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Deletion Mutations Affecting Autonomously Replicating Sequence *ARS1* of *Saccharomyces cerevisiae*

S. E. CELNIKER,† K. SWEDER, F. SRIENC, J. E. BAILEY, AND J. L. CAMPBELL*

Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125

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DNAs that contain specific yeast chromosomal sequences called *ARS*s transform *Saccharomyces cerevisiae* at high frequency and can replicate extrachromosomally as plasmids when introduced into *S. cerevisiae* by transformation. To determine the boundaries of the minimal sequences required for autonomous replication in *S. cerevisiae*, we have carried out in vitro mutagenesis of the first chromosomal *ARS* described, *ARS1*. Rather than identifying a distinct and continuous segment that mediates the *ARS*$^+$ phenotype, we find three different functional domains within *ARS1*. We define domain A as the 11-base-pair (bp) sequence that is also found at most other *ARS* regions. It is necessary but not sufficient for high-frequency transformation. Domain B, which cannot mediate high-frequency transformation, or replicate by itself, is required for efficient, stable replication of plasmids containing domain A. Domain B, as we define it, is continuous with domain A in *ARS1*, but insertions of 4 bp between the two do not affect replication. The extent of domain B has an upper limit of 109 bp and a lower limit of 46 bp in size. There is no obvious sequence homology between domain B of *ARS1* and any other *ARS* sequence. Finally, domain C is defined on the basis of our deletions as at least 200 bp flanking domain A on the opposite side from domain B and is also required for the stability of domain A in *S. cerevisiae*. The effect of deletions of domain C can be observed only in the absence of domain B, at least by the assays used in the current study, and the significance of this finding is discussed.

As in higher organisms, chromosome replication in *Saccharomyces cerevisiae* initiates at multiple sites and is bidirectional. The DNA is replicated only during the S phase, and activation of individual replicons is thought to occur according to a specific temporal program, with each replicon activated only once per cell cycle (for review, see reference 3). There is no direct evidence in any eucaryote that chromosomal replication initiates at the same chromosomal sequence during each cell cycle. However, indirect evidence is available in *S. cerevisiae*. Yeast sequences have been isolated that confer on any colinear DNA the ability to be maintained in *S. cerevisiae* as autonomously replicating, albeit highly unstable, plasmids (20, 41, 44). The sequences responsible for autonomous replication are designated *ARS*. Estimates of the number of *ARS* elements in the *S. cerevisiae* genome (1, 7) agree with the number of initiation sites for DNA replication estimated by fiber autoradiography and electron microscopy (reviewed in reference 3). *ARS* elements have been classified by nucleic acid hybridization as either unique or repetitive (6, 8, 41, 45). In addition to *S. cerevisiae* DNA, DNAs from a wide variety of eucaryotes including *Neurospora crassa*, *Dictyostelium discoideum*, *Caenhorabditis elegans*, *Drosophila melanogaster*, *Zea mays* (42), humans (31), *Xenopus* mitochondrial DNA (51), and *Tetrahymena* rDNA (25) also promote high-frequency transformation of recombinant DNA plasmids in yeasts. Thus it is even possible that initiation of DNA replication occurs at sequences which are similar in many eucaryotes. But are such *ARS* elements origins of replication?

A eucaryotic chromosomal origin of replication should contain sequences that allow the initiation of DNA synthesis, initiation of bidirectional replication, and the temporal regulation of initiation within the S phase. We have shown, by in vitro replication, that *ARS* plasmids contain the necessary information to direct the initiation of DNA synthesis at a unique site (5) and that subsequent synthesis is sequential and bidirectional. Fangman et al. (11) have recently shown that the yeast *ARS* plasmids replicate only once during each S phase and also contain the information necessary to determine the time of activation of replication within the S phase. These sequences also replicate under the control of the *CDC* genes that are required for chromosomal replication (52). The only substantive evidence against *ARS* elements being origins of replication comes from the fact that specific sequences do not seem to be required in *Xenopus* embryos for cell-cycle-regulated DNA replication (e.g., see reference 17).

Hoping to gain a better understanding of the function of *ARS*s, we have investigated the organization of the *S. cerevisiae ARS1* element, first identified by Struhl et al. (44). Stinchcomb et al. (40, 41) located *ARS1* to an 838-base-pair (bp) *Eco*RI-*Hin*dIII fragment. Further studies showed that a smaller fragment of 600 bp was not sufficient for *ARS* function (40, 48). We have extended these studies by systematically generating mutations in vitro in the cloned *ARS1* element and then introducing these mutant DNAs into *S. cerevisiae* cells by transformation. We have assayed *ARS* activity by the ability of the plasmid to transform the *S. cerevisiae* cells at high frequency and to be maintained as extrachromosomal DNA. Instead of finding, as expected by analogy to *oriC* of *Escherichia coli*, that *ARS* activity can be described in terms of a single continuous minimal DNA segment, we have found that *ARS1* consists of different domains, each of which affects only part of the *ARS*$^+$ phenotype. We also describe a new method for determining the plasmid copy number.

