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Genetics

Properties of a *Saccharomyces cerevisiae* mtDNA segment conferring high-frequency yeast transformation

(DNA replication/yeast extrachromosomal element/*petite* mutant/recombinant DNA)

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ABSTRACT **The bakers' yeast *Saccharomyces cerevisiae* is a facultative anaerobe, tolerant to mutations in its mitochondrial genome. Individual cytoplasmic *petite* mutants retain genetic information derived from any portion of the parental mtDNA, prompting questions concerning distribution of the DNA replication origin(s) on the yeast mitochondrial genome. The experiments described in this paper were designed to test the possibility of using high-frequency yeast transformation as a selection for yeast mtDNA sequences conferring autonomously replicating function. A complete *petite* mitochondrial genome was inserted into the yeast vector YIp5, and the hybrid plasmid (YRMp1) was used to transform yeast. YRMp1 promoted high-frequency transformation of both wild-type yeast cells and *petite* mutant hosts lacking mtDNA and was maintained in each of these strains as a high-copy-number extrachromosomal element. The stability and copy-number properties of YRMp1 are similar to those of YRp12, a recombinant plasmid containing a yeast chromosomal autonomously replicating sequence.**

Higher eukaryotic cells contain multiple copies of a 5-μm circular mitochondrial genome, and much of the available information on mtDNA synthesis is derived from studies on animal cell systems (1). Initiation of mtDNA replication is confined to a unique region of the mitochondrial genome referred to as the D-loop origin. The question of whether unique replication origins are present on yeast (*Saccharomyces cerevisiae*) mtDNA arises when the cytoplasmic *petite* mutation (2) is considered. These mutations are often large deletions of parental wild-type (*grande*, ρ^+) mtDNA sequences, resulting in a respiratory-deficient phenotype. Because virtually any portion of the *grande* sequences can be deleted to form a *petite* derivative (ρ^-) mtDNA, the distribution of DNA replication initiation sites on the yeast mitochondrial genome is not understood. Explanations for these observations include (*i*) that multiple origins are present on *grande* mtDNA (3), (*ii*) that a sequence common to all ρ^- *petites* and *grande* mtDNA provides origin function (4), or (*iii*) that nonspecific initiation sites may become active when a ρ^+ origin becomes deleted.

With the development of a yeast transformation system (5, 6), it is now possible to study replication processes by introducing recombinant plasmids directly into yeast cells. Most plasmids transform yeast at low frequency (1–10 colonies per μg of DNA) as a result of stable integration into host cell chromosomal DNA. A few yeast sequences transform at high frequency (500–20,000 transformants per μg of DNA) and are maintained within the cell as extrachromosomal elements. These properties result from the presence on the transforming plasmid of a DNA sequence conferring autonomously replicating functions (7–9). Hybrid molecules that combine a low-frequency transforming plasmid with an autonomously replicating sequence (*ars*) are able to transform yeast at high frequency, a readily selectable property (10). This rationale has been used to isolate putative DNA replication origins from yeast chromosomes (11, 12) and from a wide variety of heterologous eukaryotic genomes (13).

The experiments described here were undertaken to isolate and map yeast mtDNA sequences that are *ars* elements as assayed by high-frequency yeast transformation. Since many individual *petite* mtDNAs exist *in vivo* as amplified tandemly repeated segments (2), it was anticipated that the repetitive nature of these mitochondrial genomes might provide an enriched source of DNA replication origins. We have shown that a cloned intact *petite* genome is capable of conferring *ars* characteristics to a nonreplicating vector and that *ars* properties of this cloned segment, such as mitotic stability and copy number, are similar to those of the chromosomal DNA sequence *TRP1-ars1* (7, 8, 10).

MATERIALS AND METHODS

Strains and Culture Conditions. The ρ^- *petite* mutant A17-10 was isolated after ethidium bromide mutagenesis of *S. cerevisiae* strain A10 (*a his ade* ρ^+). JHC8-24C (*α ura3-52 his3-11 his3-15 leu2-3 leu2-112 ino1-13 ino4-8* ρ^+) and an ethidium bromide-induced ρ^0 derivative served as hosts in yeast transformation experiments. Bacterial cloning was carried out in a thymine-requiring derivative of *Escherichia coli* HB101 (14). Yeast culture media were used as described (15). Bacteria were propagated on Penassay broth (Difco) supplemented with thymine and necessary antibiotics.

