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GENETIC CHANGES IN YEAST CELLS COTRANSFORMED WITH MITOCHONDRIAL DNA AND PLASMID YEp13 ARE NOT ELICITED BY RECOMBINANT MOLECULES MADE BY COVALENT INSERTION OF MITOCHONDRIAL DNA INTO YEp13

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SUMMARY: *Saccharomyces cerevisiae* strain DC5-E2 that lacks mtDNA (*leu2 rho°*) can be cotransformed with a mixture of mtDNA and the plasmid YEp13 (*LEU2*/2μ/pBR322) to produce Leu$^+$ transformants which, on being mated to *mit*$^-$ tester strains, generate respiratory competent diploids (such events are denoted marker rescue). In this work strain DC5-E2 was transformed with recombinant molecules consisting of a mtDNA segment including the *oli2* gene inserted into YEp13. The Leu$^+$ transformants made with the recombinant plasmids were unable to rescue *mit*$^-$ testers carrying mutations in the *oli2* region, in contrast to Leu$^+$ cotransformants made with mixtures of YEp13 and *oli2* mtDNA. We conclude that marker rescue events occur as a result of interactions between the mtDNA of the *mit*$^-$ tester and the mtDNA sequences introduced by transformation. Such interactions cannot occur when the latter mtDNA is forced to replicate in covalent association with YEp13, probably in the nucleus.

We are currently developing strategies for the transformation of yeast cells with mitochondrial DNA (mtDNA), which lead to genetic changes in the mitochondrial genome of the recipient cell [1-4]. In a previous study we introduced a cotransformation procedure [3] which involved treatment of cells with a mixture of mtDNA and an independently replicating yeast plasmid vector YEp13 carrying the nuclear *LEU2* gene. The recipient strain DC5-E1 was leucine-requiring (*leu2*) and also devoid of mtDNA [*rho°*]. The transformants were first recognised by their conversion to the Leu$^+$ phenotype through their uptake of the plasmid YEp13; such Leu$^+$ transformants were tested for the presence of mtDNA by mating each haploid clone to a series of haploid *mit*$^-$ tester strains carrying mutations in particular mitochondrial genes. Respiratory competent diploid progeny were observed in crosses between many cotransformants and certain *mit*$^-$ tester strains. (The appearance of such respiratory competent diploids is denoted marker rescue.) When a mtDNA

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segment carrying the *oli2* gene was used in cotransformation experiments of this type, the rescue of *oli2 mit*$^-$ testers was observed to occur in the crosses [3] but the marker rescue patterns were not those expected if the transforming mtDNA was now replicating in mitochondria with the properties of a petite mtDNA genome.

The sequences of YEp13 that are responsible for its autonomous replication in yeast lie within its 2μ plasmid segment [5,6]. The most probable site of replication of YEp13 in yeast is the nucleus, together with the resident population of 2μ circular yeast plasmids [reviewed in ref. 7]. In the cotransformation experiments [3] involving mtDNA and YEp13, it was not clear whether there was any physical interaction between the mtDNA sequences and the YEp13 indicator vector. We address this point in this communication by asking the following question. If a segment of mtDNA is physically integrated into YEp13, can it elicit the same type of genetic effects seen in the cotransformation experiments? To answer this question we constructed derivatives of YEp13 carrying covalent inserts of mtDNA including the *oli2* gene. It was found that, in contrast to the marker rescuing properties exhibited by cotransformants produced by mixtures of mtDNA and YEp13, the Leu$^+$ transformants produced by recombinant plasmids containing mtDNA inserts could not rescue the *mit*$^-$ tester strains.

EXPERIMENTAL

Strain DC5-E2 *a leu2-3 leu2-112 his3 can1-11* [*rho*^{0}] was constructed in the same manner as DC5-E1 [3]. Transformation of strain DC5-E2 with DNA and mating of Leu$^+$ transformants to *mit*$^-$ tester strains were as described previously [3] except that Leu$^+$ clones were selected directly in regeneration-agar lacking leucine [8].

