CLONING OF MITOCHONDRIALLY DETERMINED OLIGOMYCIN RESISTANCE, *oli-1* IN YEAST

TADASHI MABUCHI and KAZUHIKO WAKABAYASHI

Department of Biochemistry, Medical School, The University of Yamanashi
Tamaho, Nakakoma, Yamanashi 409-38, Japan

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An *Escherichia coli*-yeast shuttle vector pOL 221 is described, which consists of pBR 322 and a yeast mitochondrial fragment. The mitochondrial insert in pOL 221 possessed a mitochondrial autonomously replicating sequence (*ARS*) and a single *Sst* II site. The *ARS* function of pOL 221 was demonstrated by insertion of *LEU2* gene into pOL 221 (giving pOL 2211) followed by the transformation of leu$^-$ yeast cells.

By use of this vector, chimeric plasmids pOL 37 and pOL 379 (a derivative of pOL 37 inserted with *LEU2* gene), carrying the yeast mitochondrial *oli-1* gene were constructed. Marked instability was observed on the maintenance of pOL 37 in *E. coli* under non-selective conditions. pOL 37 can, however, be maintained in *E. coli* in the presence of tetracycline. pOL 2211, pOL 379 and other pOL plasmids showed mitotic instability in yeast. Transformants of oligomycin sensitive yeast cells with pOL 37 and pOL 379 did not show oligomycin resistance. The absence of recombination between the cloned mitochondrial gene and the host mitochondrial gene is discussed.

Extrachromosomal oligomycin resistance in yeast has been reported previously[1,2] and the resistant cells were shown to have altered ATPase activity[2]. From a petite mutant O-111 of yeast which retained mitochondrial oligomycin resistance, *oli-1*^{r}, a 3.9 kb fragment of yeast mtDNA was isolated harboring the *oli-1*^{r} gene[3,4]. Its physical map has been reported[3,4]. The base sequence of the *oli-1* gene has been elucidated[5,6]. The protein coded by this gene plays an important role in the transfer of energy of oxidative phosphorylation in the mitochondrial membrane. Cloning of the *oli-1* gene will contribute to the study of such mechanisms. Cloning genes of F_0 subunits of ATPase in *Escherichia coli* demonstrated that its transformants suffered subsequent inhibition of cell growth[7]. Difficulties were encountered in cloning of *oli-1* gene, since such recombinant plasmids were prone to deletion, possibly due to a strong disadvantage for the host cells.

Due to the high content of AT in yeast mtDNA, only a limited number of restriction sites are available within fragments of suitable length for the cloning of mitochondrial genes. Accordingly yeast-*E. coli* shuttle vectors with a suitable cloning site are convenient. We have constructed such vectors using a combination of pBR 322 and a mitochondrial *ARS*.

We report here the cloning of the *oli-1*^{r} gene. The recombinant is unstable in *E. coli*, but can transform yeast cells and the phenotype of yeast transformants is described.

Materials and Methods

Strains and Media

E. coli, WA802 (F$^-$, *metB*, *lac*, *gal*, *sup*E44, *hsd*R), and C600 (F$^-$, *thi*, *thr*, *leuB6*, *rK*$^-$, *mK*$^-$, *Nal*^{r})

Abbreviations: *oli-1*, oligomycin resistance; *tet*, tetracycline resistance; bp, base pair; kb, kilo base; mtDNA, mitochondrial DNA; *ARS*, autonomously replicating sequence; SSC, saline-sodium citrate.

were used. *Saccharomyces cerevisiae*, O-111 (a, *ade*, *oli-1*^{r}, *rho*$^-$), 706 (a, *his*, *leu*, *thr*, *rho*$^+$), SHY2 (α, *ste*, *ura*, *trp*, *leu*, *his*, *ade*, *rho*$^+$) and AH22 (a, *leu*, *his*, *can1*, *rho*$^+$) were used. L broth and M9 media[8], YPG and YPY media[9] were as described. L broth was supplemented with tetracycline at 10 μg/ml for selection of tetracycline resistant transformants. Minimal media, used for regeneration of yeast spheroplasts after transformation are as described[4].