DNA Preparation. Recombinant plasmids YIp1, YIp5, and YRp12 (7) were obtained from R. W. Davis. Plasmid DNA was isolated as described (16). mtDNA was purified by repeated Hoechst dye 33258/CsCl gradient centrifugation (17).

Rapid Yeast DNA Preparation. High-molecular-weight total cellular DNA was prepared from cultures grown selectively in the absence of uracil. Zymolyase-generated spheroplasts were lysed with Sarkosyl and treated with RNase/α-amylase (each, 50 μg/ml), after which Pronase was added to 1.7 mg/ml. The lysate was extracted with chloroform/isoamyl alcohol (24:1) and dialyzed against 15 mM NaCl/1.5 mM Na citrate.

Bacterial and Yeast Transformation. Bacterial transformations were done as described by Lederberg and Cohen (18). Yeast transformations were carried out using the procedure of Hinnen *et al.* (6), except that Zymolyase 60,000 (16 μg/ml) was used.

Electrophoresis and Hybridization. Gel electrophoresis, Southern transfer, and DNA·DNA hybridization were carried out according to published procedures (19–21).

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Abbreviation: kb, kilobase(s).

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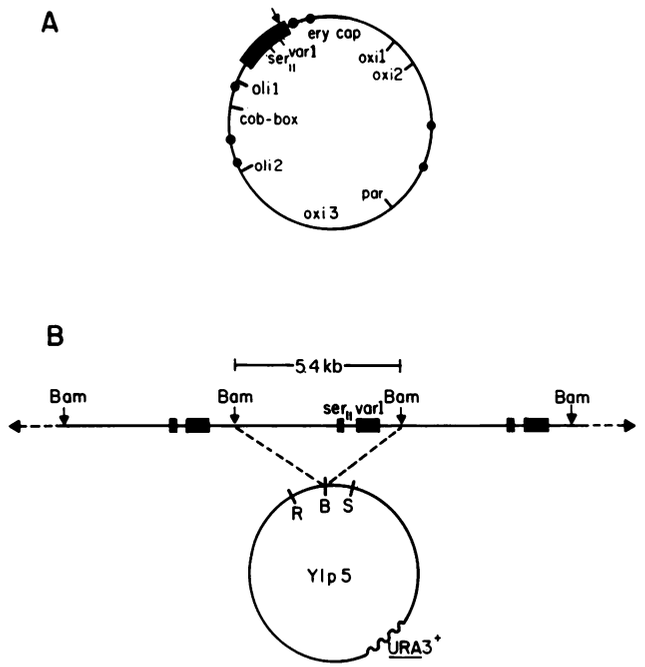

FIG. 1. Molecular cloning of a complete *petite* mitochondrial genome. (*A*) Simplified genetic map of *S. cerevisiae grande* mtDNA. ■, Approximate map location of parental ρ^+ sequences retained in *petite* strain A17-10 (25); ●, approximate map position of hypersuppressive *rep* (22) or *ori* (23) loci; →, *Bam*HI cleavage site used in cloning A17-10 mtDNA. (*B*) Tandemly repeated structure of A17-10 *petite* mtDNA. *Bam*HI-digested A17-10 mtDNA was ligated to *Bam*HI-cleaved YIp5 DNA (7), and the mixture was used to transform *E. coli* to ampicillin resistance or JHC8-24C yeast host to Ura prototrophy. B, *Bam*HI; R, *Eco*RI; S, *Sal* I.

Enzymes. Restriction enzymes and T4 ligase were obtained from New England Biolabs or Bethesda Research Laboratories (Gaithersburg, MD) and used as recommended by the source. Zymolyase was obtained from Kirin Brewery, Takasuki, Japan.

