GSyV and GlySyV and the plates were incubated and scored as above.

Sporulation and Ascus Dissection. Cultures to be induced to sporulate were grown in GYP liquid medium to late exponential phase and then 0.3 ml was spread on KAc agar. After 4 days at room temperature (23°) asci were dissected with a de Fonbrune micromanipulator after brief treatment with snail digestive tract enzymes (14). Dissected ascospores were incubated for 3 days at 30°, and the samples were resuspended in water and then inoculated onto selective plates to determine nutritional requirements.

Media. GYP and glucose synthetic vitamin medium (GSyV) have been described previously (13). Media containing glycerol (Gly) have 4% w/v glycerol in place of glucose, and GGYP plates have 4% glycerol and 0.2% w/v glucose. Ethanol YP liquid medium was 4% v/v of 96% ethanol in place of glucose. KAc sporulation agar has 5 g/liter anhydrous potassium acetate. All plates are solidified with 1.5% Difco agar.

RESULTS

In any study involving complementation between *petites*, all possible causes of ρ^+ cells other than those arising from complementation between two *petites* need to be eliminated. Possible sources of ρ^+ are the contamination of *petite* colonies by a few viable ρ^+ cells, or the presence of nuclear *petites* with intact ρDNA. Additionally the act of mating itself may induce recombination between separate but reciprocally deleted ρDNA molecules present in a single *petite* cell to form a complete ρDNA which produces a ρ^+ phenotype.

Control experiments

When putative *petite* colonies were sampled and tested for the presence of ρ^+ contaminants, one-third of the colonies (9/29) from strain F yielded ρ^+ cells, whereas strain T gave no contaminated colonies in this experiment and rarely has them. The presence of these ρ^+ contaminants is interpreted as due to a residual core of viable ρ^+ cells at the center of some *petite* colonies.

The other control was performed by mating the individual spontaneous *petite* colonies to neutral *petites* of opposite mating type which completely lack ρDNA. In one case ρ^+ cells were produced from an uncontaminated "*petite*" isolate. Subsequent analysis of this diploid showed that the *petite* phenotype segregated 2:2 in 16 tetrads dissected, indicating that the original phenotype was due to a nuclear mutation. No other "rescue" events besides that due to a nuclear *petite* have been observed in crosses of spontaneous *petites* to neutral ones of the opposite mating type, indicating that the formation of ρ^+ due to recombination of reciprocally deleted ρ DNA present in a single *petite* cell is not a complication.

Experiment 1

Once sources of ρ^+ contamination had been eliminated the remaining isolates of strains F and T were crossed *en masse*, and after overnight growth were plated on selective media to determine if complementation had occurred. Two further controls were carried out. When the pooled *petites* of each particular strain were again analyzed for ρ^+ contamination by inoculating them onto GlyYP media, no ρ^+ cells occurred in 6.6×10^8 viable cells tested in strain F nor in 6.05×10^8 cells of strain T. In the second control the pooled samples of each strain were again crossed to the opposite mating type neutral *petites* and the resulting diploids were scored for the presence of ρ^+ cells. Once again none were detected in 6.9×10^5 and 5.8×10^8 diploids, respectively, from strains F and T.

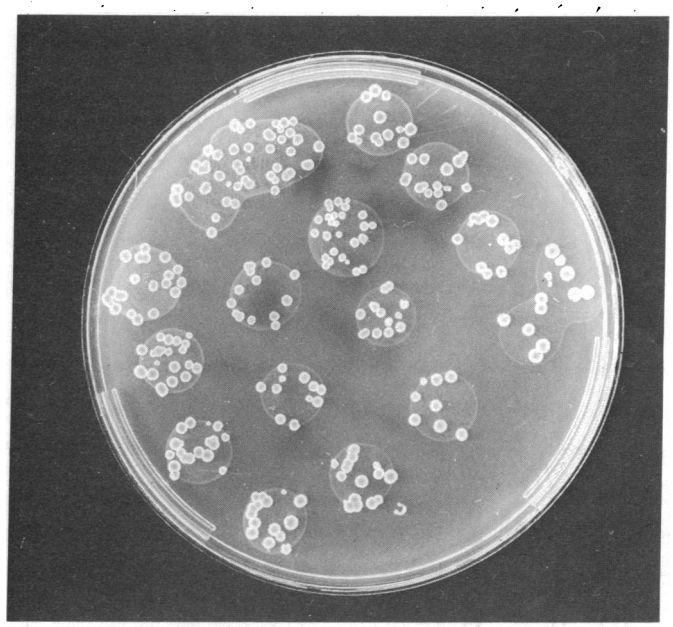

FIG. 1. ρ^+ colonies growing on GlySyV medium after mass mating of ρ^- isolates as described in experiment 1.

