Vol. 96, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 30, 1980 Pages 580-586

TRANSFORMATION OF *SACCHAROMYCES CEREVISIAE* WITH YEAST MITOCHONDRIAL DNA LINKED TO TWO MICRON CIRCULAR YEAST PLASMID

Bentley A. Atchison, Rodney J. Devenish, Anthony W. Linnane and Phillip Nagley

Department of Biochemistry, Monash University,
Clayton, Victoria, 3168, Australia

Received August 11,1980

SUMMARY: Mitochondrial DNA from a petite mutant of yeast carrying an oligomycin resistance determinant has been ligated *in vitro* to 2 μm yeast plasmid DNA. The recombinant DNA so produced has been used to transform an oligomycin sensitive strain of *Saccharomyces cerevisiae* to oligomycin resistance at a frequency approaching 50 times the spontaneous mutation rate to oligomycin resistance. The majority of transformants showed genetic properties suggesting that recombination between the transforming DNA and the resident mtDNA has occurred. The properties of a subclass of oligomycin resistance transformants suggested that in these cells the transforming DNA has not become stably integrated into the mitochondrial genome of the recipient cell.

INTRODUCTION

Transformation of *S. cerevisiae* using segments of yeast nuclear DNA cloned in bacterial plasmids has recently been achieved in experiments involving several nuclear genes [1-4]. The efficiency of transformation was greatly enhanced if the segment of yeast nuclear DNA was linked to DNA sequences from 2 μm circular yeast plasmid [2,3,5]. As part of a continuing study of the structure and function of mitochondrial DNA (mtDNA) in yeast [6,7], we have applied the technique of yeast transformation to experiments involving mitochondrial genes. The transformation of yeast with mtDNA sequences should provide a novel approach to studying the replication and expression of mtDNA in yeast. In this communication we report the transformation of oligomycin sensitive cells of yeast to oligomycin resistant cells using mtDNA linked *in vitro* to 2 μm circular yeast plasmid.

MATERIALS AND METHODS

Yeast Strains: Strain J69-1B α *ade1 his* [*rho+*] has been described previously [8]. The oligomycin sensitive strain X4005-11A *a leu2 met5* [*rho+*] was obtained from the Donner Laboratory, Berkeley. The petite strain 70M-J

0006-291X/80/180580-07$01.00/0

[*rho*⁻ *oli2*3-*r*] was derived from strain 70M *a ade1 lys2 trp1* [*rho*⁺ *oli2*3-*r*][8]. The *oli2*3-*r* mutation lies in the *oli2* gene coding for the 20,000 dalton (subunit 6) polypeptide of the mitochondrial ATPase [9].

Growth Media: Media for culture of yeast strains, were as described [10]; in brief: YEPD contained yeast extract (1%), peptone (2%), glucose (1%); YEPE contained yeast extract (1%), peptone (2%), ethanol (2%). Where required, media were solidified by addition of agar (1.5%). YEPE-oli plates were YEPE plates containing oligomycin (2 µg/ml).

Isolation of DNA: Yeast mtDNA was purified by equilibrium centrifugation in KI gradients of DNA obtained from lysed protoplasts, as previously described [11]. Two micron circular yeast DNA was isolated by lysis of spheroplasts of strain J69-1B as outlined by Livingston and Kupfer [12] and further purified by equilibrium centrifugation in CsCl-ethidium bromide gradients.

Preparation of recombinant DNA: Samples of mtDNA of petite 70M-J, and 2 µm plasmid DNA from strain J69-1B were separately digested in a volume of 30 µl of 10 mM $MgCl_2$, 50 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, pH 7.5 for 1-2 h with sufficient *Pst*I restriction enzyme (Boehringer-Mannheim) to ensure complete digestion of the DNA. Yeast transfer-RNA (5 µg in 100 µl, 10 mM Tris-HCl, 1 mM EDTA, pH 8) was added and the mixtures extracted with 100 µl chloroform-isoamyl alcohol (24:1) for 5 min. After a brief centrifugation to clarify the two phases, nucleic acids were precipitated from the aqueous phase by the addition of two volumes of ethanol. For ligation, mixtures of *Pst*I-digested 2 µm circular yeast DNA (1 µg) and *Pst*I-digested mtDNA (2 µg) in 10 µl of 30 mM $MgCl_2$, 1 mM EDTA, 10 mM Tris-HCl, pH 8 were allowed to stand at 0°C for 2 h. ATP (final concentration 0.2 mM), dithiothreitol (10 mM) and one unit of T4-ligase (Miles) were added and the incubation continued at 15°C for 20 h. Nucleic acids were then precipitated with two volumes of ethanol (-70°C for 1 h) and were then used to transform spheroplasts of strain X4005-11A.