Biochemicals

Restriction endonucleases, *Sst* II, *Alu* I, *Eco*R I, *Bam*H I, *Hae* III, *Hinc* II, *Hinf* I, *Hpa* II and *Pst* I were from commercial sources. T4 DNA ligase was purchased from Bethesda Research Laboratory and Takara Shuzo, Japan. Zymolyase 60000 was obtained from Kirin Brewery, Japan. [α-^{32}P]dTTP was supplied from Amersham Japan. The conditions for enzyme reactions were as recommended by suppliers.

Preparation and Electrophoresis of DNA

O-111 cells were digested with Zymolyase 60000 and spheroplasts were washed with SET solution[3], suspended in 0.25 M sucrose solution and homogenized. The homogenate was centrifuged at 2,500$\times g$ for 5 minutes and the supernatant centrifuged at 12,000$\times g$ for 20 minutes. Precipitates were suspended in 0.25 M sucrose solution and centrifuged. The pelleted mitochondrial fraction was resuspended in 1$\times$SSC and sodium dodecylsulfate and $NaClO_4$ added to final concentrations of 2% and 1 M, respectively. The suspension was deproteinized with a mixture of $CHCl_3$ - isoamyl alcohol (24: 1) with gentle mixing. From the aqueous phase DNA was precipitated by the addition of cold ethanol and subjected to two successive CsCl density ultracentrifugations for 60 hours at 36,000 rpm.

A 4.3 kb fragment of YEp 13[10] containing the yeast chromosomal *LEU2* gene was used for construction of pOL 2211, pOL 2212, pOL 132, pOL 244 and pOL 379.

Preparations of DNA from transformants of *E. coli*[11] and of yeast[12] were performed as described. Isolation of the *Alu* I-*Eco*R I fragment of pOL 221 was performed by elution from an agarose gel as previously described[4]. For electrophoresis of DNA, agarose gels (0.7% and 1.0%) and composite gels (0.5% agarose+3% polyacrylamide gel) were used.

Construction of pOL Plasmids

pOL 221; 4 μg of pBR 322 was cleaved with 18 units of *Sal* I at 37°C for 1.5 hours and then cleaved with 9 units of *Hinc* II at 37°C for 1.5 hours. 1.2 μg of O-111 mtDNA was cleaved with 2.5 units of *Alu* I at 37°C for 1 hour. The digests were heated for 6 minutes at 65°C. After confirmation of the cleavage, 0.8 μg of digested O-111 mtDNA and 0.6 μg of double-digested pBR 322 were ligated with 5 units of T4 DNA ligase at 8°C for 13 hours and then at 12°C for 38 hours. The mixture was used to transform *E. coli* WA802 and nine *tet*^{r} transformants were selected. Plasmid pOL 221 was isolated from one of the transformants and purified by ultracentrifugation.

To construct pOL 2211 and pOL 2212, 2 μg of pOL 221 was cleaved with *Pst* I. Five μg of YEp 13 was cleaved with *Pst* I and *Bam*H I. Both digests were ligated with 7.1 units of T4 DNA ligase at 12°C overnight. The ligates were used to transform *E. coli* C600 and leu$^+$ transformants were selected on M9 media.

For construction of pOL 13, pOL 24 and pOL 37, 2 μg of O-111 mtDNA and 4.5 μg of pOL 221 were digested with *Sst* II and ligated with 4 units of T4 DNA ligase at 14°C for 50 hours. *E. coli* WA 802 was transformed with the mixtures and tetracycline resistant transformants were selected. 2,640 transformants were analyzed by colony hybridization.

To construct pOL 132, pOL 244 and pOL 379 from pOL 13, pOL 24 and pOL 37, these plasmids were cleaved with *Pst* I and ligated with *LEU2* fragment of YEp 13 in the same way as the construction of pOL 2211; *E. coli* C600 was then transformed.

Transformation and Assay for Oligomycin Resistance

Ttransformation of *E. coli* cells[13] and yeast[14,15] were as described.

