J. Microbiol. Biotechnol. (1998), **8**(6), 650-655

JOURNAL OF
MICROBIOLOGY AND
BIOTECHNOLOGY

Nucleotide Analysis of *Phaffia rhodozyma* DNA Fragment That Functions as ARS in *Saccharomyces cerevisiae*

CHUNG, HEE YOUNG, MIN HEE HONG, YOUNG HYUN CHUN, SUK BAI, SUHN YOUNG IM, HWANGHEE BLAISE LEE[1], JONG CHUN PARK[2], DONG HO KIM[3], AND SOON-BAI CHUN*

Department of Microbiology, [1]Department of Biology, Chonnam University, Kwang-ju 500-757, Korea
[2]Department of Biology, Seonam University, Namwon 590-170, Korea
[3]Protein Function R. U., Korea Research Institute of Bioscience and Biotechnology, Yusung, Daejon 300-605, Korea

Received: September 4, 1998

Abstract The chromosomal DNA fragment from *Phaffia rhodozyma* CBS 6938 which is able to autonomously replicate in the yeast *Saccharomyces cerevisiae* was cloned on an integrative *URA3* plasmid. Its minimal fragment exhibiting autonomously replicating activiy in the *S. cerevisiae* gave a higher frequency transformation efficiency than that found for centromere-based plasmid, and enabled extrachromosomally stable transmission of the plasmids in one copy per yeast cell under non-selective culture condition. The 836-bp DNA element lacked an ORF and did not contain any acceptable match to an ARS core consensus. Sequence analysis, however, displayed a cluster of three hairpin-loop-sequences with individual $\triangle G_{25}$°C free energy value of -10.0, -17.5, and -17.0 kcal · mol^{-1} as well as a 9-bp sequence with two base pair mismatches to the *S. cerevisiae/E. coli* gyrase-binding site. This 836-bp sequence also included one 7-bp sequence analogous to the core consensus of centromeric DNA element III (CDEIII) of *S. cerevisiae*, but CDEIII-like 7 bp sequence alone did not give a replicative function in this yeast.

Key words: *Saccharomyces cerevisiae, Phaffia rhodozyma*, replicative DNA fragment, nucleotide sequence analysis

Phaffia rhodozyma is a basidiomycetous yeast that produces astaxanthin as the principal carotenoid [3, 17]. Astaxanthin has become of great commercial interest to the fish farming industries, since it is responsible for pink color formation in salmon and trout. Furthermore, a possible role of astaxanthin in the prevention of human cancer [9] and as the most effective antioxidant [16] renders it also medically attractive.

*Corresponding author
Phone: 82-62-530-3411; Fax: 82-62-530-3419;
E-mail: sbchun@chonnam.chonnam.ac.kr

One disadvantage for the industrial application of wild-type *Phaffia* strains as a possible source of astaxanthin production appears to be its low pigment content [13]. Although mutant selection and cell fusion have been used to achieve some increase in *Phaffia* pigmentation [1, 6], the maximum amounts of astaxanthin still appears to be low. Recombinant DNA technology could overcome this drawback in strain improvement. This technique is considerably impeded by the lack of molecular approach to carotenoid biosynthesis pathways and their involved genes. Such an approach would benefit enormously from the development of an efficient transformation system. The advantages of an autonomously replicating plasmid include high frequency of transformation which would be especially valuable in gene cloning by reisolation of transforming plasmids capable of complementing mutant alleles. It would also be valuable in gene expression if it is maintained in high copy numbers from generation to generation. Using a bacterial kanamycin resitance gene as a dominant selection marker, transformation has been achieved through integration of plasmid into the rDNA of *P. rhodozyma* [26]. Recently, Kim *et al.* [14] have reported that the integration of seven copies of plasmids into rDNA of this yeast was efficiently achieved using the ribososmal protein L41 gene of *P. rhodozyma* as a dominant selection marker for cycloheximide resistance.

The yeast *S. cerevisiae* has proven to be a useful cellular system for the selection of DNA fragments which confer autonomous replication [27]. Chromosomal or mitochondrial DNA fragments isolated from a wide variety of eukaryotes have been shown to function properly as an ARS in *S. cerevisiae* [2, 7, 8, 22, 24].

In the present work, the isolation of chromosomal DNA fragment from *P. rhodozyma*, which functions as ARS in *S. cerevisiae*, and its nucleotide sequence analysis have been described.