RESULTS

Molecular Cloning of a Complete *Petite* Mitochondrial Genome. Yeast strain A17-10 carries a 5.4-kilobase (kb) *petite* mutant mitochondrial genome derived from *grande* sequences that encompass the SER_{II} and *VAR1* genetic determinants (Fig. 1*A*). A17-10 mtDNA is organized within the mitochondrion as a series of tandemly aligned repeating units (Fig. 1*B*). Each repeat contains a single *Bam*HI cleavage site, so that complete digestion with *Bam*HI produces molecules of unit genome length. This preparation was ligated into the *Bam*HI cloning site of the yeast vector YIp5 (ref. 7; Fig. 1*B*) and used to transform an *E. coli* host to ampicillin resistance. Several transformants were isolated that contained plasmids that yielded linear YIp5 and a 5.4-kb insert after digestion with *Bam*HI. The cloned insert was then shown to generate fragments expected from an A17-10 *Bam*HI-cleaved repeating unit (data not presented). Fig. 2 depicts the plasmids YRMp1 and YRMp1′ obtained by molecular cloning in *E. coli*. These plasmids contain the mtDNA insert in opposite orientations. We have adopted the nomenclature YRMp (yeast replicating mtDNA plasmid) to distinguish these plasmids from YRp vectors (24), which contain yeast chromosomal DNA.

To confirm that the cloned insert was derived from the yeast mitochondrial genome, ^{32}P-labeled YRMp1 was hybridized to restriction fragments of ρ^+ mtDNA from strain A10 (Fig. 3*A*) or from A17-10 ρ^- mtDNA and restricted whole cell DNA from A17-10 (Fig. 3*B*). Fig. 3*A* shows that only those restriction fragments occupying the region of the *grande* mitochondrial genome from which A17-10 *petite* mtDNA was derived (*Hpa* I bands 4 and 5, *Hha* I bands 2 and 6, *Bam*HI/*Sal* I bands 4 and 5, *Bam*HI bands 1 and 4; ref. 25) share homology with YRMp1. Both A17-10 mtDNA and the prominant ρ^- mtDNA component of cellular A17-10 DNA display strong hybridization with YRMp1 (Fig. 3*B*). The physical and hybridization data indicate that YRMp1 contains a single *petite* mtDNA repeating unit.

Yeast Transformation with mtDNA-Containing Plasmids. Yeast strains carrying a chromosomal *ura3* mutation can be transformed to Ura prototrophy by recombinant plasmids that contain the yeast *URA3* gene (7). In a host containing the *ura3-52* allele, YIp5 is inert because the *URA3* gene does not contain an *ars* element and chromosomal integration is rare due to a DNA sequence rearrangement at the endogenous *ura3* locus (13). The presence of an *ars* element ligated into YIp5 enables such a hybrid plasmid to transform a *ura3-52* strain to Ura$^+$ at high frequency.

The *ura3-52* yeast host JHC8-24C and an isogenic ρ^0 derivative were transformed with purified YRMp1, YRMp1′, and the vector YIp5. In addition, YRp12, which contains the 1.4-kb yeast chromosomal sequence *TRP1-ars1* in YIp5 (ref. 13; Fig. 2) was used in these experiments. Molecules containing the cloned mtDNA *petite* genome in either orientation transformed this yeast strain to Ura prototrophy at high frequency, as did YRp12. YRp12 produced a 10-fold enhancement in transformation frequency over YRMp1 and YRMp1′ in either ρ^+ or ρ^0 host backgrounds. In addition, transformation frequency was reduced in ρ^0 hosts when compared with numbers of transformants obtained using the isogenic ρ^+ parent. This property was