MATERIALS AND METHODS

Materials. Acrylamide, bisacrylamide, and bisacrylylcystamine were obtained from Bio-Rad Laboratories; Sea-

* Corresponding author.
† Present address: Division of Biology, California Institute of Technology, Pasadena, CA 91125.

Kem agarose and low-melting-temperature agarose (Sea Plaque) were obtained from FMC Corp., Marine Colloids Div.; [α-^{32}P]dNTPs (400 to 3,000 Ci/mmol) were obtained from Amersham Corp.; [γ-^{32}P]ATP (8,000 Ci/mmol) was obtained from ICN Pharmaceuticals Inc.; restriction enzymes were obtained from New England Biolabs; DNA modifying enzymes were obtained from Boehringer Mannheim Biochemicals, Bethesda Research Laboratories, and Sigma Chemical Co.; and T4 polynucleotide kinase and T4 DNA ligase were gifts from C. C. Richardson, Harvard University, Boston, Mass. *Eco*RI and *Hin*dIII linkers were from Collaborative Research, Inc.

Strains, plasmids, and media. The *S. cerevisiae* strain used for yeast transformation, SS111 (**a** *trp1-289 ura3-1 ura3-2 his3-532 ade2-10 gal2 ino can*^{R}) was a gift from S. Scherer, California Institute of Technology, Pasadena. The yeast strain used to study the pLG plasmids (see Fig. 5) was YM603 (**a** *ura3-52 lys2-801 met his3 ade2-101 reg1-501*), which was a gift from M. Johnston, Washington University, St. Louis, Mo., and which contains a mutation (*reg1-501*) that renders the strain insensitive to catabolite repression by glucose. Strain D603 is a homozygous **a/a** diploid prepared from strain YM603. Plasmid pLGSD5 is described by Guarente et al. (15). All plasmids used in these studies are described below (see Fig. 1 and 6).

Complete medium contained 1% yeast extract, 2% peptone, and 2% glucose (YPD medium). Minimal medium was 0.64% yeast nitrogen base–2% glucose–0.002% adenine sulfate–0.002% uracil and was supplemented with purified amino acids (SD medium).

E. coli MC1061 (4) was grown in either Vogel-Bonner medium (50) or L broth (28). For selective growth, ampicillin was added to 35 to 50 μg/ml.

DNA preparations. Yeast plasmid DNA was prepared by the method of Nasmyth and Reed (34). To identify yeast plasmids, a sample of the DNA was electrophoresed on a 0.8% agarose gel as described below, transferred to nitrocellulose, and then localized by hybridization with pBR322 [^{32}P]DNA (32, 37).

Plasmid DNA was prepared on a large scale (1 to 4 liters) from stationary phase *E. coli* cells grown in Vogel-Bonner medium (10).

Yeast transformations. All yeast transformation frequencies were determined with 4 μg of plasmid DNA purified as described in reference 10. Transformation was by a modification of the method of Ito et al. (21) as described by Kuo and Campbell (26).

Gel electrophoresis. DNA was electrophoresed in 0.8 to 3% agarose cells containing TAE buffer (40 mM Tris-hydrochloride [pH 7.5], 10 mM sodium acetate, 2 mM EDTA) at 30 to 60 V for 12 to 16 h. The DNA was visualized by staining with ethidium bromide.

Polyacrylamide gels were used to resolve DNA fragments less than 600 bp in size. The acrylamide–*N*,*N*'-methylenebisacrylamide ratio was 29:1, and the concentration of acrylamide varied from 8 to 25%. Gels were prepared, and electrophoresis was carried out in Tris-borate buffer (TBE: 88 mM Tris-hydrochloride, 89 mM boric acid, 2.5 mM Na_2–EDTA [pH 8.3]). Some DNA fragments for sequencing were isolated from acrylamide gels with the cleavable cross-linker bisacrylylcystamine in the ratio 19:1, acrylamide to cross-linker (16).