Yeast Transformation: The procedures used were modified from Hinnen et al. [1] and Gerbaud et al. [5]. Cultures of the recipient yeast strains were grown to mid-log (approximately 2 x 10^7 cells/ml) in YEPD. Samples (10 ml) of the culture were washed with 1 ml of water and then resuspended in 1 ml 1.2 M sorbitol containing 0.2 mg zymolase 60,000 (Kirin, Japan). The suspension was then incubated at 28°C for 30-45 min; the formation of spheroplasts was followed optically by lysis of diluted portions in 0.5% sodium dodecyl sulphate. Spheroplasts were collected by centrifugation, washed three times with 1.2 M sorbitol, and then once with 1 ml 1.2 M sorbitol, 5 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5. The spheroplasts were resuspended in 50 µl of a solution containing DNA dissolved in 1.2 M sorbitol, 5 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5. After incubation of this mixture for 10 min at 28°C. 500 µl of 40% polyethylene glycol 4000, 5 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5 was added and the incubation continued for a further 15 min. Spheroplasts were centrifuged, resuspended in 500 µl 1.2 M sorbitol, 0.4% yeast extract, 0.6% peptone, 0.6% glucose, 5 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5 and then incubated at 28°C for 1 h. Samples (100 µl) of the spheroplasts were then added to 7 ml regeneration agar (1.2 M sorbitol, 0.67% yeast nitrogen base, 0.03% yeast extract, 0.06% peptone, 2% glucose, 3% agar) maintained at 45°C and poured immediately onto YEPD plates containing 1.2 M sorbitol. After 2-3 days incubation at 28°C, the agar was chopped into pieces about 2 mm^3 and suspended in water, using 100 ml for 2 plates. The agar fragments were removed by filtration through three layers of muslin and the cells collected by centrifugation before being plated on selective media.

RESULTS AND DISCUSSION

The segment of mtDNA that was used for transformation experiments was derived from a petite mutant 70M-J carrying an oligomycin resistance mutation that affects a polypeptide (subunit 6) of the mitochondrial ATPase. This petite retains a segment of the mitochondrial genome 8.6 kilobase pairs (kb) in length, representing about 11% of the wild-type genome. There is one *PstI* cleavage site in this segment of mtDNA (C. Bingham, unpublished data). After cleavage of 70M-J mtDNA with *PstI*, it was ligated *in vitro* to 2 μm yeast plasmid DNA (cleaved at its unique *PstI* site [2]) to produce recombinant DNA.

Recombinant DNA was incubated with spheroplasts of the oligomycin sensitive yeast strain, X4005-11A, under conditions previously described to allow transformation of yeast cells [1,5]. After regeneration of spheroplasts in non-selective medium, cells were collected and plated onto YEPE-oli plates at about 10^6 cells per plate for selection of oligomycin resistant cells. Other samples of the regenerated cells were diluted and spread on YEPE plates for measurement of the number of respiratory competent cells. The results of three independent experiments of this type are shown in Table 1; frequencies of oligomycin resistant cells ranging from 2.5 to 6 per 10^5 respiratory competent cells were observed amongst the cells regenerated from the spheroplasts treated with recombinant DNA. By contrast, spheroplasts of strain X4005-11A treated with 2 μm plasmid DNA alone did not give rise to detectable oligomycin resistant cells (less than 0.3 per 10^5 cells). The spontaneous mutation rate to oligomycin resistance of vegetative cells of strain X4005-11A was found to be 0.12 per 10^5 cells as determined by plating portions of cells directly from control liquid cultures in YEPD medium. The results of two other controls are shown in Table 1, experiment 3. Spheroplasts treated with 70M-J mtDNA that had been *PstI*-cleaved then ligated, did not give rise to detectable oligomycin resistant cells. Only one oligomycin resistant colony was obtained when spheroplasts of strain X4005-11A was

TABLE 1: TRANSFORMATION OF STRAIN X4005-11A TO OLIGOMYCIN RESISTANCE[a]