For construction of the recombinant plasmids pSW1 and pSW2 (see Fig. 1), YEp13 was linearized by BamHI cleavage within the tetracycline resistance gene of the pBR322 segment of this plasmid [5]. The largest HaeIII fragment of DS14 (2.2 kb) was blunt-end ligated to BamHI linkers (Collaborative Research, Inc.) and was then separated by agarose gel electrophoresis from the bulk of linkers. After electroelution from the gel, and purification on DEAE-cellulose (DE52) the mtDNA fragment was digested with BamHI, mixed with the linearized YEp13, ligated, and the mixture used to transform *E. coli* ED8654. Ampicillin resistant, tetracycline sensitive transformants were selected following cycloserine enrichment. Colony hybridization using nick-translated ^{32}P-labelled DS14 mtDNA as probe was used to identify those bacterial clones carrying the desired mtDNA inserts. From these clones plasmid DNA was prepared. The orientation of the mtDNA segment was determined by digestion

with EcoRI. Details of methods are described in ref. 9. Recombinant DNA experiments were carried out under CI containment conditions.

Rapid yeast DNA preparations were carried out essentially as described by Davis et al. [10]. Conditions for Southern blots of DNA digests and hybridization to nick-translated labelled probes were described previously [11].

RESULTS

The source of the *oli2* gene used in this work was the mtDNA of the petite DS14 (see Fig. 1). As shown in Table 1 the cotransformation of strain DC5-E2 with YEp13 accompanied by DS14 mtDNA produced Leu^+ cells that rescued *oli2 mit*$^-$ testers M18-5 and M44. The rescue events with these co-transformants occurred at a level three to four times the background of reversion events observed with untreated DC5-E2 cells or Leu^+ transformants made with YEp13 alone. By contrast the cotransformants made with YEp13 and DS14 mtDNA showed no significant rescue of the *oli1 mit*$^-$ tester 2422, or the *oxi3 mit*$^-$ M21 (Table 1).

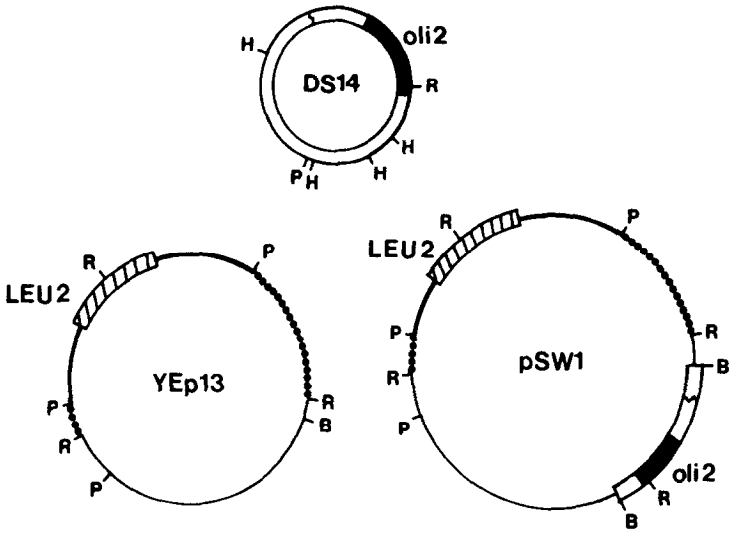

Fig. 1. DNA molecules used in this work. The mtDNA segment from petite DS14 [ref. 17] is shown as a unit circle, although most molecules are arranged as head-to-tail repeats in longer molecules. For construction of pSW1 (12.9 kb), the largest HaeIII fragment (2.2 kb) of the petite DS14 (4.1 kb) was joined to BamHI linkers and cloned into the BamHI site of YEp13 (10.7 kb). Restriction digestion of pSW1 confirmed the structure shown here. pSW2 is equivalent to pSW1 except that the orientation of mtDNA segment is inverted. DNA sequences (not drawn to scale) are represented as follows: ▭ mtDNA; ▬ *oli2* gene; •••• 2μ DNA; ⎯ nuclear DNA; ▨ *LEU2* gene; — pBR322 DNA; ⚡ indicates the site in the mtDNA segment where the deletion event occurred that gave rise to the petite DS14 from parental grande mtDNA. Restriction sites are as follows: H, HaeIII; R, EcoRI; p, PstI; B, BamHI.

TABLE 1: RESCUE OF *MIT*$^-$ TESTER STRAINS BY Leu$^+$ TRANSFORMANTS[a]

DNA used in transformation	Tester *mit*$^-$ strain			
	M18-5 (*oli2*)	M44 (*oli2*)	2422 (*oli1*)	M21 (*oxi3*)
None[b]	4	4	0	0
YEp13	4	4	0	0
YEp13 + DS14	15	14	1	1
pSW1 (YEp13/*oli2*)	4	3	0	0
YEp13 + DS14 HaeIII	14	13	1	0
YEp13 + DS14 PstI	21	11	1	0

[a] Spheroplasts of DC5-E2 were incubated with the DNA indicated. The masses of DNA in each transformation mix were 10 µg for YEp13, pSW1 and 20 µg for DS14 (with or without prior restriction enzyme digestion). Leu$^+$ regenerated cells were selected, then mated to *mit*$^-$ testers. Data shown are the number of respiratory competent diploid patches arising from 122 tested haploids in each case.