Construction of deletion and insertion mutants. DNA molecules were linearized with the appropriate restriction enzyme and extracted with phenol and then chloroform-isoamyl alcohol (24:1); the DNA was ethanol precipitated. Linear DNA (1 μg) was incubated for 30 min at 37°C in a reaction mix (0.3 ml) containing 280 mM NaCl, 30 mM sodium acetate (pH 4.4), 4.5 mM $ZnCl_2$, and 30 to 67 U of S1 nuclease (12). The reaction was terminated by the addition of sodium acetate to a final concentration of 0.3 M. The DNA was precipitated with EtOH and resuspended in a 200-μl ligation reaction mixture that contained 50 mM Tris-hydrochloride (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM ATP, and 1 μl of ligase. The reaction was incubated for 16 h at 12°C.

DNA molecules were linearized and prepared as described for the S1 nuclease digestion. DNA (1 μg) was incubated for 30 min at 25°C in a reaction mix (25 μl) containing 0.5 M Tris-hydrochloride (pH 7.8), 5 mM $MgCl_2$, 5 mM dithiothreitol, 50 μg of bovine serum albumin per ml, 0.1 mM each dATP, dTTP, dCTP, and dGTP, and 1 U of the Klenow fragment of *E. coli* DNA polymerase I. The reaction was terminated, and the DNA was prepared and ligated as described above.

Construction of plasmids containing *Bal*31-generated deletions. Plasmid DNA (10 μg) was cut with a 10-fold excess of restriction enzyme at 37°C for 2 h in an 0.05-ml reaction mix containing 60 mM NaCl, 7 mM Tris-hydrochloride (pH 7.4), 7 mM $MgCl_2$, and 150 μg of bovine serum albumin per ml. The restriction enzyme was inactivated by incubation at 70°C for 10 min. The reaction mix was then made 12 mM $CaCl_2$, 12 mM $MgCl_2$, 20 mM Tris-hydrochloride (pH 8.0), 1 mM EDTA, and 0.2 M NaCl, and the final volume was brought to 0.1 ml. The appropriate concentration of the double-strand exonuclease *Pseudomonas Bal*31 was added (27). At 1-min intervals, 10-μl samples were removed and the reaction was stopped by the addition of EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid] to a final concentration of 0.02 M. A 20% portion of the sample was digested with restriction enzymes and analyzed by gel electrophoresis. The remainder was stored at −70°C in EtOH.

The *Bal*31-digested DNA was collected by centrifugation and resuspended in 0.05 ml of 0.05 M Tris-hydrochloride (pH 9.0). The DNA was dephosphorylated by the addition of 0.01 U of calf intestinal alkaline phosphatase, followed by incubation for 30 min at 37°C. The phosphatase was inactivated by the addition of 1 μl of 0.2 M sodium nitrilotriacetate for 10 min at 37°C, and DNA was precipitated with ethanol. Oligonucleotide linkers (*Eco*RI or *Hin*dIII) were phosphorylated in a reaction mixture containing 50 mM Tris-hydrochloride (pH 9.0), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM spermidine, 1 mM dithiothreitol, 0.05 mM ATP, and 1 μl of T4 polynucleotide kinase for 30 min at 37°C. The reaction was stopped by freezing at −20°C.

Phosphorylated linker (100 ng) was then ligated to the *Bal*31-digested, dephosphorylated DNA in a 0.25-ml reaction mix which contained 50 mM Tris-hydrochloride (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 mM ATP for 3 h at 12°C. The ligase was inactivated by heating for 10 min at 70°C. The reaction mix was then made 50 mM NaCl, 50 mM Tris-hydrochloride (pH 7.5), 10 mM $MgCl_2$, and 1 mM dithiothreitol in a final volume of 0.05 ml. Restriction enzyme (30 U) was added, and the DNA was digested at 37°C for 1 h. The free pentanucleotide generated by this digest was removed by EtOH precipitation with ammonium acetate as described by Maxam and Gilbert (29). The restricted DNA was then ligated in 0.2 ml overnight, using the buffer described above. Samples (5, 10, and 20 μl) of the ligation reaction were used for transformation of competent *E. coli* MC1061 cells, as described above.