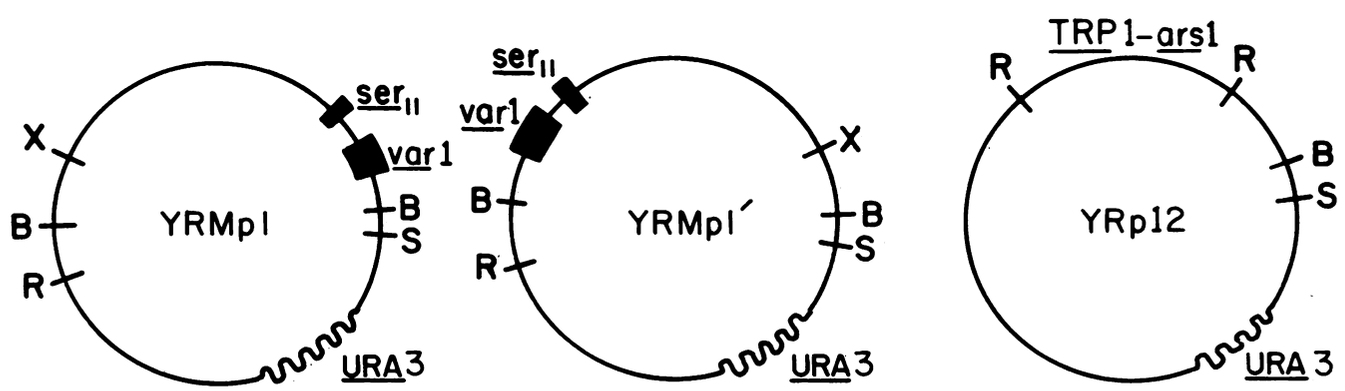

FIG. 2. Structure of plasmids obtained by molecular cloning in *E. coli*. YRMp1 and YRMp1′ differ only with respect to the orientation of the cloned 5.4-kb mtDNA insert. YRp12 is YIp5 containing the yeast chromosomal DNA sequence *TRP1-ars1* (13). B, *Bam*HI; R, *Eco*RI; S, *Sal* I; X, *Xho* I.

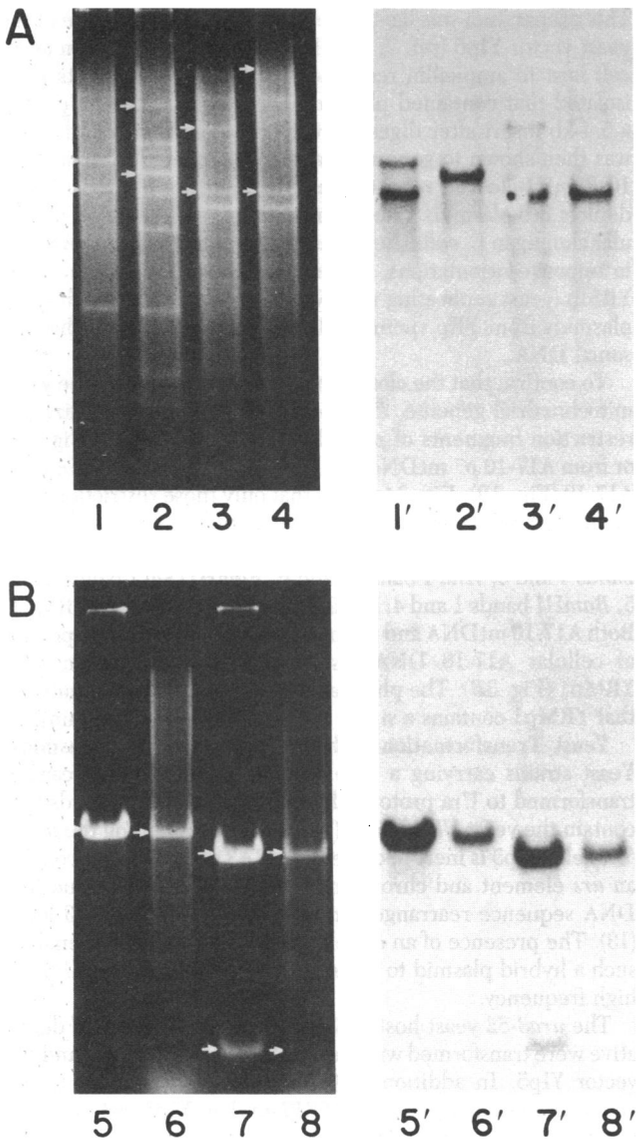

FIG. 3. YRMp1 hybridizes to yeast *grande* and *petite* mtDNA. Restriction fragments of purified A10 ρ^+ mtDNA (*A*) or purified A17-10 ρ^- mtDNA and A17-10 whole cell DNA (*B*) were electrophoretically resolved on 0.7% agarose gels. (*Left*) Ethidium bromide-stained gels. Lanes: 1, Hpa I-digested A10 ρ^+ mtDNA; 2, Hha I-digested A10 ρ^+ mtDNA; 3, *Bam*HI/*Sal* I-digested A10 ρ^+ mtDNA; 4, *Bam*HI-digested A10 ρ^+ mtDNA (intact *grande* mtDNA cannot be isolated on a preparative scale and therefore the largest restriction fragments in each digest are not present in stoichiometric yields); 5, *Bam*HI-digested A17-10 mtDNA; 6, *Bam*HI-digested A17-10 cellular DNA; 7, *Bam*HI/*Xho* I-digested A17-10 mtDNA; 8, *Bam*HI/*Xho* I-digested A17-10 cellular DNA. Amplified *petite* mtDNA appears as distinct bands superimposed on a background smear of cellular DNA in lanes 6 and 8. White arrows, fragments expected to show homology with A17-10 mtDNA based on previously established restriction maps (25). (*Right*) Autoradiogram after hybridization with YRMp1. DNA was transferred to nitrocellulose filters and hybridized to ^{32}P-labeled YRMp1. Variations in band intensities in lanes 1′–4′ reflect differential recovery of individual *grande* restriction fragments and their levels of homology with A17-10 mtDNA. Lanes 1′–8′ correspond to lanes 1–8.

