Proc. Natl. Acad. Sci. USA
Vol. 77, No. 11, pp. 6329–6333, November 1980
Biochemistry

Autonomously replicating sequences in *Saccharomyces cerevisiae*

(chromosomal replicating segments/baker's yeast/repetitive sequences/transformation)

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Communicated by I. Robert Lehman, July 7, 1980

ABSTRACT **A method is presented for isolating DNA segments capable of autonomous replication from *Saccharomyces cerevisiae* chromosomal DNA based on the differential transforming ability of autonomously replicating plasmids and nonreplicating plasmids. DNA plasmids that are capable of self-replication in yeast transform yeast spheroplasts at about 1000-fold higher frequency than nonreplicating plasmids. We have cloned from total yeast DNA a number of DNA segments that permit the pBR322 plasmid carrying the yeast *LEU2* gene to replicate autonomously. These plasmid clones are characterized by their ability to transform Leu$^-$ spheroplasts to Leu$^+$ at a high frequency and their ability to replicate autonomously. Analysis of the insert DNAs carried in some of these self-replicating plasmids divides them into two categories: those that are unique in the yeast genome and those that are repetitive.**

For the past two decades, the study of the mechanism for DNA replication has been focused mainly on the chromosomes of the simple prokaryotes and their viruses. The complexity of the eukaryotic genome has until recently prevented similar studies. Eukaryotes have multiple chromosomes, each of which contains multiple replicons (replication units). These replicons are believed to be activated in a specific temporal order (1, 2). Experiments using *Drosophila melanogaster* and *Triturus* suggest that the different rates of DNA replication in rapidly dividing embryonic cells compared to slowly dividing somatic cells are regulated not by the rate of DNA chain growth but rather by the utilization of initiation sites (replicators) (3). The mechanism that determines the site and time within the cell cycle when replication begins is unknown. A first step towards understanding the control mechanism that regulates eukaryotic chromosomal replication is to study the structure, organization, and complexity of these chromosomal initiation sites. We describe here a method that allows the isolation of chromosomal DNA segments capable of autonomous replication from *Saccharomyces cerevisiae*, a simple eukaryote with 17 chromosomes and a genome 4 times the complexity of *Escherichia coli*.

Hinnen *et al.* (4) have shown that spheroplasts of a *leu2* yeast strain can be transformed to *LEU2* by a chimeric ColE1 plasmid carrying the *LEU2* gene. These Leu$^+$ transformants are stable, and the transforming plasmid DNA is integrated into the yeast genome. The transformation frequency for the *LEU2* gene is low (1–10 transformants per μg of DNA). The same low frequency of transformation has been observed for the *HIS3* gene (5) and the *URA3* gene (6). Yeast chromosomal DNA segments capable of autonomous replication transform yeast spheroplasts at much higher frequencies (2000–5000 transformants per μg of DNA), as shown for the *TRP1* gene (5), the *ARG4* gene (7), the *Bgl* II B fragment from DNA coding for rRNA (rDNA) (8), and the 2μ circle (5, 9, 10). These transformants, however, are unstable, and the transforming DNA replicates autonomously inside the cell.

We have used the differential transformation frequency between replicating and nonreplicating plasmid DNA to isolate individual chromosomal DNA segments capable of autonomous replication from yeast by cloning total *Sal* I-restricted yeast DNA into a chimeric pBR322 plasmid carrying the yeast *LEU2* gene (pSZ58) and selecting for Leu$^-$ → Leu$^+$ transformants. These chromosomal segments are presumed to carry chromosomal sites for the initiation of replication. It has been estimated by electron microscopy and fiber autoradiography (11) that there are about 200 replicons per haploid yeast genome. If the yeast genome [1.5×10^4 kilobases (kb)] is cleaved into fragments of 8 kb average size, then approximately 1 out of 10 fragments should contain a replicator. Because self-replicating plasmids transform yeast spheroplasts at a frequency about 1000-fold higher than nonreplicating plasmids, one would expect >90% of the cells transformed by a nonreplicating plasmid carrying random yeast DNA inserts of 8 kb to harbor self-replicating plasmids. This was the basis of the isolation scheme.