Construction of subclones for copy number analysis. The 2μ

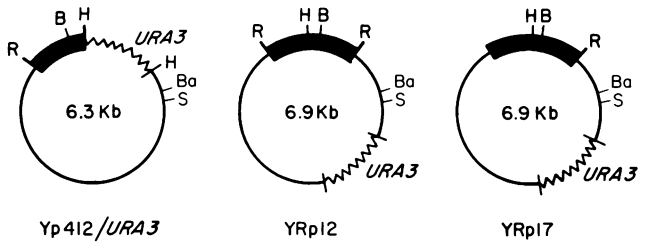


FIG. 1. Plasmids used for in vitro mutagenesis. Yp412/*URA3* is a derivative of plasmid YRp7 which contains the first identified *ARS*, *ARS1*, in the yeast fragment Sc4101 (41). Plasmid Yp412/*URA3* was constructed by digesting YRp7 with *Hin*dIII, followed by religation. This resulted in the removal of a 630-bp *Hin*dIII fragment. The *URA3* gene, contained in a 1.1-kb yeast DNA fragment, was then inserted into the *Hin*dIII site. YRp12 and YRp17 (gifts from S. Scherer) are derivatives of YIp5 and have the *ARS1* fragment Sc4101 inserted into the *Eco*RI site of pBR322 in the opposite orientation to YRp7 (35, 44). The 1.1-kb *URA3* DNA fragment was inserted into the *Ava*I site of pBR322. YRp17 has a single *Eco*RI site as indicated. Symbols: ——, pBR322 DNA; ■, yeast chromosomal sequence Sc4101 or a derivative thereof; ≈, yeast chromosomal DNA containing *URA3*. Restriction sites: R, *Eco*RI; B, *Bgl*II; H, *Hin*dIII; Ba, *Bam*HI; S, *Sal*I.

fragment of pLGSD5 (see Fig. 6) was replaced by the *Eco*RI-*Hin*dIII fragment either from YRp7, containing the complete *ARS1* region, or from various deletion plasmids (see Fig. 2 and 5). All constructions were carried out by the same procedure, with pLGSD5/2, which is a derivative of pLGSD5 in which we eliminated the *Hin*dIII site within the 2μ region by partially digesting pLGSD5 with *Hin*dIII and filling in and ligating the blunt ends by standard procedures. To replace 2μ DNA with *ARS1* sequences between the *Eco*RI site at the end of the 2μ sequences and the unique remaining *Hin*dIII site, pLGSD5/2 was digested partially with *Eco*RI and ligated with a complete *Eco*RI digest of YRp7 or the variants of YRp17. The ligation mixture was digested completely with *Hin*dIII and religated. To avoid a high frequency of unwanted plasmids among the transformants, the mixture was digested with *Xba*I (which has a unique site in the 2μ fragment) and *Sal*I (which has a unique site in the undesired regions of YRp7 or YRp17 plasmids) before transformation. Desired transformants were identified by the presence of a characteristic *Bgl*II-*Xho*I fragment (see Fig. 6). All resulting plasmids, pLGARS1, pLGR8, pLGR88, pLGH103, and pLGH200, carry the *ARS1* fragment as the *Eco*RI-*Hin*dIII fragments (Figs. 2 and 5) and are in the same orientation. Finally a plasmid, pLG2ARS1, was constructed that carried two copies of *ARS1*.

β-Galactosidase assay. β-Galactosidase activity was measured spectrophotometrically with whole cells after permeabilization with isopropanol as previously described (38). The reaction mixture (1 ml) contained 0.1 M sodium phosphate [pH 7.0], 0.02 M KCl, 2 mM $MgSO_4$, 25 to 200 μl of permeabilized cells, and 1 mg of *O*-nitrophenyl-β-D-galactopyranoside per ml. The reaction was carried out at 37°C and stopped by the addition of 0.3 ml of Na_2CO_3 (1 M). The suspension was centrifuged in an Eppendorf microfuge for 1 min, and the absorbance of the supernatant was measured at 420 nm. Initial rates were measured, and activity was normalized to the cell count of the permeabilized cell suspension which was determined in a Coulter Counter (Coulter Electronics, Inc.). One unit represents an increase in absorbance at 420 nm of 1.0 per min.

RESULTS

Assay for *ARS* function. We have investigated the sequences necessary for *ARS* function by in vitro mutagenesis of the *ARS*-containing plasmids (Fig. 1). These plasmids are derivatives of YIp5, a nonself-replicating vector that contains the entire sequence of the bacterial plasmid pBR322, for efficient replication and selection in *E. coli*, and the yeast *URA3* gene, for selection in yeasts (35, 44). YRp12 and YRp17 contain the 1.4-kilobase (kb) *Eco*RI *ARS1* yeast fragment, Sc4101, and are identical except that YRp17 has lost the *EcoRI* site indicated in Fig. 1. Yp412/*URA3* contains the 855-bp *Eco*RI-*Hin*dIII fragment of Sc4101 (41). These plasmids define the ARS^+ phenotype, as we shall use it in discussing our results. That is, they transform *S. cerevisiae* at high frequency (300 to 2,000 transformants per μg of DNA) and can replicate extrachromosomally. The ARS^+ plasmids are mitotically unstable, however, losing the plasmid at a rate of ca. 10^{-1} per cell per generation. Chromosomes are lost at a rate of 10^{-5} per cell per generation for comparison (9, 18). Under selective conditions, the generation time for strain SS111 carrying an ARS^+ plasmid is 2.5 h as compared with 1.5 h in complete medium. Based on these properties, our initial assay for *ARS* function after mutagenesis was for high-frequency transformation as defined above. Mutants that did not give more than one or two transformants per μg of DNA were called ARS^-. Mutants that did give transformants were further characterized by comparing parent and mutant as to the size of transformant colonies, the rate of plasmid loss in nonselective medium, and the generation time in selective medium. We have chosen in most cases to report only the generation time as a qualitative indication of *ARS* function, since no additional, reproducible quantitative information was obtained from plasmid loss studies. The weaker the *ARS*, the longer the generation time, with the most defective mutants unable to grow at all in liquid medium. Others have previously used the same criterion to characterize *ARS*s for similar reasons (23, 41, 48). Our only attempt at quantitation is the copy number analysis presented in the second section of the paper.