[b] Where no DNA was used, 122 single clones of DC5-E2 were mated to each *mit*$^-$ tester.

Leu$^+$ transformants were obtained from DC5-E2 treated with the recombinant plasmid pSW1 carrying the *oli2* gene integrated into YEp13 (Fig. 1). The data in Table 1 indicate that mating these Leu$^+$ cells to *mit*$^-$ testers did not result in frequencies of grande diploids above the background of reversion in control diploids made with Leu$^+$ transformants produced by YEp13, or in diploids made with untreated DC5-E2 cells. The inability to rescue *oli2 mit*$^-$ testers was also observed with Leu$^+$ transformants made with plasmid pSW2 which is similar to pSW1 except that the 2.2 kb mtDNA segment carrying the *oli2* gene has the opposite orientation with respect to YEp13 (data not shown). These results indicate that incorporation of mtDNA into the plasmid YEp13 prevents the manifestation of *mit*$^-$ rescue events that occurs in cotransformation experiments.

It was necessary to verify the presence of the mtDNA sequences in Leu$^+$ transformants produced by pSW1, as the possibility existed that the mtDNA sequences may have been lost from the Leu$^+$ transformants through intermolecular recombination events [see e.g. refs. 12,13]. Cellular DNA was extracted from Leu$^+$ clones, digested with BamHI and the resulting DNA

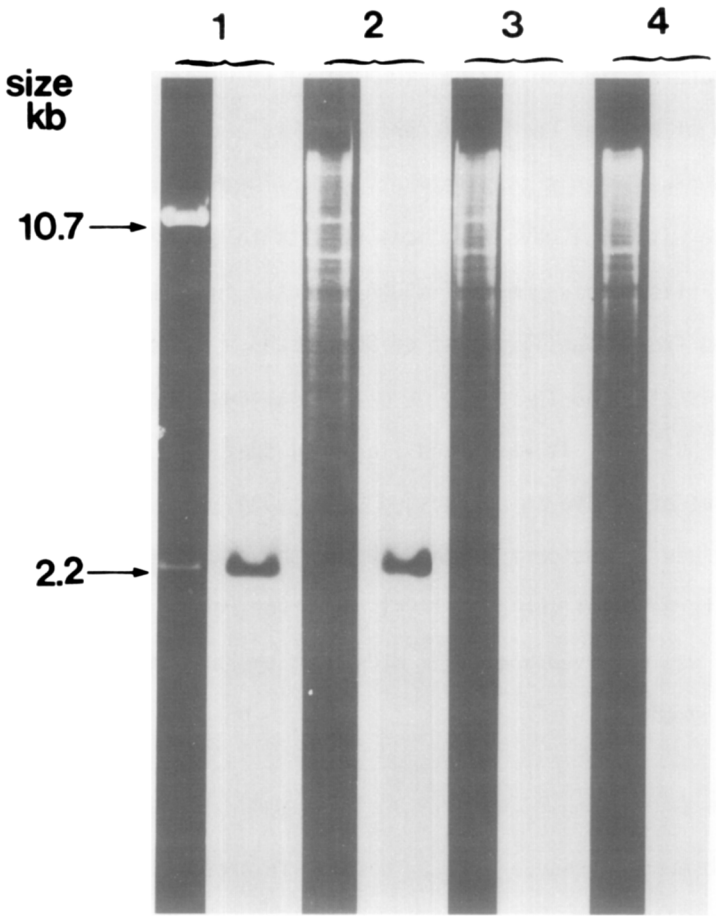

Fig. 2. Demonstration of mtDNA segments inside cells transformed with plasmid pSW1. DNA samples were digested with BamHI and the fragments were separated on 1% agarose gels. In each lane, the left portion shows the ethidium bromide staining pattern; the right portion shows the autoradiogram obtained after Southern blotting and hybridization to ^{32}P-labelled DS14 mtDNA probe. DNA samples were from purified plasmid pSW1 (lane 1) or from the following cellular DNA preparations: Leu$^+$ transformant of DC5-E2 made with pSW1 (lane 2); Leu$^+$ transformant of DC5-E2 made with YEp13 (lane 3); DC5-E2 not transformed (lane 4).