MATERIALS AND METHODS

Strains. *E. coli* strain 5346 (*thr1 leuB thi1 lacY1 hsm1 hsr1*) or SF8 (C600 *thr6 leuB thy$^-$ hsr$^-$ hsm$^-$ lop11 recBC*) was used as the host for the amplification of all hybrid plasmids. Yeast strain LL20 (*α leu2-1 leu2-112 his3-11 his3-15*) was obtained from G. Fink and was used as the recipient for yeast transformation. Strain LL20-1 is derived from strain LL20, and it carries an integrated copy of the plasmid pSZ53 in the genome (8). Yeast DNA used for construction of the yeast DNA bank was prepared from strain S228C (*α mal gal2*).

Plasmids. Plasmids pSZ57, pSZ58, and pSZ53 were constructed by J. Szostak. Plasmid pSZ58 was constructed by insertion of the *Sal* I/*Xho* I fragment containing the yeast *LEU2* gene into the *Sal* I site of pBR322 (Fig. 1A). Plasmid pSZ57 is the same as pSZ58 except that the *Sal* I/*Xho* I fragment is in the reverse orientation. Plasmid pSZ53 was constructed by insertion of the *Eco*RI/*Hin*dIII fragment (containing the 5S RNA gene) of the rDNA repeats into pSZ58.

DNA Preparation. Yeast DNA was isolated as described (12). DNA enriched in plasmid was isolated from yeast by the rapid procedure (5) except that cells were grown on agar plates and zymolyase was used in place of lyticase. Total *E. coli* DNA was prepared by Marmur's procedure (13). Plasmid DNA from *E. coli* was prepared essentially as described (14, 15) or by a rapid procedure described by Davis *et al.* (16).

Yeast Transformation. The procedure for yeast transformation has been described by Hinnen *et al.* (17).

***E. coli* Transformation.** Competent cells were prepared by Ca^{2+} treatment (18) and transformation was carried out as described (19).

Other Techniques. DNA fragments were eluted from agarose gels by the perchlorate method (20). ^{32}P-Labeled probes were prepared by nick translation (21). DNA was transferred

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Abbreviations: kb, kilobases; rDNA, DNA coding for rRNA.

from agarose gel to nitrocellulose filter as described by Southern (22). DNA hybridization was performed at 65°C in 0.75 M NaCl/0.075 M sodium citrate, pH 7.0, containing 1% Sarkosyl. Screening by colony hybridization was done as described by Grunstein and Hogness (23).

Enzymes. *E. coli* DNA ligase was a gift from I. R. Lehman and was used as described (24). All restriction endonucleases were purchased from New England BioLabs and used as directed. DNase I and lysozyme were purchased from Sigma. DNA polymerase I was purchased from Boehringer Mannheim. Zymolyase was a gift from Kirin Brewery, and glusulase was purchased from Endo Laboratory.

Media. All media used have been described (4, 25).

Biohazard Considerations. This work was carried out under P2 containment conditions specified by the National Institutes of Health Guidelines for Recombinant DNA Research.

RESULTS

Isolation of Replicating Segments from Yeast Genome. The vector used to clone yeast DNA fragments was the chimeric plasmid pSZ58 (Fig. 1A). Because we were interested in the cloning of chromosomal *a*utonomously *r*eplicating *s*equences (*ars*), we wanted to avoid cloning both the extrachromosomal (mitochondrial and 2μ) *ars*s and the *ars*s from rDNA. The 2μ and ribosomal DNA could be avoided by cloning *Sal* I-restricted fragments from the total yeast genome because there are no *Sal* I sites in either 2μ circles or the rDNA repeat (26, 27). Although there is a single *Sal* I site in mitochondrial DNA (28), this DNA is too large to be cloned into pSZ58.