***Bal*31-generated deletions to determine the left boundary of *ARS1*.** (The left and right designations refer to the orientation of *ARS1* [Fig. 2].) To localize *ARS1* we have introduced deletions or insertions of sequences in or surrounding an 11-bp consensus sequence proposed to be necessary for autonomous replication in *S. cerevisiae* (2, 40). The consensus sequence as refined by Broach et al. (2) is $\overset{A}{T}TTTATPuTTT\overset{A}{T}$ and is found within the small *Bgl*II-*Eco*RI fragment of Sc4101 (Fig. 1), including 1 bp of the *Bgl*II recognition site. Another sequence designated *ARS1'* by Stinchcomb et al. (40) and differing from the consensus by only 2 bp, TCTTGTATTTA, is also found on this fragment (position 315 to 325 of Sc4101; see Fig. 2 for an explanation of the numbering system).

A series of deletions was constructed from the *Eco*RI site closest to the *Bgl*II site of Sc4101 (Fig. 1). Methods are described in detail above. In summary, DNAs were digested with *Eco*RI and treated with the exonuclease *Bal*31, and *Eco*RI linkers were ligated to the deleted fragments. The fragments were digested with *Eco*RI, and the DNA was ligated to form circular molecules and used to transform *E. coli*. Transformants were screened for deletion mutations by analysis of plasmid DNA by gel electrophoresis. Digestion with the exonuclease *Bal*31 results in the deletion of pBR322 sequences as well as yeast sequences.

A map depicting nine deletion mutants, designated ΔR1 to ΔR9, from the left side of *ARS1*, is shown (Fig. 2A). The