observed with plasmids containing either mitochondrial or chromosomal DNA segments (Table 1).

YRMp1 is Maintained in Yeast as an Extrachromosomal Element. To demonstrate that high-frequency yeast transformation by YRMp1 is associated with an *ars* element, total cellular DNA was prepared from four independent Ura$^+$ transformants, resolved without cleavage by restriction enzymes on agarose gels, and hybridized to ^{32}P-labeled pBR322. This experiment showed that all four transformants contained an element indistinguishable in size from the input YRMp1 DNA (Fig. 4). The observation that plasmids containing pBR322 sequences are not associated with high-molecular-weight cellular DNA indicates that YRMp1 is being maintained as an autonomous extrachromosomal extra-mtDNA element.

Table 1. Yeast transformation with hybrid plasmids containing chromosomal or mtDNA

Plasmid	Host mtDNA phenotype	Selection	Transformants,* no. Per μg of DNA	Transformants,* no. Per μg per 10^6 regenerating spheroplasts
YRp12	ρ^+	*URA3*	>5000	>380
YRp12	ρ^0	*URA3*	>600	>35
YRMp1	ρ^+	*URA3*	>500	>38
YRMp1	ρ^0	*URA3*	>150	>9
YRMp1′	ρ^+	*URA3*	>500	>38
YRMp1′	ρ^0	*URA3*	>150	>9
YIp5	ρ^+	*URA3*	0	0
YIp5	ρ^0	*URA3*	0	0
YIp1†	ρ^+	*HIS3*	<10	<0.1
YIp1	ρ^0	*HIS3*	<10	<0.1

* Absolute values of transformants vary among experiments. Only ranges of transformant counts are presented.
† YIp1 (pBR322 containing the yeast *HIS3* gene) was used as an integrating DNA control.

Phenotypic Properties of YRMp1. Properties such as stability and copy number, which may vary among plasmids depending on their individual replicons, were examined for yeast extrachromosomal elements that contain *ars* segments.

Stability. Grande or ρ^0 yeast transformants harboring either YRp12 or YRMp1 were tested for mitotic stability of the Ura$^+$ phenotype (Table 2). Equivalent samples of logarithmic- or stationary-phase cultures grown selectively in the absence of uracil were plated onto media with or without uracil and the numbers of colonies were compared. The percentage of Ura$^+$ cells from these eight cultures varied from 5 to 32. When these same

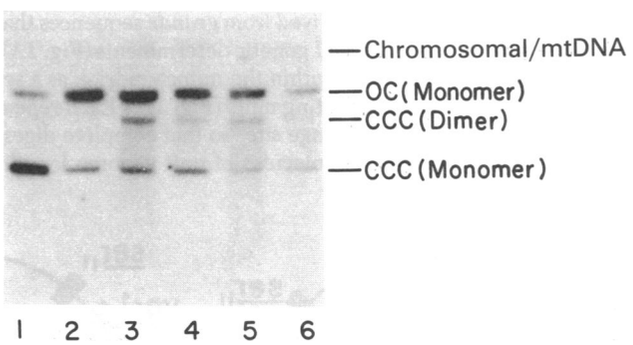

FIG. 4. YRMp1 is maintained in yeast as an extrachromosomal element. YRMp1 prepared from *E. coli* was used to transform the yeast host JHC8-24C to Ura prototrophy. Whole cell DNA was prepared from individual transformants. Undigested DNA was fractionated on a 0.8% agarose gel, transferred to nitrocellulose paper, and hybridized with ^{32}P-labeled pBR322. Migration position of high-molecular-weight chromosomal DNA/mtDNA is indicated. Lanes: 1, purified YRMp1 size standard; 2, DNA prepared from a Ura$^+$ transformant obtained after directly transforming yeast with the original ligation mixture (this suggests that passage through *E. coli* is not responsible for *ars* properties of YRMp1); 3–6, DNAs prepared from Ura$^+$ transformants after transformation with YRMp1 purified from *E. coli*. CCC, covalently closed circles; OC, open circles.