Forty micrograms of *Sal* I-restricted total yeast DNA purified from strain S288C was ligated with 10 μg of *Sal* I-restricted pSZ58 plasmid DNA. The ligation mixture was used to transform strain LL20 to the Leu$^+$ phenotype. The parental plasmid DNA (pSZ58) transformed LL20 to Leu$^+$ at a frequency of about 1–2 Leu$^+$ transformants per μg of DNA. However, in the same experiment, 5 μg of the ligation mixture (4 μg of yeast DNA and 1 μg of pSZ58 plasmid DNA) yielded 45 Leu$^+$ transformants, which is a 5- to 10-fold increase in the frequency of transformation over the control experiment. Of the 45 transformants examined, two-thirds were unstable for the *LEU2* marker on the plasmid, a property which is characteristic of transformants carrying replicating plasmids. In this way, we obtained approximately 150 independent Leu$^+$ transformants unstable for the *LEU2* marker.

Correlation Between Instability of the *LEU2* Marker and the Presence of Self-Replicating Plasmid. The correlation between the instability of the *LEU2* marker and the presence of self-replicating plasmids was examined by analysis of the DNA content of the unstable transformants. DNA from these Leu$^+$ transformants was extracted by the rapid procedure. These DNA preparations were subjected to electrophoresis on 0.7% agarose gel and then transferred to nitrocellulose filters (22). ^{32}P-Labeled pBR322 DNA probe was prepared by nick translation (21). The controls used were pSZ58 plasmid DNA, DNA extracted from the untransformed LL20 strain, and DNA from the LL20-1 strain, which carries a copy of the integrated pSZ53 plasmid. All Leu$^+$ transformants showed positive hybridization with the pBR322 probe. Because the DNA on these gels was not cut, the chromosomal DNA migrated to one position (at the top of the gel) and the plasmid DNA migrated to a different position (further down). The DNA from some transformants showed hybridization at positions on the gel suggesting the presence of pBR322 DNA in the form of a plasmid—e.g., nos. 15, 20, and 21 (Fig. 2)—whereas others showed hybridization at positions close to the chromosomal DNA bands. Every strain that showed a plasmid band was an unstable transformant.

Recovery of Plasmid DNA from Yeast. The best evidence that these cloned segments confer the property of autonomous replication comes from the behavior of these plasmids when they were reintroduced into yeast in subsequent transformation experiments. These plasmids were isolated from the unstable transformants by the rapid procedure and were used to transform *E. coli* to ampicillin resistance. Plasmid DNA was amplified in *E. coli* and purified. Nineteen independent plasmid

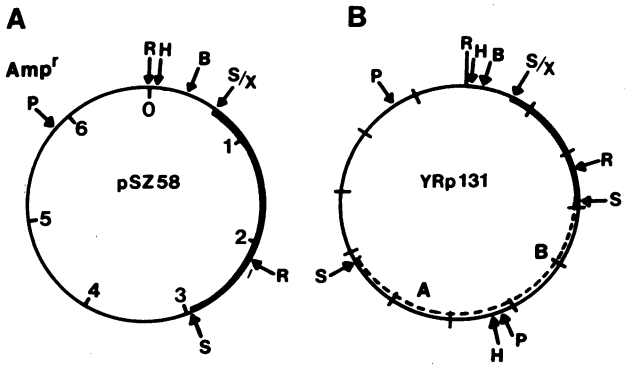

FIG. 1. (*A*) Restriction map of the cloning vector pSZ58 (6.6 kb). (*B*) Restriction map of YRp131 (≈11.45 kb). —, pBR322 DNA; ▬, DNA carrying the yeast *LEU2* gene; ═, yeast *Sal* I insert DNA. R, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; S, *Sal* I; X, *Xho* I; P, *Pst* I.