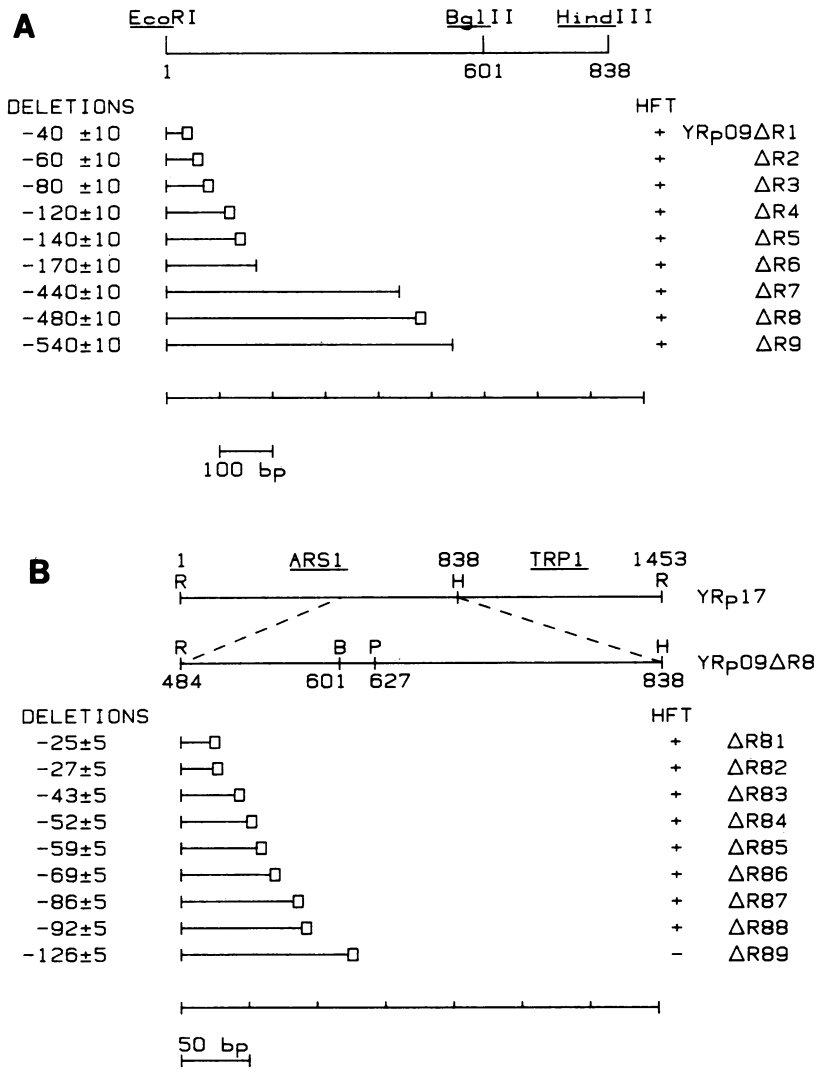


FIG. 2. (A) Map of the deletions from the *Eco*RI sites of Yp412/*URA3* and YRp17. Deletions were generated by *Bal*31 digestion of *Eco*RI-cleaved Yp412/*URA3* and YRp17. Shown are the deletions in relation to the *Eco*RI-*Hin*dIII fragment common to both YRp17 and Yp412/*URA3* (Fig. 1). YRp09ΔR1 to ΔR5 are derivatives of Yp412/*URA3*, and YRp09ΔR6 to ΔR9 are derivatives of YRp17. Open boxes indicate clones that have an *Eco*RI linker at the end of the deletion. The deletions were sized by agarose gel electrophoresis to the nearest 10 bp. HFT, High-frequency transformation. Restriction sites: R, *Eco*RI; B, *Bgl*II; H, *Hin*dIII; P, *Pst*I. Numbers above or below restriction sites are the distance in nucleotides from the *Eco*RI site, designated position 1. Position 1 is position 1453 in reference 48. (B) Map of the *Bal*31-generated deletions of YRp09ΔR8. Yeast plasmid YRp09ΔR8 (A) was linearized at the synthetic *Eco*RI site, and deletions were generated by using *Bal*31. Shown are the size of the deletions as determined by restriction enzyme digestion and electrophoresis on acrylamide gels. All the DNA clones contain synthetic *Eco*RI sites. The endpoint of the deletion in ΔR88 was determined by nucleotide sequencing (29). HFT, High-frequency transformation.

deletions range from 40 to 540 bp. The first five deletions, 40 to 140 bp, were constructed with plasmid Yp412/*URA3*. YRp17 was used to generate longer deletions, 440 to 540 bp, to maintain an intact β-lactamase gene and thus the ability to select for ampicillin resistance in *E. coli*. To obtain further deletions, a third series of mutants was constructed with plasmid YRp09ΔR8 (Fig. 2), a deletion mutant with 117 bp of yeast sequence between *Bgl*II and the endpoint of the deletion. (Sequences to the right of *Bgl*II are intact.) These deletions range in size from 25 to 126 bp and are designated ΔR81 to ΔR89 (Fig. 2B). We were somewhat surprised to find that deletions to within 18 bp of *Bgl*II and within 4 bp of the consensus sequence are still capable of high-frequency transformation. In contrast, the 126-bp deletion that removes the *Bgl*II site and an additional 11 nucleotides to the right completely abolishes *ARS* activity (i.e., no high-frequency transformation). Thus, deletion of the consensus makes the plasmids ARS$^-$, and at most, 18 bp to the left of the *Bgl*II site are necessary to give high-frequency transformation.

Transformation frequencies and stabilities of deletion mutants. To examine more carefully whether there is any effect at all on the *ARS* function of the *Eco*RI deletions up to but not including the consensus the relationship between the size of the deletions and the frequency of transformation was determined. DNA from clones YRp09ΔR1 to 8 and YRp09ΔR81 to 88 was used to transform strain SS111. The frequency of transformation ranged from 195 to 707 transfor-

mants per μg of DNA, similar to the frequencies determined by Tschumper and Carbon (49) for *ARS1*. The number of transformants, however, does not correlate under the conditions tested with the size of the deletions (data not shown), and we conclude that these results merely reflect inherent variability in the transformation system and that there is no difference in the ability of these deletion mutants to mediate high-frequency transformation.

The stability of the plasmids was next estimated by several means. First, the doubling time of cells containing the plasmids was measured. Plasmids YRp09ΔR1 to 8 and YRp09ΔR81 to 88 all give doubling times indistinguishable from that of YRp17, 2 to 2.5 h. This is similar to the generation times found by Stinchcomb et al. (40) for the entire 850-bp *Eco*RI-*Hin*dIII fragment of Sc4101. In contrast, the generation time for strain SS111 carrying the defective mutant *ARS* plasmids that will be described below is more than 12 h. Thus again, the *Eco*RI deletion plasmids appear fully ARS$^+$.