Oligomycin resistance was assayed as described[1]. The oligomycin resistance of yeast cells transformed with pOL 37 was tested by growing transformants in regeneration agar supplemented with

oligomycin at 15 μg/ml using glycerol as a carbon source.

Plasmids pY*leu* 2 or pY*leu* 5 into which *LEU2* gene of YEp 13 was inserted at the *Pst* I site of pBR 322 were used as a reference for *ARS* function.

Hybridization

The procedure for colony hybridization described by GRUNSTEIN and HOGNESS[16] was followed up to the immobilization of DNA, except that cells grown on membrane filters were transferred together with the filter onto solid L broth supplemented with chloramphenicol to amplify plasmids in the transformants. Southern blotting of DNA and hybridizations are as described[17]. Nick translation was performed according to RIGBY *et al.*[18]. Specific activities of the probes of O-111 mtDNA used for colony hybridization was from 9.1 to 4.6×10^7 cpm/μg. Schleicher and Schuell No. 85 filters were used.

Results

Construction of pOL 221

For construction of a vector capable of replication in yeast cells, we used a mitochondrial *ARS* instead of chromosomal *ARS* or 2 μ *ARS*. Mitochondrial DNA of the petite O-111 strain[3,4] was digested with *Alu* I and fragments were ligated to *Hinc* II site of pBR 322. Since pBR 322 has two *Hinc* II sites, its digestion with *Hinc* II does not give a single fragment. Accordingly, plasmid pBR 322 was first digested with *Sal* I to give staggered ends. The digested pBR 322 was then cleaved with *Hinc* II to give two fragments carrying a single blunt-end. An *Alu* I digest of O-111 mtDNA was ligated to two blunt ended *Hinc* II fragments of pBR 322. The cleaved *Sal* I site was ligated at the same time. *E. coli* WA802 was transformed and *amp*^{s} *tet*^{r} transformants were isolated. Plasmids were then extracted and purified from transformants by ultracentrifugation. One of the plasmids isolated was named pOL 221 (5.8 kb), into which a 1.5 kb fragment was inserted. The size of the insert was not, however, identical either of the two *Alu* I fragments of O-111 mtDNA.

An *Alu* I-*Eco*R I fragment, which contained the 1.5 kb insert was cleaved from pOL 221 and isolated. Its physical map showed that it had a single *Hinc* II site at its upstream end and a cluster of *Hae* III, *Hpa* II, *Hinf* I and *Sst* II sites (termed as a cluster site) in the middle. The *Hinc* II site is located very close to the junction site between the insert and pBR 322 DNA. A similar cluster site exists in the Alu A fragment of O-111 mtDNA. However, the distance between the latter cluster site and *Hinc* II site in Alu A fragment differed from that in the insert of pOL 221. Also no *Hinf* I site was present in the insert, which is located rightward to the cluster site in the Alu A fragment. These data showed that the insert in pOL 221 is derived from a fragment other than that of O-111 mtDNA. However, labeled mtDNA hybridized with pOL 221, indicating its mitochondrial origin.

Cloning of *Sst* II Fragments of O-111 mtDNA

Since the *oli-1*^{r} gene in O-111 mtDNA is reportedly located between two *Sst* II sites[4], *Sst* II fragments of O-111 mtDNA, Sst A (2.4 kb), Sst B (0.8 kb) and Sst C (0.7 kb) fragments were cloned into pOL 221. The sum of these fragments covered most (>97%) of the region of O-111 mtDNA. After ligation of *Sst* II digests of O-111 mtDNA to a *Sst* II site of pOL 221, *E. coli* WA802 was transformed and *tet*^{r} transformants were selected. Since colonies of transformants of *E. coli* which has pOL 221 did not hybridize with labeled O-111 mtDNA, the selection of transformants with pOL 221 inserted with *Sst* II fragments was performed by colony hybridization using labeled O-111 mtDNA as a probe.