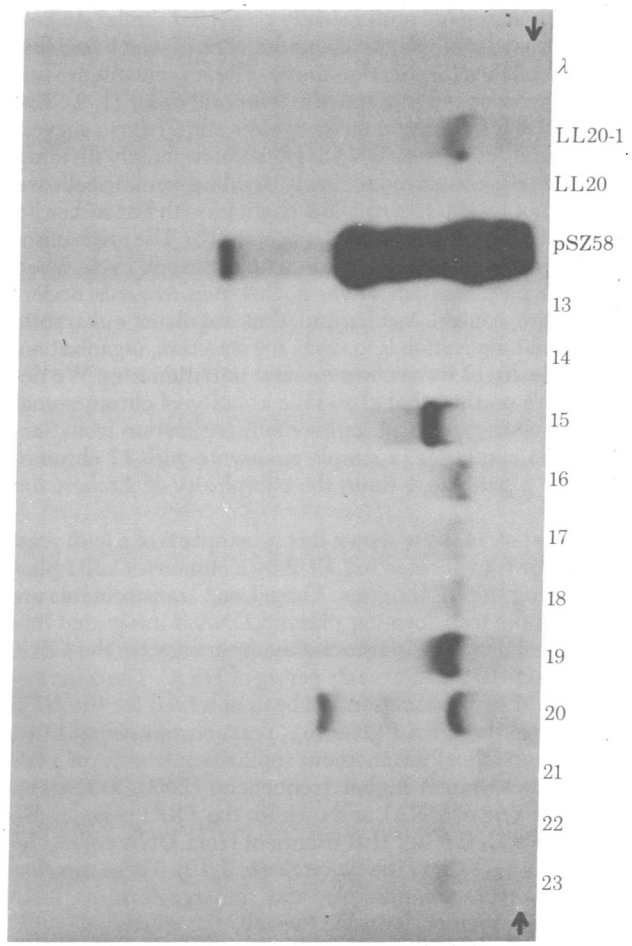

FIG. 2. States of the transforming plasmids in Leu$^+$ yeast transformants. DNA was extracted from *LEU2* transformants grown on selective medium. These DNA extracts were separated on 0.7% agarose gel, transferred to nitrocellulose filters, and hybridized with ^{32}P-labeled pBR322 DNA.

clones isolated from *E. coli* were characterized by restriction analysis (Table 1). Ten of them retained the entire pSZ58 vector with inserts at the *Sal* I site. The sizes of these inserts varied from 2.3 kb to 27 kb. All of these plasmids transformed Leu$^-$ yeast spheroplasts at frequencies significantly higher than that of pSZ58. These frequencies ranged from 500 transformants per μg of DNA to over 4000 transformants per μg of DNA. These transformants are all unstable for the *LEU2* allele and show the serrated colony morphology characteristic of transformants carrying replicating plasmids. The transforming DNA exists as free plasmids in each of these transformants, as detected by hybridization (data not shown). Two plasmids (YRp111 and YRp220) are the same size as pSZ58 and may either have inserts too small to be determined on a 0.7% agarose gel or they may have been derived from pSZ58 through some minor sequence rearrangements. Four plasmids (YRp157, YRp158, YRp175, and YRp180) seem to have lost one *Sal* I site as though deletion of one of the *Sal* I sites had taken place. The insert from one such plasmid (YRp175) hybridized to a 6.2-kb *Sal* I fragment from yeast, thus suggesting that a deletion of 3.6 kb had occurred. Three other plasmids (YRp208, YRp210, and YRp230) seem to have resulted from large deletions because these plasmids are smaller than the 6.6 kb of the original vector. These three plasmids can no longer complement the *E. coli leuB* allele. Moreover, none of the plasmids that carry deletions can transform Leu$^-$ yeast cells at high frequency, suggesting that deletion might have taken place during growth in *E. coli*.

Hybridization of Insert DNA to *Sal* I-Restricted Total Yeast Genome. In order to identify the source of these DNA inserts, the insert DNA (*Sal* I restriction fragment) of 10 of the plasmid clones was labeled with ^{32}P by nick translation. They were used for hybridization to *Sal* I-restricted total yeast DNA that had been separated on 0.7% agarose gel and transferred to nitrocellulose filters. They all hybridized to the yeast genome under moderately stringent hybridization conditions (65°C; 0.75 M NaCl/0.075 M sodium citrate). Six of the insert DNAs hybridized to unique bands, and four of them hybridized to multiple bands (Fig. 3). Of the four independent isolates that hybridized to multiple bands, three of them (YRp131, YRp120, and YRp206) crosshybridized with each other and they seemed to hybridize to a similar set of bands (Fig. 3). The transposable element TY1, which is a known repetitive sequence in yeast (29), did not hybridize to these three inserts but hybridized to the insert of YRp245. None of the inserts studied hybridized to mitochondrial DNA (gift from M. Rabinowitz) or 2μ plasmid DNA. They are therefore sequences derived from the chromosomal DNA of the yeast genome.