The stability of the plasmids was further measured by the rate of loss of plasmid from transformants. YRp17, YRp09ΔR8, and YRp09ΔR88 (see Fig. 2 for plasmid designations) were grown overnight in minimal medium without uracil. The cells were then transferred to complete minimal media (SD plus required amino acids or nucleotides), and the rate of plasmid loss was estimated by determining, after every generation, the number of cells that could grow with and without uracil. Determinations were made both by replica plating and by plating onto duplicate selective and nonselective plates with similar results. As reported by others, 10% of the cells in the culture grown under selective conditions contained *ARS1* plasmids, and after three generations in nonselective medium, this number had dropped to 3%, i.e., the same 30% loss per generation noted by others (43). No differences in the rates of loss of the three plasmids were detected. Thus, this criterion does not allow distinction among these mutants and wild type either. It is interesting to note that the sequence, *ARS1'* (40), that differs by only 2 bp from the consensus sequence, has been deleted in plasmids YRp09ΔR7 to 9 and YRpΔR81 to 88, suggesting that this sequence, unlike the consensus, is not essential for function. Furthermore, the nature of sequences flanking the *S. cerevisiae* insert, although different in each construction, shows no effect on *ARS* function.

Insertion, deletion, and point mutations in and around the consensus sequence. To further investigate the role of the consensus sequence in *ARS* function, small deletions and insertions were created at the *Bgl*II site. To generate the deletion mutants, YRp12 was digested with the restriction enzyme *Bgl*II. The DNA was treated with S1 nuclease, and the deleted fragments were circularized by blunt-end ligation. Transformants were obtained and analyzed as described for the *Bal*31 experiments. DNAs that had lost the *Bgl*II restriction site were subsequently characterized by DNA sequence analysis as described by Maxam and Gilbert (29) (data not shown). YRp12S9 has a 20-bp deletion, and YRp12S1 has a 11-bp deletion.

Insertion mutations were created by digesting YRp12 with *Bgl*II, the cohesive ends were filled in by using the Klenow fragment of DNA polymerase I, and the plasmid was circularized by blunt-end ligation. This procedure creates a 4-bp insertion (confirmed by DNA sequencing) at the *Bgl*II site.

A comparison of the deletion and insertion mutations is shown (Fig. 3). The sequence of YRp12 is shown in Fig. 3A; the box outlines the consensus sequence, and the arrows indicate the *Bgl*II site. The 11-bp deletion of YRp12S1 is shown in Fig. 3B. The deletion not only removed the 4-bp cohesive end but also an additional 3 to 4 bp on either side of the restriction site. Note that 4 bp of the consensus sequence have been deleted. An unexpected result of this construction is that the deletion followed by religation generates a sequence TTTTATGTTAT, which can be interpreted as having a single bp point mutation in the consensus sequence ($^{A}_{T}$TTTATPuTTT$^{A}_{T}$). This plasmid is incapable of high-frequency transformation; that is, it is completely ARS$^-$. The results of Kearsey (23) with the *ARS* near the *HO* gene suggest that the core of that *ARS* contains 3 bp to the right of the consensus essential for *ARS* function. We have no deletions removing any one of the last 4 bp of the *Bgl*II site and therefore do not know whether they are essential at *ARS1*. Therefore it is not clear whether the reconstituted site is inactive because of the deletion or the point mutation, although we favor the latter since the sequence found in the 4 bp of the *Bgl*II site (597 to 601) is not conserved at any other

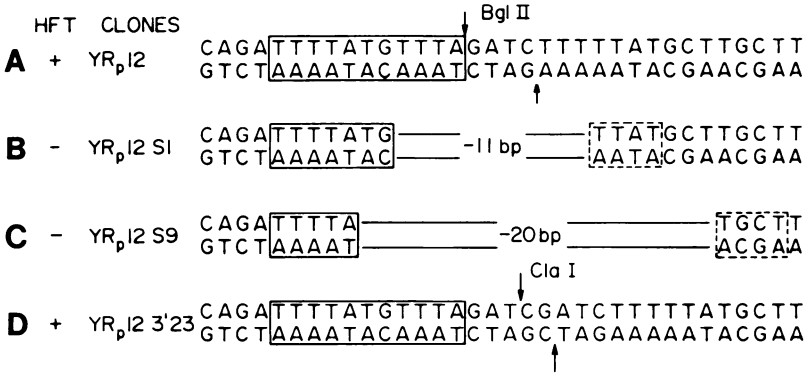
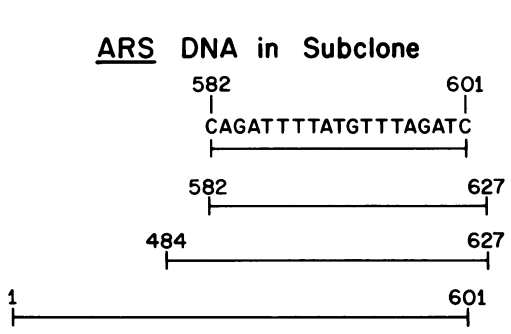