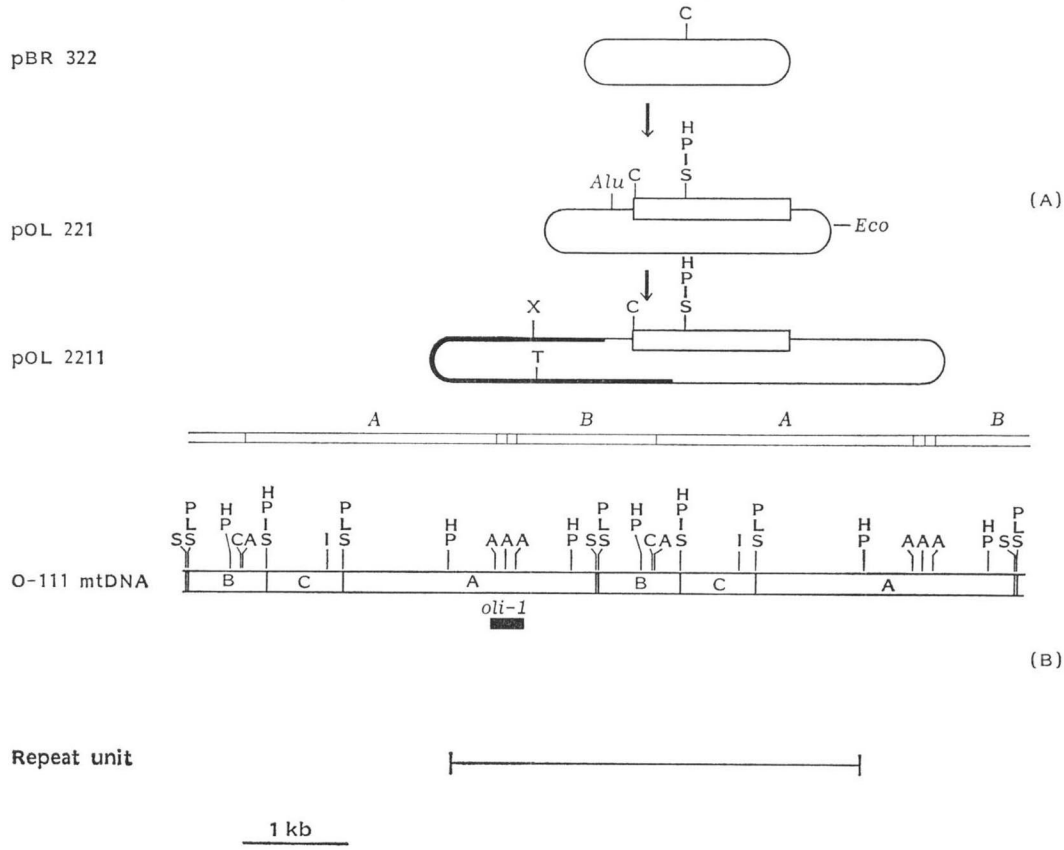
Nineteen strongly hybridizing colonies were isolated and plasmid DNA were extracted from each

Fig. 1. Construction of pOL 221 and pOL 2211 (A) and restriction sites in the insert in pOL 221, pOL 2211 and O-111 mtDNA (B).

Restriction sites are abbreviated as A for *Alu* I, C for *Hinc* II, H for *Hae* III, I for *Hinf* I, L for *Hha* I, P for *Hpa* II, S for *Sst* II, T for *Sal* I and X for *Xho* I.

(A) Thin lines in circles indicate pBR 322 DNA. A thick black line in pOL 2211 indicates the inserted *LEU2* gene. pOL 2212 is the same as pOL 2211 except that the orientation of the inserted *LEU2* gene is reversed. Only the *Alu* I site and *Eco*R I site are shown in pBR 322.

(B) A map of O-111 mtDNA is shown in the lowest figure as described in the previous paper[4]. Note that the junction site is present in Sst A and in the Alu A fragment.



clone. The size of the plasmids and cleavage with *Sst* II showed that the Sst B or Sst C fragment or a combination was inserted in pOL 221. The cloned inserts were shown to be derived from O-111 mtDNA. Two plasmids pOL 13 (6.6 kb) and pOL 24 (6.5 kb) were cleaved with *Sst* II, subjected to electrophoresis, and hybridized with labeled O-111 mtDNA. As seen from Fig. 2, inserts in pOL 13 and pOL 24 gave bands identical to the sizes of Sst B and Sst C fragments of O-111 mtDNA. The *Alu* I site in Sst B and *Hinf* I site in Sst C were used to determine orientation. pOL 13 had the Sst B fragment in the orientation as depicted in Fig. 3. pOL 24 has the Sst C fragment in the reverse orientation (Fig. 3). Although various plasmids were obtained, we found no transformants which had plasmids with an inserted Sst A fragment.