fragments were separated by electrophoresis on agarose gels. Southern blots of these gels were probed with labelled DS14 mtDNA. The results in Fig. 2 show data for one such transformant produced by pSW1. In the digest of DNA of the Leu$^+$ transformant made with pSW1 (Fig. 2, lane 2) the *oli2*-specific mtDNA probe hybridized to a 2.2 kb band that corresponded to the mtDNA segment of plasmid pSW1 itself (Fig. 2, lane 1). No such band of hybridization was observed in DNA from Leu$^+$ transformants made with YEp13 (lane 3), or in DNA from untreated DC5-E2 cells (lane 4). The 10.7 kb YEp13 vector BamHI frag-

ment is clearly visible in the stained gel of DNA of Leu$^+$ cells made with pSW1 (Fig. 2, lane 2) and the 2.2 kb mtDNA BamHI fragment is just visible above the background of nuclear BamHI fragments. This attests to the high copy number of plasmid pSW1 in this transformant. Ten further Leu$^+$ transformants of DC5-E2 made with pSW1 gave identical results to those shown in lane 2.

The particular fragment of mtDNA inserted into YEp13, to produce pSW1, was derived from a HaeIII digest of DS14 mtDNA. We tested the ability of this HaeIII digest to lead to rescue of *mit*$^-$ mutations after cotransformation with YEp13 into DC5-E2. It was found (Table 1) that such a HaeIII digest of DS14 mtDNA was as effective as undigested DS14 mtDNA, when mixed with YEp13, in generating Leu$^+$ transformants that rescue the *oli2 mit*$^-$ testers. It is of passing interest that the same level of marker rescue in a cotransformation experiment was observed with DS14 mtDNA cut by PstI into single genome length (4.1 kb) segments.

DISCUSSION

The absence of rescue of *mit*$^-$ testers in crosses to Leu$^+$ transformants of DC5-E2 made with pSW1 indicates that the mere presence of mtDNA inside a yeast cells is not a sufficient condition to achieve marker rescue. We have not only used pSW1 and pSW2 which have the *oli2* mtDNA inserted into the BamHI site of YEp13 (i.e. in the pBR322 segment), but we have also constructed a series of recombinant plasmids carrying the same 2.2 kb *oli2* mtDNA segment ligated into the HpaI or BclI sites of the 2μ portion of other *LEU2*/2μ/pBR322 composite plasmids [14]. Leu$^+$ derivatives of DC5-E2 made by transformation with each of these vectors were unable to rescue *oli2 mit*$^-$ testers [14]. As it is likely that plasmids such as YEp13 containing 2μ sequences replicate in the nucleoplasm together with the resident 2μ plasmid population [7], the mtDNA segment in the recombinant plasmids would also be confined to a nuclear environment. This would prevent accessibility of these mtDNA segments to the mtDNA of the *mit*$^-$ tester strains. By contrast, in the cotransformation process where mtDNA and the YEp13 plasmid are not joined physically, the

mtDNA would not be constrained in this way and would be free to interact with the *mit*$^-$ mtDNA so as to lead to the production of respiratory competent diploids. The patterns of marker rescue [3] resulting from these interactions are suggested to reflect an increased mutation frequency within the mtDNA genome of the *mit*$^-$ tester strain in the *oli2* region [4].

We have carried out a series of mtDNA transformation experiments using an oligomycin sensitive *rho*$^+$ recipient strain, X4005-11A. Initially, this strain was found to be transformed to oligomycin resistance using a ligation mix consisting of an *oli2* mtDNA segment and 2μ DNA (both linearized with PstI) that had been treated with DNA ligase [1]. We subsequently found [4] that mixtures of mtDNA and 2μ DNA were able to generate oligomycin resistant transformants but recombinant plasmid molecules containing 2μ DNA and covalently linked mtDNA lacked this ability. This finding is consistent with the data presented here and supports the view that genetic changes in the mitochondrial genome of recipient yeast cells can be elicited through cotransformation with mtDNA and molecules containing 2μ DNA.

Finally, we point out that our studies on the mitochondrial genetic effects of mtDNA inserted into yeast plasmids described here are quite distinct from the studies on DNA replication reported by Blanc and Dujon [15] and by Hyman et al. [16]. In these experiments, various mtDNA segments were inserted into plasmids containing only pBR322 and the yeast nuclear *URA3* gene; these particular mtDNA sequences were found to confer upon the recombinant plasmids the ability to be autonomously replicated extrachromosomally but not in mitochondria. There was no reported change in the mitochondrial genome of the transformants carrying these recombinant molecules.

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