Table 2. Phenotypic properties of yeast plasmids

Host mtDNA phenotype	Culture growth phase	Mitotic stability*	Copy number	
			Per haploid genome	Per Ura$^+$ cell
		YRp12		
ρ^+	Logarithmic	16	6	38
ρ^+	Stationary	5	5	92
ρ^0	Logarithmic	11	10	88
ρ^0	Stationary	8	8	99
		YRMp1		
ρ^+	Logarithmic	12	8	66
ρ^+	Stationary	12	1	10
ρ^0	Logarithmic	26	11	43
ρ^0	Stationary	32	7	64

* Expressed as percentage of *Ura*$^+$ cells in selectively grown cultures.

strains were grown overnight (approximately five generations) in the presence of uracil, <0.5% of the cells were Ura$^+$ when scored in a similar fashion.

Copy Number. We have developed a simple procedure for determining the number of plasmids present per haploid yeast genome. The nucleotide sequences of the pBR322 and *TRP1-ars1* components of the hybrid plasmid YRp7 (ref. 7; Fig. 5A) have been determined (26, 27), and each has a defined level of homology with both transforming plasmid and single-copy yeast genomic sequences present in host cell DNA preparations. Hybridization of uniformly labeled YRp7 to each of these sequences, when quantitated and corrected for homology levels, generates a copy-number estimate for a plasmid of interest in a yeast cell culture population. Examples of this procedure are shown in Fig. 5*B*.

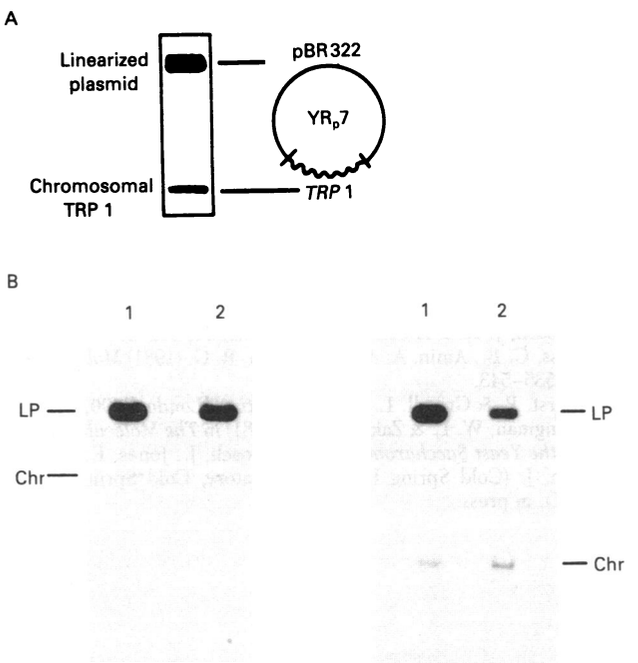

FIG. 5. (*A*) Composite structure of YRp7 DNA. *TRP1* and pBR322 components sharing homology (1453 and 4362 base pairs, respectively) with transformant DNA sequences are indicated. (*B*) Examples of yeast plasmid copy-number determination. The yeast host JHC8-24C (ρ^+) was transformed with YRp12 (*Left*) or YRMp1 (*Right*). Ura$^+$ transformant whole cell DNA was prepared from either logarithmic (OD_{650} = 0.2) (lanes 1)- or stationary (OD_{650} = 2.0) (lanes 2)-phase cultures grown selectively without uracil. YRp12 transformant DNA was digested with *Sal* I/*Sst* I/*Xho* I; YRMp1 transformant DNA was cleaved with *Eco*RI. After electrophoresis on 1.0% agarose gels, the DNA was transferred to nitrocellulose paper and hybridized with ^{32}P-labeled YRp7. Hybridized bands were localized by autoradiography and removed from the filter, and radioactivity was quantitated by liquid scintillation counting.

