5. SUMMARY AND CONCLUSIONS

The work involves two aspects; transformation of *Saccharomyces cerevisiae* with (1) isolated mitochondria, and (2) mitochondrial DNA (mtDNA). Extensive natural polymorphism of the mitochondrial genes seen among different *S. cerevisiae* strains has enormously contributed to a better understanding of the mitochondrial gene structure, organization and expression (Dujon, 1981). This polymorphism can be further increased if the mitochondrial genomes of related yeast species can be transferred into *S. cerevisiae*. An efficient mitochondria mediated transformation technique is essential for this purpose. In order to develop an efficient procedure, attempts were made to repeat a paper (Tuppy and Wildner, 1965) where a very high transformation frequency was claimed, and when that failed, to improve upon the procedures reported for mitochondria-spheroplast fusion (Gunge and Sakaguchi, 1979; Yoshida, 1979).

Tuppy and Wildner (1965) have reported the transformation of respiratory-deficient (*rho−*) spheroplasts
to respiratory sufficiency with mitochondria isolated from a respiratory sufficient (rho+) strain. They have claimed a transformation frequency of 2.6% without using any fusogen and without employing the optimal conditions reported for spheroplast regeneration. We could not successfully repeat their work. They have failed to mention certain crucial details in their paper and therefore it is not clear as to what extent the procedures used by us are similar to the method employed by them.

Gunge and Sakaguchi (1979) and Yoshida (1979) have employed the PEG-CaCl₂ procedure for mitochondria-spheroplast fusion. The PEG solutions used by them also contained 0.8 M sorbitol. Sorbitol was present apparently as an osmotic support. However, for fungal protoplast fusion, the presence of additional osmotic supports in PEG solutions was found inhibitory (Ferenczy et al., 1976). In our mitochondria-spheroplast fusion experiments, we examined the effect of additional osmotic supports on transformation frequency. About 20 fold more number of transformants were obtained when osmotic supports were omitted from the PEG solution.
DNA mediated transformation techniques facilitate reverse genetics. In classical genetics, mutants affecting the phenotype of an organism is first isolated followed by further studies until gene expression is understood at the molecular level. On the other hand, in reverse genetics the DNA is mutated in vitro and the phenotypic consequences of the mutation is examined after introducing the mutant DNA into the organism. The development of a transformation technique for the yeast nuclear genes has ushered in a new era of yeast molecular biology (Struhl, 1983). Unfortunately, no reliable transformation technique is available for the mitochondrial genes. We have attempted to transform *S. cerevisiae* with isolated mtDNA.

Our experimental design was such that (1) the problem posed by the presence of 50-100 mtDNA molecules in each cell would be partially alleviated and (2) true transformants could be distinguished from spontaneous mutants. The mtDNA donor strain was a rho− petite which retained, in an amplified form, a 4 kb long segment of the large rRNA region of the mitochondrial genome. It had three closely linked mitochondrial
markers: $C_{321}^R$ (chloramphenicol resistance), $E_{354}^S$ (erythromycin sensitivity) and $\omega^+$. It could recombine its markers at high frequency with $\rho^+$ mitochondrial genomes in crosses. The mtDNA was purified through CaCl$_2$-bisbenzimide gradients and the identity of the purified preparation was confirmed through restriction endonuclease mapping.

A mtDNA-less ($\rho^0$) and a $\rho^+, C_{321}, E_{354}^R, O_1^+$, $\omega^-$ strain were used as recipients. The $\rho^0$ strain (DC5-0) was obtained from a highly transformable $\rho^+$ strain (DC5), through ethidium bromide mutagenesis. The $\rho^+$ strain used in transformation (5-841) was constructed through cytoduction; its nuclear genome was that of DC5-0 and the mitochondrial genome was from a strain which had the desired mitochondrial markers. Its $\omega^+$ allele was determined through standard crosses with $\omega^+$ and $\omega^-$ testers. The spontaneous mutation of 5-841 to chloramphenicol resistance was checked. The frequency was low when the selection was done on plates containing 4 mg/ml chloramphenicol compared to the plates with 2 mg/ml chloramphenicol. The $C^R$ mutants were sensitive to
4 mg/ml chloramphenicol and it was apparently due to the *cis* effect of $E_{354}^R$ mutation on $C^R$ mutations.

A cotransformation strategy was used. Transformation was done with a mixture of mtDNA and YEpl3 plasmid DNA. YEpl3 carried the yeast LEU2 gene and could transform the leu2 recipient strains to leucine prototrophy at high frequency ($5-50 \times 10^3$ transformants per µg plasmid DNA). The leu$^+$ transformants were first selected and then they were checked for the simultaneous transformation of mitochondrial genes. The leu$^+$ transformants of 5-841 were checked for chloramphenicol resistance. The $C^R$ clones obtained were subsequently checked for erythromycin sensitivity. The leu$^+$ clones of DC5-0 were first crossed with a rho$^+$ $C_{321}^S E_{354}^R$ tester strain. The diploids obtained were checked for chloramphenicol resistance. The $C^R$ diploids were subcloned and checked for erythromycin sensitivity.

The $C^R$ clones obtained could best be explained as spontaneous mutants rather than as true transformants for the following two reasons: (1) The number of $C^R$ clones obtained was not correlated with the amount of mtDNA used in the experiments, and (2) all
the $C^R$ clones were found to be resistant to erythromycin. Since the transforming mtDNA carried the closely linked $C^R_{321}$ and $E^S_{354}$ markers, most if not all of the true transformants were expected to be sensitive to erythromycin. The negative results were not due to the possible presence of any nuclear suppressor of $C^R_{321}$ mutation. By constructing a strain with the DC5-0 nuclear genome and a $\text{rho}^+ C^R_{321}$ mitochondrial genome, we could show the expression of $C^R_{321}$ in DC5-0 nuclear background.

The contradiction between our results and the positive results of Linnane's group could not be reconciled unless the transformants obtained by Linnane's group were explained as spontaneous mutants. In most of their experiments, they employed the cotransformation strategy and used a $\text{rho}^0$ recipient strain. Transformation was done with a mixture of YEpl3 DNA and mtDNA. A petite strain which had retained the entire $\text{oli2}$ gene was the source of mtDNA. The $\text{leu}^+$ transformants were crossed with $\text{mit}^-$ testers (the $\text{mit}^-$ mutations were in the $\text{oli2}$ gene). The diploids were checked for respiratory sufficiency (marker rescue). Positive marker rescue events were often considered as due to true
mitochondrial transformation. Marker rescue experiments of Linnane's group could be considered as unreliable for the following reasons: (1) Marker rescue was observed even for clones obtained from no-DNA control experiments (Woo et al., 1982; Nagley et al., 1983). (2) The \textit{leu}^+ clones which initially rescued one set of \textit{mit}^- testers, on subcloning rescued a different set of \textit{mit}^- testers (Vaughan et al., 1980). (3) Some \textit{leu}^+ clones which initially failed to rescue any \textit{mit}^- testers, on subcloning rescued several of the testers (Vaughan et al., 1980). (4) Rescue was observed even for \textit{mit}^- mutations for which the transforming mtDNA did not have any wild type equivalent (Nagley et al., 1983). All the above features could be explained only if it were assumed that the rescue events were all due to spontaneous reversion of \textit{mit}^- testers; no other explanation could consistently account for the above rescue events.