Cloning of *oli-1*^{r} Gene and Instability of the Plasmids

From transformants, ten or eleven hybridizing colonies were cultured together. DNAs were

Fig. 2. Homology of O-111 mtDNA and *Sst* II fragments inserted in pOL 37, pOL 13 and pOL 24.

The plasmids pOL 37, pOL 13 and pOL 24 were digested with *Sst* II, and subjected to electrophoresis. A O-111 mtDNA probe (specific activity 3.7×10^7 cpm/μg) was used. Lanes A and B, *Eco*R I and *Hind* III+*Eco*R I fragments of lambda DNA used as markers; lane C, pOL 24; lane D, pOL 13; lane E, pOL 37. These plasmids are stained with ethidium bromide. Lanes F, G and H are the same as in lanes C, D and E with the plasmids hybridized with labeled O-111 mtDNA. Note that pOL 37 in lane E, which was isolated from the transformants grown in the presence of tetracycline showed no deletion product.

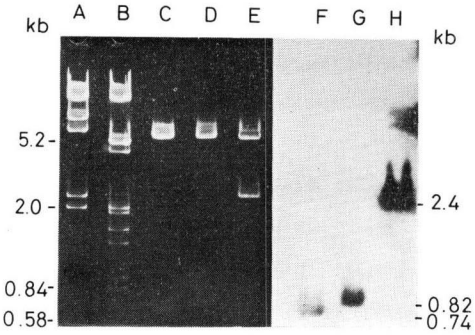


Fig. 3. Construction of plasmids with inserted *Sst* II fragments of O-111 mtDNA.

Thin lines in circles indicate pBR 322 DNA. Thick lines indicate mitochondrial inserts. Arrows indicate orientations of the inserts. Letters A, B and C indicate Sst A, Sst B and Sst C fragment of O-111 mtDNA, respectively.

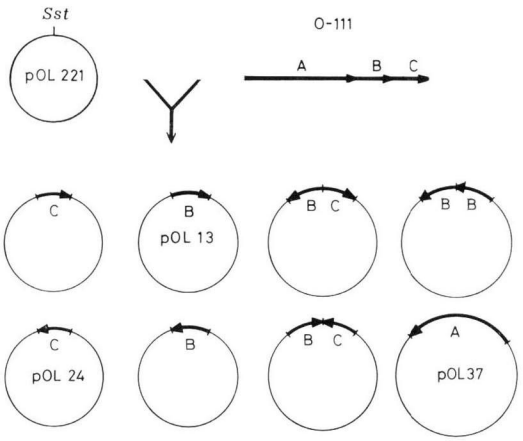
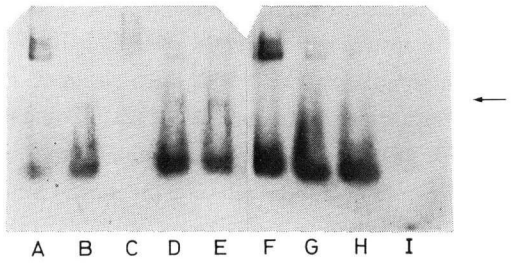


Fig. 4. Detection of the Sst A fragment in transformants.

Lanes A, B, C, D, E, F, G, H and I show extracts from different groups of colonies cultured together as explained in the text. The arrow indicates the band of 2.4 kb. Labeled O-111 mtDNA (specific activity 2.3×10^7 cpm/μg) was used as a probe. The extracts in lanes B, D and E show bands of Sst A fragment. The transformant with pOL 37 was found in the group applied to lane E. The bands larger than 2.4 kb are probably due to incomplete digestion of chimeric plasmids cleaved with *Sst* II at one of the two *Sst* II sites.