Copy numbers for YRp12 and YRMp1 in ρ^+ and ρ^0 backgrounds were determined for logarithmic- and stationary-phase cultures (Table 2). Both YRp12 and YRMp1 exhibit low copy number (1–10 per haploid genome), similar to values reported for the related plasmid YRp7 (7), when calculated on the basis of the total culture population. To accurately estimate the copy number *per cell*, it is necessary to consider the mitotic instability of these elements. The copy number of these plasmids on a per cell basis, when corrected for the percentage of plasmid-containing cells in individual cultures, increases to 40–100 copies, approximating levels described for yeast elements such as the 2-μm circular plasmid (28) and the *TRP1*-RI circle (V. A. Zakian and J. F. Scott, personal communication).

DISCUSSION

The bakers' yeast *S. cerevisiae* is a facultative anaerobe that can tolerate mutations of its mtDNA resulting in a respiratory-deficient phenotype. The large deletions associated with these ρ^- mutations suggest the possibility that more than one unique DNA replication origin may be present on the yeast mitochondrial genome. High-frequency yeast transformation was used as a positive selection for a yeast mtDNA segment exhibiting possible origin function. This approach requires expression of a selectable yeast chromosomal gene (*URA3*) in an assay designed to test the function of a *cis*-acting element derived from mtDNA sequences. To enhance our chances of success, a cloned *petite* genome expected to have a DNA replication origin was used as a potential source of *ars* elements.

The observations that YRMp1 (and YRMp1′) transform yeast at a high frequency and are maintained as unstable extrachromosomal elements indicate that the cloned yeast mtDNA can provide *ars* characteristics to the YIp5 vector. Novel cloning junctions between mtDNA and YIp5 are not responsible for these properties, since plasmids containing the cloned mtDNA insert in both orientations behave in a similar fashion. There is no evidence for plasmid integration into nuclear or mtDNA. Recombinant plasmids containing heterologous mtDNA from *Xenopus laevis* (29) and the minicircular component of *Leishmania tarentolae* kinetoplast DNA (G. Kidane, J. Scott, T. Spithill, and L. Simpson, personal communication) also transform yeast at high frequency.

DNA sequences representing specific replication origins have not been positioned on the yeast mitochondrial genome. However, yeast mtDNA loci providing possible origin function have recently been defined on a genetic basis by using a group of *petite* mutants exhibiting a hypersuppressive (HS) phenotype (22, 30, 31). Crosses of HS *petites* with ρ^+ cells give rise to nearly 100% ρ^- diploids containing amplified mtDNA contributed by the HS parent. One interpretation of these results is that HS mtDNA may contain an enriched source of origin sequences, thereby conferring a replicative advantage over *grande* genomes in a heteroplasmic zygote. However, many *petite*-derivative genomes, such as the A17-10 mtDNA used in this study, do not contain such *rep* (22) or *ori* (23) sequences (Fig. 1A).

Despite the fact that A17-10 ρ^- mtDNA confers *ars* properties to a nonreplicating vector, it remains to be determined whether this segment actually contains a unique replication origin used in either *grande* or *petite* mtDNA synthesis. The question of whether *ars* elements as defined by yeast transfor-

mation represent authentic replication origins used at their native location remains open. However, this assay appears to define some eukaryotic origins. Plasmids containing known DNA replication origins from the yeast 2-μm plasmid (5, 7, 32), *X. laevis* mtDNA (29), and *Tetrahymena thermophila* extrachromosomal rDNA (33) all participate in high-frequency yeast transformation.

The cellular location of YRMp1 has not yet been determined, although indirect evidence suggests that the mitochondrion is an unlikely site for its maintenance. Unique features of the mitochondrial genetic code (34) may not permit expression of the chromosomal *URA3* gene and, in preliminary experiments, we found that YRMp1 is not cured by ethidium bromide under conditions in which host mtDNA is eliminated (unpublished data). The stability and copy-number behavior of YRMp1 are also similar to properties exhibited by YRp12, which contains a chromosomal *ars* segment. Even if maintained in the nucleus, plasmids containing an *ars* element from the yeast mitochondrial genome may use nuclear-encoded components responsible for mtDNA replication. Since yeast mtDNA synthesis is easily uncoupled from chromosomal DNA replication (35), it should be straightforward to inquire whether plasmids containing yeast mtDNA sequences respond to cellular controls that sponsor chromosomal DNA or mtDNA synthesis.

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