MATERIALS AND METHODS

Microbial Strains and Media

E.coli JM83 (*ara, delta(lac-proAB), rpsL,* Φ80d*lacZ ΔM15*) was used for all bacterial transformation and gene library construction. *S. cerevisiae* SHY3 (*Mat a, ste*-VC9, *ura3*-52, *trp1*-289, *leu2*-112, *his3* Δ1, *ade1*-101, *can1*-100) and *P. rhodozyma* CBS 6938 were used as hosts for ARS cloning and as sources of genomic DNA. LB medium with ampicillin was used for cultivation and selection of *Escherichia coli* harboring plasmid of ampicillin resistance. YEPD (1% yeast extract, 2% Bactopeptone, 2% dextrose) and YNB (0.67% Yeast Nitrogen Base without amino acids [Difco]) were used for yeast culture.

DNA Analysis and Transformation

Digestion of plasmid and yeast chromosomal DNA by restriction enzymes, electrophoresis of DNA fragments, Southern hybridzation, and *E. coli* transformation were carried out as described by Sambrook *et al.* [23].

S. cerevisiae was transformed as described previously by Park *et al.* [20]. *P. rhodozyma* chromosomal DNA was extracted from the 36 h grown cells by the method of Nagy *et al.* [18]. The DNA sequencing was performed by the dideoxy chain termination method using the sequencing kit supplied by the United States Biochemical Company and appropriate oligonucleotide primers.

Plasmid Copy Number Per Cell

Yeast cells were transformed with vector. The plasmid DNA was isolated from transformant and restricted with *Sal*I as described in method of Sambrook *et al.* [23]. Several quantities of genomic or plasmid DNA were loaded. After electrophoresis, the DNA fragments were blotted and hybridized using the *URA3* gene fragment (1.2 kb) derived from YEp352 vector as probe. The plasmid copy number per cell was estimated by comparison of the autoradiogram of the plasmid versus chromosomal band intensity, corrected according to the percentage of the cells containing the plasmid at the moment of harvesting.

Plasmid Stability Assay

A single colony of the transformants was precultured on the selective YNB-glucose medium and then inoculated (1%) into 50 ml YPD medium. At the start and after an appropriate period of cultivation, a suitable dilution (100~300 colonies/plate) was spread onto a selective plate without uracil and a non-selective plate with uracil. The ratio between the numbers of colonies on the two kinds of plates was taken as a measurement of plasmid stability.

RESULT AND DISCUSSION

Isolation of *P. rhodozyma* ARS

To isolate the *P. rhodozyma* DNA fragment capable of functioning as ARS in *P. rhodozyma* and *S. cerevisiae*, *P. rhodozyma* genomic DNA was partially digested with *Sau*3AI and ligated into the unique *Bam*HI site of the YIp5 plasmid which lacks a yeast replication origin. The resulting recombinant plasmids were used to transform *S. cerevisiae* in order to select DNA fragments with replicative activity in this yeast. *S. cerevisiae* transformants were checked for their growth on YNB plate without uracil. The extrachromosomally replicating plasmid DNA of each transformant was rescued from total DNA by transformation of *E. coli* JM83. All recombinant plasmids recovered by this procedure were tested for the ability to transform *S. cerevisiae*. Recombinant plasmids were recovered from the same transformants, and reintroduced into the *S. cerevisiae* cells. All *S. cerevisiae* transformants grew well on YNB plate without uracil. We selected three clones; of these, two contained a common 1.8-kb insert able to efficiently transform *S. cerevisiae* while the other (approximately 3.8 kb) showed low transformation frequency. This 1.8-kb insert cloned in YIp5 was designated as YRp1. Plasmid YRp1 can be readily recovered from *S. cerevisiae* by transforming total genomic DNA of the *S. cerevisiae* recipient cells into *E. coli* and isolating plasmid DNA from individual ampicillin resistant *E. coli* transformants. When the transformation of *S. cerevisiae* with recovered plasmids was repeated, plasmid YRp1s prepared from these *E. coli* retransformants showed the same band patterns on agarose electrophoresis (data not shown). This suggests that YRp1 exists in *S. cerevisiae* as an extrachromosomal self-replicating plasmid.

Southern analysis of *P. rhodozyma* genomic DNA using this 1.8-kb insert as probe showed that this was derived from *P. rhodozyma* genomic DNA (data not shown).