The YRp131 Plasmid. The insert of YRp131 hybridized to between 10 and 15 sites in the yeast genome (Fig. 3). It is about 4.8 kb long and can be cleaved into two approximately equal halves (A and B) by the *Hin*dIII restriction enzyme. A restriction map of the YRp131 plasmid is shown in Fig. 1*B*. When each half of the DNA insert was labeled with ^{32}P and used in

Table 1. Properties of plasmid clones isolated from Leu$^+$ transformants

Plasmid	Insert size, kb	Complementation to *E. coli leuB* allele	Transformation frequency	No. bands hybridized
YRp120	4.3	+	500	>10
YRp121	27.0	+	1600	1
YRp131	4.8	+	2500	>10
YRp132	6.0	+	3000	1
YRp137	2.3	+	4150	1
YRp166	9.7	+	1450	1
YRp191	6.8	+	960	1
YRp206	5.0	+	2600	>10
YRp245	4.4	+	4400	>10
YRp249	5.7	+	1250	1
YRp111	?*	+	60	ND
YRp220	?*	+	0	ND
YRp157	ND†	+	0	ND
YRp158	ND†	+	10	ND
YRp175	ND†	+	10	1
YRp180	ND†	+	0	ND
YRp208	ND‡	−	0	ND
YRp210	ND‡	−	0	ND
YRp230	ND‡	−	50	ND
pSZ58	0	+	3	0

Transformation frequency is expressed as the number of Leu$^+$ transformants per μg of transforming plasmid used. ND, not determined.

* Plasmids appear to be very similar to pSZ58. Inserts are either too small to be detected on 0.7% agarose gel or they are not present.

† Plasmids are 9.2 kb long and contain only one *Sal* I site.

‡ Plasmids are 5.1 kb long and contain only one *Sal* I site.