Experiment No.	DNA added to spheroplasts	Oligomycin resistant cells per plate[b]	Grande cells per plate ($\times 10^{-5}$)[c]	Frequency of *oli-r* cells (per 10^5 grande cells)
1	70M-J/2 μm DNA (recombinant DNA)	34	6.2	5.5[d]
	2 μm DNA, *Pst*I cleaved and ligated	0	4.8	<0.21
2	70M-J/2 μm DNA (recombinant DNA)	35	5.8	6.0
	2 μm DNA, *Pst*I cleaved and ligated	0	5.2	<0.19
3	70M-J/2 μm DNA (recombinant DNA)	12	4.7	2.5
	2 μM DNA, *Pst*I cleaved and ligated	0	4.1	0.24
	70M-J mtDNA, *Pst*I cleaved and ligated	0	3.4	<0.29
	70M-J mtDNA, undigested	1	5.5	0.18

a Cells of strain X4005-11A were converted to spheroplasts, treated with DNA, then regenerated on non-selective medium as described in Materials and methods. Suspensions of regenerated cells recovered from two duplicate regeneration agar plates were plated on YEPE-oli plates, and after dilution, onto YEPE plates.

b Mean of two duplicate YEPE-oli plates. All oligomycin resistant cells were tested for nuclear markers and were found to be auxotrophic for leucine and methionine.

c The number of respiratory competent cells calculated to have been plated on the YEPE-oli plates, as determined from diluted samples plated on YEPE plates.

d In this experiment, the total number of colonies on each regeneration agar plate was determined to be 8×10^5. This was measured by diluting spheroplasts in 1.2 M sorbitol before addition to regeneration-agar (data not shown). Since the measured frequency of oligomycin resistant cells recovered from the plates was 5.5 per 10^5 cells, it can be calculated that each regeneration-agar plate in this experiment contained on average $(8 \times 10^5) \times (5.5 \times 10^{-5}) = 44$ oligomycin resistant transformed colonies.

treated with undigested 70M-J mtDNA, representing a calculated frequency of oligomycin resistant cells of 0.18 per 10^5 cells, which is close to the spontaneous mutation rate. It is thus concluded that when 70M-J petite mtDNA is linked to 2 μm circular DNA, and incubated with spheroplasts of

strain X4005-11A, oligomycin resistant transformants can be reproducibly obtained at a frequency 15-50 times greater than the spontaneous rate of mutation of strain X4005-11A to oligomycin resistance.

Transformation frequencies may also be expressed in terms of the number of transformants obtained per μg DNA. Thus, in experiment 1 shown in Table 1 [see footnote (d)], 44 transformed oligomycin resistant colonies had been present on each regeneration-agar plate. Since the cells on one regeneration-agar plate arose from spheroplasts treated with 0.2 μg plasmid DNA ligated to mtDNA, a minimal estimate of the transformation frequency is 220 transformation events per μg of 2 μm plasmid DNA under the conditions used here.

Conditions for obtaining oligomycin resistant transformants were found to be stringent, and the nature of the host strain is critical. Many strains apart from X4005-11A were found, for various reasons, to be unsuitable for transformation with mtDNA [13]. Several strains failed to regenerate under any conditions tested, whilst some yeast strains were subject to almost quantitative petite induction during the regeneration procedure. This latter effect precludes experimentation where an intact mitochondrial genome is required for the function of transforming mtDNA sequences, but this would not have interfered with previously reported experiments involving nuclear genes [1-5].

One stringent condition applying in the present mitochondrial transformation experiments relates to the procedure used for selecting oligomycin resistant transformants. It was found that when recombinant DNA-treated spheroplasts of strain X4005-11A were regenerated in 3% agar containing ethanol as a carbon source, plus oligomycin as a selective agent, no transformant colonies were obtained. Although spheroplasts of strain X4005-11A regenerated efficiently in regeneration-agar containing ethanol instead of glucose [13], the presence of oligomycin apparently places potential transformants under too great a selection pressure to permit them to form colonies.

Therefore, it was necessary to regenerate spheroplasts under non-selective conditions followed by subsequent plating of cells on YEPE-oli plates.