After elimination of ρ^+ contamination, complementation was found in the mass mating with a frequency of 20.5 ρ^+ per 10^6 ρ^- diploids (Fig. 1). To verify that ρ^+ diploids were indeed derived from the input parental *petites*, 10 ρ^+ colonies were sporulated and dissection of asci showed that parental markers segregated in the expected 2:2 fashion. Thus, these respiratory competent cells must have arisen from our working, strains; furthermore, all segregants were ρ^+.

Experiment 2

Complementation having been demonstrated in systems of mass mating, it was necessary to discover whether it occurred at a low level in all matings or was characteristic of particular clones. We, therefore, picked 30 putative *petites* of each mating type, and crossed them separately to produce a 30 × 30 matrix. The experiment was performed in this way because of the advantage gained from immediate analysis and the minimization of information decay in the recently arisen spontaneous *petites*.

The results are shown in Fig. 2, where it is seen that the matrix is now 30 × 20 owing to the removal of 10 ρ^+-contaminated *petites* of strain F. Complementation was found in 54 of the 600 successful crosses, corresponding to the open and filled circles of Fig. 2, whereas, 546 crosses gave no complementation. Furthermore, six *petites* out of 30 from strain T and eight *petites* out of 20 from strain F showed no complementation whatsoever.

A repetitive pattern of complementation is also apparent; for example, *petites* 2 and 20 of strain T show complementation in exactly the same crosses. Furthermore, T 4 is a simple "reduction" of T 2 showing fewer, but no new, complementation spots. On the other hand, complementation in T 7 does

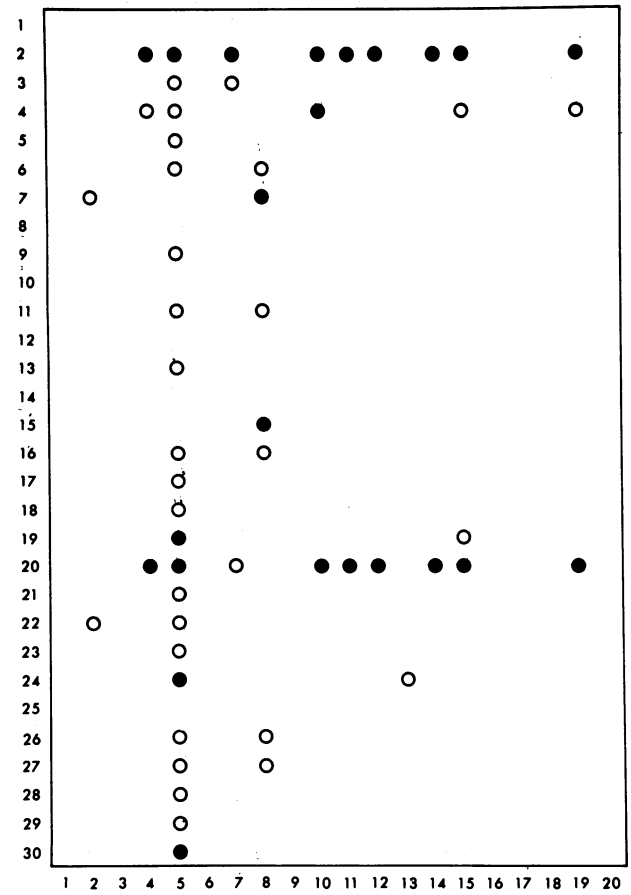

FIG. 2. Complementation pattern observed between individual crosses of ρ^- isolates from strains T (ordinate) and F (abscissa). Open circles indicate fewer than 10 ρ^+ colonies per drop; solid circles more than 10 ρ^+ colonies. Other crosses gave no ρ^+ colonies.

not overlap that in T 2, whereas, T 6 shows points in common with T 2 and T 7.

A further analysis of the data can be made by simplifying and ordering the points of Fig. 2. The 30 × 20 matrix contains a number of duplicates, and when the redundant *petites* are eliminated, a more ordered 11 × 9 matrix can be drawn (Fig. 3). From this diagram complementation maps can be constructed as shown in Fig. 4.

A limited number of ρ^+ complementation diploids was induced to sporulate and asci were dissected. In all nine cases (T parent first), 30 × 5, 24 × 13, 22 × 2, 20 × 5, 20 × 19, 9 × 5, 7 × 8, 4 × 10, and 2 × 15, the parental nuclear markers segregated in the expected 2:2 manner. Additionally it was found that the ρ^+ phenotype segregated 4:0 except in asci derived from 30 × 5, 20 × 5, and 9 × 5, where it segregated 2:2. Furthermore, F 5 did not show any ρ^+ when crossed to the neutral *petite* tester strain. These results show that F 5 is a "double" mutant, being both a nuclear and a cytoplasmic *petite*, and can, therefore, be kept in the matrix.