Transformation Efficiency and Copy Number

The 1.8-kb fragment in YRp1 was subcloned in order to define the smallest region of ARS activity in *S. cerevisiae*. By *Sal*I digestion of this insert, we obtained three fragments with sizes of 0.5, 1.0, and 0.3 kb, which were cloned in YIp5 to give YRp1-1, YRp1-2, and YRp1-3, respectively (Fig. 1). When these plasmids were tested for ARS activity, only YRp1-2 was able to transform *S. cerevisiae* and to exhibit the same transformation frequency (6.12×10^2 cfu (colony forming unit)/μg of plasmid DNA) as YRp1 plasmid, as shown in Fig. 1. This value was lower than the transformation frequency of *S. cerevisiae* ($3.5\sim4\times10^3$/μg DNA) with ARS-based plasmids [27] and of *Candida boidinii* with CARS1 (1.0×10^4/μg DNA) [22], but was comparable to the transformation frequency of

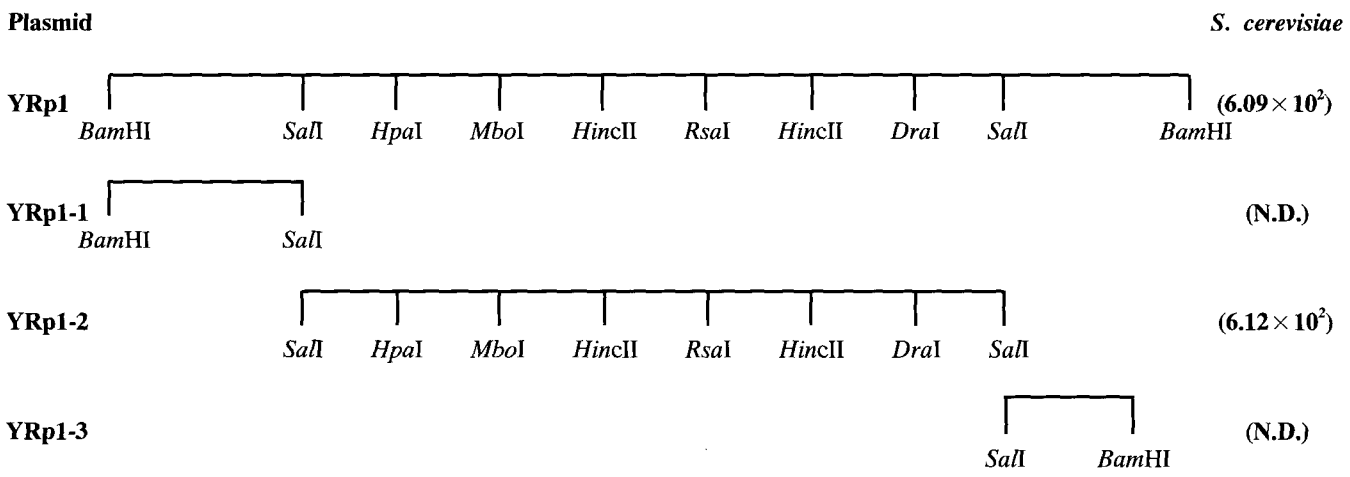

Fig. 1. Replicative funtion of different subfragments of YRp1 restricted by *SalI*. The numbers in parenthesis indicate transformation frequency; N.D., not detectable.

S. cerevisiae ($4.5\sim5\times10^2$/μg DNA) by the plasmid containing a replicative mitochondrial DNA fragment of *Papaver somniferum* L [8]. The YRp1-2 stability in *S. cerevisiae* transformants grown in YPD for 96 h, expressed as the ratio of the number of colonies on the uracil-minus plate to the number on the uracil-plus plate, was approximately 80% which was high when compared with the 5~50% found for ARS plasmids [11]. This value was comparable to the 70% found for the centromere-based plasmid of *Candida maltosa* [19]. This high stability was also encountered in plasmids containing both an ARS and a centromere as in *Kluyveromyces marxianus* [12] and for 2μ-like plasmids [27].

To determine the copy number of YRp1-2 per *S. cerevisiae* cell, Southern blots of plasmid DNA from *URA3*$^+$ transformants cut with *SalI* were probed with a P^{32}-labeled *URA3* gene sequence. The resulting intensity of bands corresponding to the *URA3* gene on the plasmid and the chromosomal *URA3* allele was compared (Fig. 2). On the assumption that the *URA3* gene is present in one copy per haploid genome, densitometric analysis using Fluor-S multiimager (Bio-Rad) showed that the copy number of YRp1-2 was approximately 1.3. This copy number of YRp1-2 corresponds to that typical of YCp in *S. cerevisiae* [19].