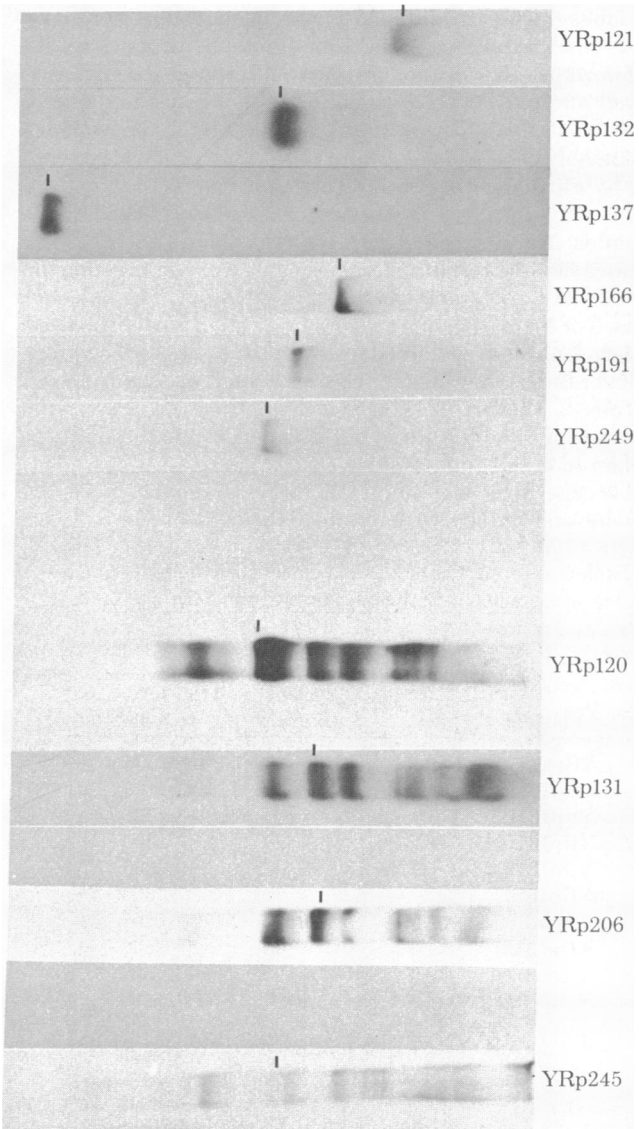

FIG. 3. Complexity of autonomously replicating segments in the yeast genome. Cloned *Sal* I inserts containing yeast replicators were labeled with ^{32}P and used to hybridize *Sal* I-restricted total yeast DNA that had been separated on 0.7% agarose gel and blotted onto nitrocellulose filters. The bars indicate the positions corresponding to the size of each of the inserts used for the hybridization.

hybridization to probe total *Sal* I-restricted yeast DNA, only the A fragment hybridized to the same set of multiple bands revealed by the complete insert in YRp131. The B fragment hybridized to only a single band, corresponding to the 4.8-kb *Sal* I restriction fragment, indicating that fragment A but not fragment B contains the repetitive DNA sequence. We have subcloned fragment A into the pSZ57 vector. The resulting plasmid retained the ability to transform Leu$^-$ yeast cells to Leu$^+$ at high frequency (1400 transformants per μg of DNA). Thus, fragment A, which is 2.4 kb long, retained both the repetitive DNA sequence and the ability to transform yeast cells at high frequency.

Are All Members of a Family of Homologous DNA Sequences Capable of Autonomous Replication? We have shown that one out of four randomly isolated *Sal* I fragments from yeast confers the ability to replicate in pSZ58 (see next section). In order to investigate the significance of these repetitive homologous DNA sequences, we examined the correlation between the occurrence of these repetitive sequences and the ability of a plasmid to self-replicate. Fragment A of YRp131 was labeled with ^{32}P and used as a probe to screen for homologous DNA sequences of the same family by colony hybridization with an *E. coli* bank carrying *Sal* I-restricted yeast DNA cloned into pSZ58. Thirteen out of 1400 independent clones showed positive hybridization. The inserts of these clones presumably correspond to the set of *Sal* I restriction fragments that hybridized to the A fragment of YRp131 on the gel. Plasmid DNA was prepared from each of these clones. All 13 of the plasmids transformed Leu$^-$ yeast spheroplasts at high frequencies, the resulting transformants were all unstable for the *LEU2* marker, and free plasmid DNA could be detected in each of them (data not shown). Restriction analysis showed that each of them contained inserts at the *Sal* I site. The properties of these plasmids and the sizes of their inserts are shown in Table 2. These results suggest a close correlation between the presence of these repetitive DNA sequences and the ability of a plasmid to self-replicate (see *Discussion*).

Average Size of a Replicon in *S. cerevisiae*. Recently, Newlon and Burke (30) determined the replicon size in *S. cerevisiae* by electron microscopy and found an average replicon size of 36 kb. We have tried to determine the number of replicons in yeast by transforming *E. coli* with the yeast genome bank that was constructed by cloning *Sal* I-restricted yeast DNA into pSZ58. Plasmids were extracted from these ampicillin-resistant clones and screened for the presence of *Sal* I inserts. Those plasmids that contained *Sal* I inserts were used to transform a Leu$^-$ strain to Leu$^+$. About one-quarter (5 out of 20) of the DNA plasmid clones thus obtained transformed yeast at a high frequency, and plasmid DNA could be detected in each of these transformants. Because the average size of the *Sal* I fragments was $\approx$8 kb, one can estimate that the average size of a replicon in yeast is about 32 kb and the average number of replicons per chromosome is about 25, excluding from the calculation the 140 copies of the rDNA repeat in the yeast genome (31). Similar results were observed in other laboratories (ref. 32; S. C. Falco and D. Botstein, personal communication) by using different restriction enzyme digests for cloning. Thus, the average size of replicons in yeast as estimated by electron microscopy measurement agrees with the value determined by genetic cloning.