That only 54 of 600 crosses resulted in some complementation could be due to rapid information decay in *petites*. An indication of this was revealed when the remaining samples of the original *petites* (which had been stored at 4° for 10 days) were regrown and crossed in the same way as before. Under these circumstances, no complementation events were found.

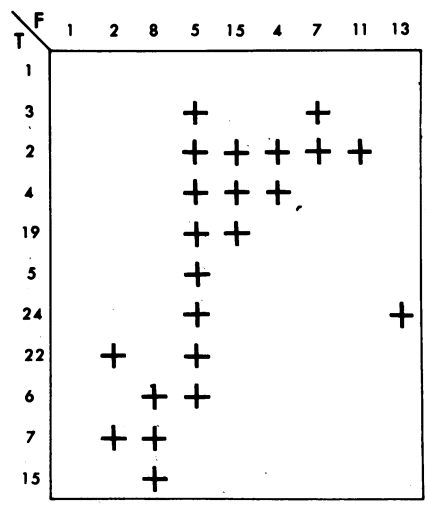

FIG. 3. Ordering of the data of Fig. 2 into a matrix of 11 × 9 *petites* after the elimination of redundant *petities* showing a duplicate pattern.

This repeat experiment, using exactly the same material, is thus an excellent internal control and clearly illustrates that there is a critical time for obtaining successful complementation.

Experiment 3

To determine whether the nuclear genotype exerts specific influences on isogenic mitochondrial DNA, strains A and B, which are closely related in their nuclear genomes, were used in a repeat of experiment 2. It was found that 3 of 150 crosses yielded complementation. The reasons for this lower level, as compared to the previous experiment, may simply be due to the chance presence of T 2, T 20, and F 5 in that experiment, together accounting for 38 of the 54 complementation events.

DISCUSSION

Although complementation to yield respiratory competent cells has been described between nuclear-nuclear (8, 15) and nuclear-cytoplasmic *petites* (1, 8, 16), it has not been reported for cytoplasmic-cytoplasmic *petites* (7–10). However, the present study indicates that in suitable circumstances this last type of complementation can occur. Conditions favoring this observation are the use of spontaneous *petites* with a minimum number of generations after initial *petite* formation and the employment of many pairwise crosses of different *petites*.

In the present study complementation could be due to either of two basic mechanisms. It may be sufficient for the cell simply to have the complete ρ^+ genome present on separate but incomplete ρDNA molecules. Alternatively, the incomplete ρDNA molecules may need to recombine before phenotypic expression is possible. In this regard recombination of antibiotic resistance genes has been demonstrated in crosses of *petites* (11) and biochemical studies have shown recombination of ρDNA from *petites* and wild type (17, 18). Additionally, there may be a necessity for transcription of an entire ρDNA genome for normal expression and, if this is so, recombination between incomplete ρDNA genomes would be a necessary prerequisite for complementation. Pertinent to this is the observation in mammalian mitochondrial DNA that the entire genome is transcribed as a single RNA molecule (19).

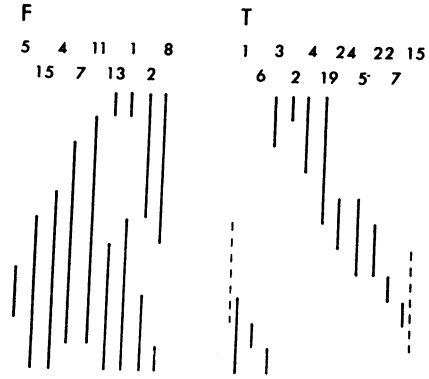

FIG. 4. Complementation maps of *petites* of strains F and T derived from Fig. 3. The solid bars represent regions of deficiency but the T diagram is not unique. The broken lines indicate possible regions of defect which are still consistent with the data. All maps are circular but have been drawn linearly for ease of representation.

Another interesting point is the low level of complementation. The repeat of experiment 2, after storage of the *petites* in the cold, in which complementation did not occur, demonstrates that ρDNA decays and does so rapidly. Thus, the complementation observed in the first part of experiment 2 is due to sampling before this decay has proceeded too far. A second factor which could be depressing the observed frequency of complementation is illustrated by *petite* T 14. This *petite* was highly suppressive when crossed to the F ρ^+ tester and this may contribute to the complete lack of complementation in this isolate. Complementation may, therefore, be potentially much more frequent between *petites*, but be decreased by further recombination between complete and incomplete ρDNA molecules, as has been discussed previously in accounting for the phenomenon of suppressiveness (5).