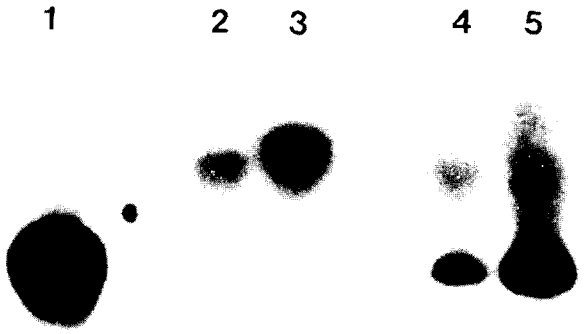

Fig. 2. Southern blot analysis of DNA from *S. cerevisiae URA3*$^+$ transformant. Chromosomal and plasmid DNA from *URA3*$^+$ transformant of *S. cerevisiae* SHY3 separated on a 0.8% agarose gel.

Lanes: 1, Intact plasmid YIp5 cut with *SalI*: 2, total genomic DNA from *S. cerevisiae* SHY3 strain (20 μg); 3, total genomic DNA from *S. cerevisiae* SHY3 strain (40 μg); 4, plasmid YRp1-2 DNA (20 μg) from *URA3*$^+$ transformant of *S. cerevisiae* cut with *SalI*; 5, plasmid YRp1-2 DNA (40 μg) from *URA3*$^+$ transformant of *S. cerevisiae* cut with *SalI*. Blots were hybridized with ^{32}P-labelled *URA3* gene fragment.

Nucleotide Analysis of ARS

The nucleotide sequence of an approximately 1.0-kb insert in YRp1-2 has been determined (Fig. 3). No open reading frame was found in the 836-bp region. One cannot find 11/11 or even 10/11 matches to the core consensus sequence[(A/T)TTT(A/G)TTTA] for *S. cerevisiae* ARS elements except for one 8/11 match at position number 237–248 in the 5' region of which no A-T rich stretches were found. Many DNA fragments with ARS activity contain the A-T rich sequences on the 5' side of the ARS core consensus sequence which lowers the free energy of the DNA helix, facilitating the binding of the initiation proteins in the ARS region [25]. Inspection of the 836-bp insert sequence revealed three hairpin-loop forming subsequences with respectively high $\triangle G_{25}$°C free energy values of -10.0, -17.5, and -17.0 kcal · mol^{-1} (Table 1) which are found as a cluster in the region delineated by position number 279–516 (Fig. 3). These free energy values were higher than $\triangle G_{25}$°C free energy values of -7.6, -6.4, and -5.2 kcal · mol^{-1} calculated from a replicative mitochondrial DNA fragment of a basidomyceteous fungus, *Pleurotus ostreatus* [21]. Sequence analysis of the 836-bp insert exhibited one putative consensus core site (position

Fig. 3. Nucleotide sequence of the chromosomal DNA fragment of *P. rhodozyma* able to autonomously replicate in *S. cerevisiae*.

The 836 bases are composed of A (224), C (216) G (182), and T (214). Numbered squares flanked by outward-pointing shaded arrowheads indicate three postulated hairpin-loop-forming sequences located between nucleotides 278 and 516.The underlined bolded sequence represents a 8/11 match to the *S. cerevisiae* ARS core consensus sequence. Inward pointing triangles below the sequence designate putative consensus sites for centromere (▷ cen◁) and gyrase binding site (▷gyr◁).

Table 1. Hairpin-loop sequence identified in YRp1-2.

No.	Energy ΔG_{25}°C: kcal · mol^{-1}	Stem (paired)	Loop (unpaired)	Position		
				—5' stem	Loop	3' stem—
1	-10.00	10	22	278-303	304-311	312-321
2	-17.50	16	25	428-443	444-468	469-484
3	-17.00	13	11	479-492	493-503	504-516

number 764-772) with two base mismatches to the *S. cerevisiae* gyrase recognition site (TATGCTTA) [10].

Also present in just the 5' side of a putative gyrase binding site is a putative cen core sequence (position number 751-757) with a single base pair mismatch to the core concensus site (TTCCGAA) of the centromere locus of chromosome III (CDE III) of *S. cerevisiae* [11], where a conserved C was replaced with G. However, no AT-rich stretches [12], a common feature of yeast centromere, was found.