The demonstration of the transformation of strain X4005-11A to oligomycin resistance raises a number of interesting questions as to the site of replication of the segment of 70M-J mtDNA in the transformed yeast cell and the manner in which the oligomycin resistance is expressed. A number of possibilities for the fate of the transforming DNA may be envisaged. First, the 70M-J mtDNA may recombine with the resident mtDNA and become integrated stably into the mitochondrial genome. Second, the 70M-J/2 μm recombinant DNA may replicate within mitochondria, but not recombine with the host mtDNA. Third, the 2 μm DNA component of the recombinant transforming DNA may promote autonomous replication of the 70M-J mtDNA segment in the nucleus (and/or cytoplasm), as has been shown for 2 μm DNA linked to fragments of nuclear DNA [2,3]. Experiments to determine the intracellular location of the 70M-J and 2 μm segments of the recombinant DNA in transformants are currently in progress. It is clearly important to establish whether DNA entering the cytoplasm of a spheroplast can be taken up by the mitochondria.

Our preliminary genetic analyses have indicated that the transformed clones may be divided into three classes. The initial transformed clones obtained from the YEPE-oli plates (Table 1) were transferred to fresh YEPE-oli plates, and it was found that about 5% of such colonies failed to grow. In the remaining transformants the oligomycin resistance was more stable to repeated sub-culturing of cells on YEPD or YEPE-oli plates. However, amongst these transformants two subclasses could be distinguished. The first subclass (comprising about 75% of transformants) are quite stable and fail to segregate oligomycin insensitive cells. Cells in this class behave as if the oligomycin resistance determinant is stably integrated into the mitochondrial genome. Thus, when these transformants were mated to a *rho*$^+$ *oli-s* haploid strain, both oligomycin resistant and oligomycin sensitive diploids were produced in almost equal proportions, which is

characteristic of mitochondrial genetic behaviour [see 14]. Moreover, when these transformants were mated to a *rho°* haploid strain (a petite mutant lacking mtDNA), only oligomycin resistant diploids were observed. By contrast, the remaining 20% of transformants were moderately unstable, and segregate oligomycin sensitive cells at frequencies of the order of 1-10% after 10 generations of growth. The resistant cells, on being crossed to both *rho*$^{+}$ and *rho°* haploids, produced mixtures (in roughly equal proportions) of oligomycin sensitive and oligomycin resistant diploids. This finding suggests that the oligomycin resistant determinant is not stably integrated into the mitochondrial genome and may have some other site of replication in the transformed cell.

Acknowledgements. The authors thank Mr. I. Macreadie and Mr. C. Bingham for the isolation and characterization of petite 70M-J. This work was supported by the Australian Research Grants Committee (D2 78/15236).

REFERENCES

1. Hinnen, A., Hicks, J.B., and Fink, G.R. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 1929-1933.
2. Beggs, J.D. (1978) Nature, 275, 104-109.
3. Struhl, K., Stinchcomb, D.T., Scherer, S., and Davis, R.W. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 1035-1039.
4. Hsaio, C-L., and Carbon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 3829-3833.
5. Gerbaud, C., Fournier, P., Blanc, H., Aigle, M., Heslot, H., and Guerineau, M. (1979) Gene, 5, 233-253.
6. Linnane, A.W., and Nagley, P. (1978) Plasmid, 1, 324-345.
7. Borst, P., and Grivell, L.A. (1978) Cell, 15, 705-723.
8. Murphy, M., Choo, K-B., Macreadie, I., Marzuki, S., Lukins, H.B., Nagley, P., and Linnane, A.W. (1980) Arch. Biochem. Biophys. (in press).
9. Roberts, H., Choo, W.M., Murphy, M., Marzuki, S., Lukins, H.B., and Linnane, A.W. (1979) FEBS Lett. 108, 501-504.
10. Linnane, A.W., Kellerman, G.M., and Lukins, H.B. (1975) In: Techniques of Biochemical and Biophysical Morphology (Glick, D., and Rosenbaum, R., eds.) Vol. 2, pp.1-98, John Wiley & Sons, New York.
11. Atchison, B.A., Choo, K-B., Devenish, R.J., Linnane, A.W., and Nagley, P. (1979) Mol. Gen. Genet. 174, 307-316.
12. Livingston, D.M., and Kupfer, D.M. (1977) J. Mol. Biol. 116, 249-260.
13. Nagley, P., Atchison, B.A., Devenish, R.J., Vaughan, P.R., and Linnane, A.W. (1980) In: Organization and Expression of the Mitochondrial Genome (Kroon, A.M., and Saccone, C., eds.) North-Holland, Amsterdam (in press).
14. Nagley, P., Sriprakash, K.S., and Linnane, A.W. (1977) In: Advances in Microbial Physiology (Rose, A.H., and Tempest, D.W., eds.) Vol. 16, pp.157-277, Academic Press, London.