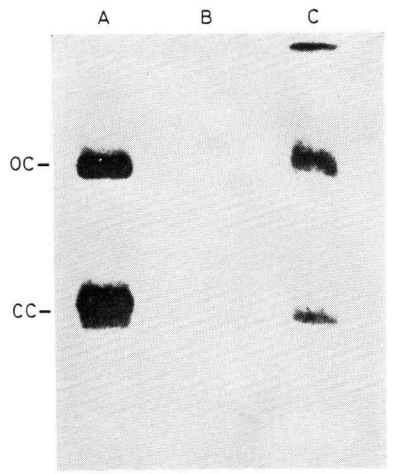


extracted from each group, purified, digested with *Sst* II, subjected to electrophoresis and hybridized with the labeled 0-111 mtDNA. Bands were found which hybridized weakly with labeled 0-111 mtDNA in the extracts from 3 groups (in lane B, D and E in Fig. 4). Strongly hybridizing bands of small size were also observed which corresponded to the Sst B and Sst C fragments (Fig. 4). The faint bands (2.4 kb) had the same size as Sst A fragment. Plasmid DNA was extracted from cells from each of 20 colonies of groups D and E. Plasmids from two colonies gave *Sst* II fragment of 2.4 kb and one was named pOL 37.

The transformant harboring pOL 37 was found in a colony which hybridized weakly, which was unexpected since the Sst A fragment was larger than the Sst B and Sst C fragments and stronger hybridization was anticipated. It was then found that the loss of plasmid DNA occurred from *E. coli* cells growing under non-selective conditions. Such loss can be prevented by growing the transformants with tetracycline selection. This explains the results of colony hybridization, since transformants were grown on membrane filters placed on solid L media in the absence of tetracycline. When transformants harboring pOL 37 grown in the absence of tetracycline and then replated on media with tetracycline, most of

Fig. 5. Recovery of pOL 379 from a yeast transformant.

Transformant of yeast SHY2 with pOL 379 was grown in the absence of leucine. Plasmid DNA was extracted and purified. Bands were hybridized with the labeled pBR 322 (specific activity 3.56×10^7 cpm/μg). Lane A, authentic pOL 379; lane B, DNAs from SHY2; lane C, recovered pOL 379 from SHY2 transformant.



the cells were *tet*^{s} and did not grow. One out of 6 *tet*^{r} colonies contained a smaller plasmid which had 2.0 kb insert. The others had a 2.4 kb insert. The mechanism of this deletion is unknown. The unstable character of plasmid pOL 37 contrasted with that of pOL 13 and pOL 24, which remained unchanged in *E. coli* cells.

The insert in pOL 37 was identified as the Sst A fragment after cleavage with *Sst* II, electrophoresis and hybridization with the labeled O-111 mtDNA as shown in Fig. 2. The restriction map of the inserted 2.4 kb fragment was identical to the Sst A fragment of O-111 mtDNA. The Sst A fragment is a junction fragment and hence has no counterpart in *Sst* II fragments of 992 mtDNA.

Autonomous Replication of pOL Plasmids in Yeast Cells

Since pOL 221 has no genetic markers for use with yeast systems, yeast chromosomal *LEU2* gene was inserted into pOL 221 to give pOL 2211 and pOL 2212. The two differ with respect to orientation of the inserted *LEU2* gene. Leu$^-$ yeast cells AH22 were transformed with these plasmids and the Leu$^+$ phenotype of the transformants was mitotically unstable as reported for other yeast mitochondrial *ARS*. The phenotype can however be maintained under selective conditions in minimal media. pBR 322 with inserted *LEU2* gene (pY*leu* 2 and pY*leu* 5) did not replicate in yeast. The transformation frequencies of pOL 2211 and pOL 2212 were 3.4×10^2/μg and 2.3×10^3/μg respectively (4.5×10^3/μg for YEp 13 taken as a reference).