P. rhodozyma shares some common features with those of a replicative mitochondrial DNA fragment of a basidomyceteous fungus, *Pleurotus ostreatus* [21], where the DNA fragment has a CDEIII-like sequence with a single base-pair mismatch with the core sequence of *S. cerevisiae* CDEIII and a hairpin-loop structure with a high free energy value as well as several putative gyrase recognition-binding sites.

In order to know the potentials for the function of putative ARS and centromere-like sequence, the 836-bp

insert was subcloned using different restriction enzymes. We ligated the 545-bp *SalI-RsaI* fragment carrying hairpin-loop structures and an 8/11 match to the core consensus sequence of *S. cerevisiae* ARS into the *SalI-NruI* sites of YIp5, and introduced this ligated vector (designated as YRp1-4) into *S. cerevisiae*. The transformation of *S. cerevisiae* with YRp1-4 gave rise to only mini colonies at high frequency. However, these colonies exhibited no further growth on YNB plate without uracil. Similarly, a 291-bp *RsaI-SalI* fragment containing the CDEIII-like sequence as well as a putative gyrase-binding site was ligated into filled-up *SphI-SalI* sites of YIp5 (designated as YRp1-5). When *S. cerevisiae* was transformed with YRp1-5, no transforamant was obtaind. Therefore, 545-bp or 291-bp alone is not sufficient to promote replication activity. Only the 836-bp insert appears to have replicative and partitioning functions in *S. cerevisiae*. Although the 836-bp insert was devoid of any structural homology to *S. cerevisiae* centromeres, including runs of alternating AT (AT content>90%) [4], the copy number and stability of YRp1-2 plasmid was closer to those of centromere-containing ARS plasmids [19] than circular ARS plasmids [5]. Although the 836-bp sequence possesses some sequence features expected for a replicon, including a high energy profile as well as a potential gyrase binding site, the detailed functional analysis of sequences able to replicate and stabilize this plasmid in *S. cerevisiae* awaits further studies. These might involve bidirectional deletion of the 836-bp insert and site-directed mutagenesis of expected sites for plasmid replicon and partition. The *P. rhodozyma* system for extrachromosmally replicating transformation offers an opportunity for gene cloning by reisolation of transforming plasmids capable of complementing mutant alleles. To determine whether the 836-bp insert is able to act as a replicon in *P. rhodozyma* as well, it is also necessary to transform *P. rhodozyma* with the vector containing this insert as a replicon and a suitable marker for screening transformants. Transformation experiments of *P. rhodozyma* using the pGB-Ph9 [26] derivative carrying the 836-bp insert are presently being attemped in our laboratory.

Acknowledgments

This study was supported by research funds from Chonnam University (1997).