Complementation has been found in the following three situations; first, between strains not isogenic for nuclear DNA which may not necessarily have identical mitochondrial DNA (experimental details not reported here); second, between non-isogenic strains which do have identical mitochondrial genomes; and third, between strains closely related for both nuclear and mitochondrial DNA. The latter two findings place a number of stringent conditions on possible interpretations and demonstrate clearly that there is no unique site which is initially deleted in the first event of *petite* formation. Furthermore, the finding of complementation in strains having the same mitochondrial DNA eliminates the possibility of each strain's having its own unique initial site involved in ρDNA degeneration.

A further analysis of the complementation data leads to a more ordered representation (Fig. 3) from which complementation maps have been constructed. The maps show that all the *petites* can be represented by single continuous regions of defect. Whether these maps reflect single deletions from the circular ρDNA or have only a diagrammatic meaning remains an interesting question.

Nuclear genes are certainly involved in the breakdown of mitochondrial DNA, since strains T and F, which are isogenic for their mitochondrial DNA, differ in their frequencies of spontaneous *petite* formation, these being 1.5 and 0.5%, respectively. These values led us to enquire whether nuclear genes were involved in determining the specificity of the breakdown of mitochondrial DNA. One possibility could be that the mitochondrial DNA in strain F has a different initial deletion site from strain T, these sites being determined by the nucleus. However, the demonstration of complementation in the third situation focuses attention on the mitochondrial genome itself and indicates that the specificity for the breakdown probably resides in the properties of the mitochondrial DNA.

Given that there is not a single site at which initial deletion can occur, does the breakdown involve a small number of specific sites or is it random? A preliminary statistical analysis of the repetitive patterns obtained in experiment 2 indicates that a small number of specific sites may be a likely possibility.

We thank Craig McArthur for skilled technical assistance and Prof. D. G. Catcheside and Dr. D. J. Daley for critical discussion of the manuscript.

1. Ephrussi, B. (1953) *Nucleo-Cytoplasmic Relations in Micro-Organisms: Their Bearing on Cell Heredity and Differentiation* (Clarendon Press, Oxford).
2. Mounolou, J. C., Jakob, H. & Slonimski, P. P. (1966) *Biochem. Biophys. Res. Commun.* **24**, 218–224.
3. Sanders, J. P. M., Flavell, R. A., Borst, P. & Mol, J. N. M. (1973) *Biochim. Biophys. Acta* **312**, 441–457.
4. Casey, J., Gordon, P. & Rabinowitz, M. (1974) *Biochemistry* **13**, 1059–1066.
5. Clark-Walker, G. D. & Miklos, G. L. G. (1974) *Genet. Res.* **24**, 43–57.
6. Clark-Walker, G. D. & Miklos, G. L. G. (1975) in *The Eukaryote Chromosome*, eds. Peacock, W. J. & Brock, R. D. (Australian National University Press, Canberra), in press.
7. Wright, R. E. & Lederberg, J. (1957) *Proc. Nat. Acad. Sci. USA* **43**, 919–923.
8. Jakob, H. (1965) *Genetics* **52**, 75–98.
9. Roodyn, D. B. & Wilkie, D. (1967) *Biochem. J.* **103**, 3c–5c.
10. Slonimski, P. P., Perrodin, G. & Croft, J. H. (1968) *Biochem. Biophys. Res. Commun.* **30**, 232–239.
11. Michaelis, G., Petrochilo, E. & Slonimski, P. P. (1973) *Mol. Gen. Genet.* **123**, 51–65.
12. Clark-Walker, G. D. (1973) *Eur. J. Biochem.* **32**, 263–267.
13. Clark-Walker, G. D. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 388–392.
14. Johnston, J. R. & Mortimer, R. K. (1959) *J. Bacteriol.* **78**, 292.
15. Sherman, F. (1963) *Genetics* **48**, 375–385.
16. Chen, S.-Y., Ephrussi, B. & Hottinguer, H. (1950) *Heredity* **4**, 337–351.
17. Shannon, C., Rao, A., Douglass, S. & Criddle, R. S. (1972) *J. Supramolecular Struct.* **1**, 145–152.
18. Carnevali, F., Morpurgo, G. & Tecce, G. (1969) *Science* **163**, 1331–1333.
19. Aloni, Y. & Attardi, G. (1971) *J. Mol. Biol.* **55**, 251–266.