Autonomous replication of pOL 13, pOL 24 and pOL 37 in yeast cells was thus anticipated, and shown by testing pOL 132, pOL 244 and pOL 379 obtained by insertion of *LEU2* gene. After transformation of leu$^-$ yeast cells with these plasmids, mitotic instabilities were again found. These plasmids gave transformation frequencies of 1.5×10^4/μg for pOL 132, 1.0×10^4/μg for pOL 244 and 5.6×10^3/μg for pOL 379, respectively (comparable to that of YEp 13 which has 2 μ *ARS*). These plasmids were stably maintained under selective pressure. Stable maintenance of pOL 379 under selective conditions was demonstrated by the recovery of the plasmid DNA from yeast transformants.

Expression of *oli-1*^{r} Gene in Yeast Transformants

Plasmids pOL 37 and pOL 379 were tested for their capability to transform oligomycin sensitive yeast to oligomycin resistance. Spheroplasts of yeast SHY2 were transformed with pOL 379 and allowed to regenerate in the absence of leucine to prevent deletion of the plasmids, and when plated on YPY media with oligomycin no resistant transformants were found, although pOL 379 was maintained in the transformants. Transformation of yeast 706 with pOL 37, which lacks an inserted chromosomal gene was also tested. No oligomycin resistant transformants were found. Both experiments indicated that recombination between exogenous (extramitochondrial) mtDNA and resident mtDNA was

hampered, even though we used mitochondrial *ARS* instead of commonly used 2 μ *ARS*. This contrasted with the high frequency recombination seen between the wild type mitochondrial genome and the petite mitochondrial genome.

Discussion

The mitotic instability of pOL plasmids in yeast is a characteristic feature of cloned yeast mitochondrial *ARS*s. This contrasts to the stable transmission of the mitochondrial genome of petite cells to their daughter cells. The reduced propagation of the cloned mitochondrial gene is probably due to the biased delivery of plasmids, which lack association with the mitochondrial membrane capable of mediating the transmission of mitochondrial DNA to daughter cells. This instability suggested the extramitochondrial localization of the cloned *oli-1* gene. The failure to accumulate cloned genetic elements into the mitochondrial structure prevents the two genetic elements, (newly introduced and resident mitochondrial allelic genes) from the close association necessary to undergo recombination.

Deletions and rearrangements of cloned yeast mtDNA have been reported by others[19]. The decreased amount of pOL 37 found in transformants of *E. coli* and their selective growth bear comparison with the difficulties in cloning F_0-ATPase genes of *E. coli*[7]. We assume that suicide of the *E. coli* pOL 37 transformant is due to the loss of protons through the altered ATPase system of the cell membrane. The possible interference of *oli-1* gene product in the energy transfer system in *E. coli* is implied.

Although the insert in pOL 221 differed from the fragment of O-111 mtDNA, labeled mtDNA hybridized with pOL 221. pOL 221 did not hybridize with 2 μ DNA. In order to assess the site of the insert in pOL 221, labeled pOL 221 was hybridized with *Alu* I fragments of mtDNA of 992, from which O-111 was derived. While the *Alu* I fragment of 1.3 kb (=Alu B) did not hybridize, an *Alu* I fragment of 6.3 kb size hybridized strongly and hybridization of labeled pOL 221 with *Sst* II fragments of mtDNA gave two *Sst* II fragments of 2.2 kb and 8.0 kb, respectively (NISHIKAWA, unpublished observation). The former contained the *oli-1* gene, since this fragment hybridized with the 342 bp fragment[20] located 500 bp downstream from *oli-1* gene (data not shown). Accordingly, the mitochondrial insert in pOL 221 was probably derived from a region which overlaps the junction of the 2.2 kb and 8.0 kb *Sst* II fragments, upstream from the *oli-1* gene.

In the petite strain O-111 it is possible that heterogeneity exists in the upstream flanking region of *oli-1* gene. The subcloning of the resistant petite by oligomycin resistance alone may be insufficient to distinguish variants, since both contained the *oli-1* gene. We have not attempted to assign the locus of the insert of pOL 221 precisely and the possibility of the creation of a rearranged O-111 mtDNA during cloning cannot be completely excluded.

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