REFERENCES

1. An, G.-H., D. B. Schuman, and E.A. Johnson. 1989. Isolation of *Phaffia rhodozyma* mutants with increased astaxanthin content. *Appl. Environ. Microbiol.* **55:** 116–124.
2. Amati, B. B. and S. M. Gassser. 1988. Chromosomal ARS and CEN elements bind specifically to the yeast nuclear scaffold. *Cell* **54:** 967–978.
3. Andrews, A. G. and M. P. Starr. 1976. 3 R, 3' R-astaxanthin from the yeast *Phaffia rhodozyma*. *Phytochemistry* **15:** 1009–1011.
4. Bloom, K. S., M. Fitzgerald-Hayes, and J. Carbon. 1983. Structural analysis and sequence organization of yeast centromeres. *Cold Spring Harbor Symp. Quant. Biol.* **47:** 1175–1185.
5. Celniker, S. E., K. Sweder, F. Srienc, J. E. Bailey, and J. L. Cambell. 1984. Deletion mutation affecting autonomously replicating sequence ARS1 of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **4:** 2455–2466.
6. Chun, S. B., J. H. Chin, S. Bai, and G.-H, An. 1992. Strain improvement of *Phaffia rhodozyma* by protoplast fusion. *FEMS Microbiol. Lett.* **93:** 221–226.
7. Fabiani, L., M. Aragona, and L. Frontali. 1990. Isolation and sequence analysis of a *K. lactis* chromosomal DNA element able to autonomously replicate in *S. cerevisiae* and *K. lactis*. *Yeast* **6:** 69–76.
8. Farkasovska J. 1993. Sequence analysis of a *Papaver somniferum* L. mitochondrial DNA fragment promoting autonomous plasmid replication in *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. *Curr. Genet.* **24:** 366–367.
9. Giovannucci, E., A. Ascherio, E. B. Rimm, M. J. Stampfer, G. A. Colditz, and W. C. Willet. 1995. Intake of carotenoids and retinol in relation to risk of prostrate cancer. *J. Natl. Cancer Inst.* **87:** 1767–1776.
10. de Haas, J. M., J. Hille, F. Kors, B. van der Meer, A. J. Kool, O. Folkerts, and H. J. Nijkamp. 1991.Two potential hybrida mitochondrial DNA replication origins show structural and *in vitro* functional homology with the animal mitochondrial DNA heavy and light strand replication origins. *Curr. Genet.* **20:** 503–513.
11. Hieter, P., D. Pridmore, J. H. Hegeman, M. Thomas, R. W. Davis, and P. Philippsen. 1985. Functional selection and analysis of yeast centromeric DNA. *Cell* **42:** 913–921.
12. Iborra, F. and M. M. Ball. 1994. *Kluyveromyces marxianus* small DNA fragments contain both autonomous replicative and centromeric elements that also function in *Kluyveromyces lactis*. *Yeast* **10:** 1621–1629.
13. Johnson, E. A. and M. J. Lewis. 1979. Astaxanthin formation by the yeast *Ph. rhodozyma*. *J. Gen. Microbiol.* **115:** 173–183.
14. Kim, I.-G., S.-K, Nam, J.-H. Sohn, S.-K. Rhee, G.-H. An, S.-H. Lee, and E.-S. Choi. 1998. Cloning of the ribosomal protein L41 gene of *Phaffia rhodozyma* and its use as a drug resistance marker for transformation. *Appl. Environ Microbiol.* **64:** 1947–1949.
15. Kornberg, A. and T. A. Bake. 1992. *DNA Replication*, 2nd ed. pp. 504–505. W. H. Freeman and Company, New York, U.S.A.
16. Miki, W. 1991. Biological functions and activities of animal carotenoids. *Pure Appl. Chem.* **63:** 141–146.
17. Miller, M. W., M. Yoneyama, and M. Soneda. 1976. *Phaffia*, a new yeast genus in the *Deuteromycotina*. *Int. J. Syst. Bacteriol.* **26:** 286–291.

18. Nagy, A. N., N. Garamszegi, C. Vagrolgyi, and L. Ferenczy. 1994. Electrophoretic karyotypes of *Phaffia rhodozyma* strains. *FEMS Microbiol. Lett.* **123:** 315–318.
19. Ohkuma, M., K. Kobayashi, S. Kawai, C. W. Hwang, A. Ohta, and M. Takagi. 1995. Identification of a centromeric activity in the autonomously replicating TRA region allows improvement of the host-vector system for *Candida maltosa*. *Mol. Gen. Genet.* **249:** 447–455.
20. Park, J.-C., S. Bai, and S. B. Chun. 1990. Cloning and expression of *Schwanniomyces castellii* starch gene. *Kor. J. Appl. Microbiol. Biotech.* **18:** 653–659.
21. Peng, M., P. A. Lemke, and N. K. Singh. 1993. A nucleotide sequence involved in replicative transformation of a filamentous fungus. *Curr. Genet.* **24:** 114–121.
22. Sakai. Y., T. K. Goh, and Y. Tani. 1993. High-frequency transformation of a methylotrophic yeast, *Candida boidinii*, with autonomously replicating plasmids which are also functional in *Saccharomyces cerevisiae*. *J. Bacteriol.* **175:** 3556–3562.
23. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning*: *A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., U.S.A.
24. Teixeira, S. M. R., A. C. S. Frascino, E. V. Galenbeck, M. O. Azevedo, and S. A. Filho. 1986. Isolation of *Trypanosoma cruzi* DNA fragments which function as ARS elements in *Saccharomyces cerevisiae*. *Gene* **44:** 171–175.
25. Umek, R. M. and D. Kowalski. 1988. The *ease of DNA* unwinding as a determinant of initation of yeast replication origins. *Cell* **52:** 559–567.
26. Wery, J., D. D. Gutker, A. C. H. M. Renniers, J. C. Verdoes, A. J. J. van Ooyen. 1997. High copy number integration into the ribosomal DNA of the yeast *Phaffia rhodozyma*. *Gene* **184:** 80–97.
27. Williamson, D. H. 1985. The yeast ARS element, six years on: A progress report. *Yeast* **1:** 1–14.