Table 2. Properties of plasmid clones that showed sequence homology to the *Sal* I insert from YRp131

Plasmid	Insert size, kb	Complementation to *E. coli leuB* allele	Transformation frequency
YRp131	4.8	+	2500
YRp131A	10.0	+	2100
YRp131B*	6.9	+	800
YRp131C	4.3	+	2600
YRp131F*	6.9	+	2000
YRp131J	12.0	+	2300
YRp131L*	6.9	+	1200
YRp131M	4.2	+	2800
YRp131N	10.0	+	1500
YRp131O*	6.9	+	1000
YRp131P*	6.9	+	2000
YRp131Q*	6.9	+	4500
YRp131R	5.9	+	3400
YRp131S	5.2	+	4000
YRp120	4.3	+	500
YRp206	5.0	+	2600

Transformation frequency is expressed as the number of Leu$^+$ transformants per μg of transforming plasmid used.
* These plasmids appear to carry identical inserts by restriction analysis.

DISCUSSION

We have described a method to isolate segments of yeast chromosomal DNA that confer self-replicating ability to nonreplicating plasmids in yeast. There is no direct evidence that these DNA segments are the chromosomal sites for initiation of replication. Our procedure provides a functional assay for DNA segments capable of conferring autonomous replication. However, the close agreement between the replicon size as determined by electron microscopy measurement (36 kb) and that determined by the genetic cloning method described here (32 kb) suggests that our procedure has isolated segments of DNA that function as sites for normal chromosomal replication initiation. Because the yeast genome is estimated to be approximately 1.5×10^4 kb long, there are about 450 replicons in the haploid yeast genome, excluding the replicons located in the rDNA repeat units. The fact that three-quarters of the hybrid plasmids isolated are unable to replicate autonomously in yeast suggests that the sites of chromosomal replication initiation are not random DNA sequences. This conclusion was also reached by Stinchcomb *et al.* (33), who isolated the first yeast chromosomal replicator adjacent to the *TRP1* gene.

The technique described here may be extended to the study of chromosomal replication of other eukaryotes. Preliminary experiments show that self-replicating plasmids can be isolated by cloning DNA from other eukaryotes such as *Echinus esculentus* (unpublished results), *Neurospora crassa*, *Dictyostelium discoideum*, *Ceanhorabditis elegans*, *Drosophila melanogaster*, and *Zea mays* (34) into nonreplicating vectors. However, the nature of these inserts has not been extensively characterized.

Information about the complexity of replicators in eukaryotic chromosomes has not been available due to the limitation of conventional techniques. We have isolated self-replicating DNA segments as probes to determine the complexity of replicators in the yeast genome. The 10 replicating segments examined fell into two categories: 6 of them hybridized to unique sequences in the yeast genome and 4 of them showed homology to families of sequences in the genome. Because the yeast genome has very little redundant DNA (35), the frequent occurrence of these reiterated sequences among randomly selected self-replicating segments suggests an association of these repetitive sequences with the ability to self-replicate. This suggestion is greatly strengthened by the finding that all 16 members (6 of them may be identical) of one of these families of homologous DNA seg-

ments are capable of autonomous replication (Table 2). In view of the frequency of occurrence of self-replicating segments (about 25%) in plasmid clones carrying random yeast DNA inserts, this strong bias suggests a high probability (>99%) of association between these repetitive sequences and the ability to self-replicate. Whether these repetitive sequences are the functional replicators or signals adjacent to a set of replicators is not known. The significance of these repetitive sequences can be revealed only by further functional delimitation or subcloning experiments.

We are grateful to Dr. G. Fink and Dr. J. Roberts for their valuable comments. We thank Dr. J. Szostak for helpful discussions and Mrs. Connie Delwiche for her technical assistance. We thank Kim Muzzy for preparation of the manuscript. This work was supported in part by research grants from the National Institutes of Health (AI 1498001) and the National Science Foundation (PCM 78-02341), and in part by a grant from the U.S. Department of Agriculture (Hatch-NYC-181412).

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