FIG. 3. Analysis of the insertion and deletion mutations generated at the *Bgl*II site of YRp12. HFT, High-frequency transformation. (A) Sequence surrounding the *Bgl*II site of YRp12. The arrows indicate the *Bgl*II restriction enzyme cleavage pattern. The box outlines the 11-bp *ARS* consensus sequence of YRp12 (40). (B) YRp12S1 has an 11-bp deletion created by cleaving YRp12 with *Bgl*II and treating with S1 nuclease, followed by blunt-end ligation. The deleted sequences are diagrammed by the double lines. The box shows what remains of the consensus sequence. (C) YRp12S9 has a 19-bp deletion, constructed as in (B). The sequences deleted are diagrammed by the double lines. The box shows the remaining consensus sequence. (D) YRp123'23 has a 4-bp insertion created by cleaving YRp12 with *Bgl*II and filling in the ends, followed by blunt-end ligation. This insertion creates a new restriction enzyme site *Cla*I; the cleavage pattern is indicated by the arrows. The consensus sequence is boxed as in (A).



Plasmid Designation	HFT	Generation Time (h)
YRp09sb25	−	—
YRp09sb54	+	—
YRp09sb117	+	>12
YRp522	+	7

FIG. 4. Subclones of the consensus sequence and deletion mutants. The fragments shown in YRp09sb25 and YRp522 were cloned by replacing the *Eco*RI-*Bam*HI fragment of YRp12 with the indicated fragment of YRp09ΔR88 or YRp17. The subclones sb54 and sb117 were cloned by replacing the 637-bp *Pst*I-*Eco*RI fragments of YRp12 with the indicated fragments from YRp09ΔR8 and YRp09ΔR88, respectively. The regions shown are the only yeast DNA on the plasmid (exclusively of the selectable marker, located more than 1 kb away).

ARS element and since Kearsey has shown that point mutations in the consensus sequence do give the ARS$^-$ phenotype (23). A third possible explanation of the ARS$^-$ behavior is that the spacing is changed between the consensus and the elements in domain B (see below) in YRp12S1 and that this inactivates the *ARS*. This is rendered less likely, however, by the fact that insertions which change spacing without altering the consensus do not inactivate the *ARS* (see below).

The 20-bp deletion of YRp12S9 is shown (Fig. 3C). The deletion is asymmetrical, removing 6 of the 11 bp of the consensus sequence. However, the fusion generated by the construction produces the sequence TTTTATGCTTTT, in which a different point mutation of the consensus arises. This plasmid is also incapable of high-frequency transformation, i.e., completely *ARS*$^-$. Shown in Fig. 3D is the 4-bp insertion mutant, YRp123'23. The insertion does not disrupt the consensus sequence but generates a *Cla*I site as indicated by the arrows. Unlike the deletion mutants YRp12S1 and YRp12S9, the insertion mutant YRp123'23 is ARS$^+$. Four additional insertion mutants (identified by the loss of the *Bgl*II site) were tested for *ARS* function and also found to be ARS$^+$.

The consensus sequence is necessary but not sufficient for ARS function. Although the above experiments strongly suggest that the consensus sequence is necessary for *ARS* function, they do not determine whether it is sufficient. To determine whether the *ARS* consensus sequence was sufficient, we subcloned the *Eco*RI-*Bgl*II fragment of YRp09ΔR88, containing 19 bp to the left of *Bgl*II (nucleotides 582 to 601, Fig. 4), into the *Eco*RI-*Bam*HI sites of the pBR322 sequence of YIp5 (44). The resultant plasmid, YRp09sb25, is incapable of high-frequency transformation, suggesting that the consensus is not sufficient to give high-frequency transformation. (Although abortive transformants are observed when large amounts of DNA are used in the transformation, such abortive transformants are also observed with YIp5.) We note that this subclone contains the entire 14-bp core defined at the *ARS* near *HO* by Kearsey (23). Thus this core alone is not sufficient for function at *ARS1*. A subclone containing only the *HO* core sequence, which is different at three nucleotide positions from the *ARS1* site, and no leftward flanking DNA has not been studied and therefore cannot be compared.

The stability of plasmids YRp09ΔR1 to 88, all of which contain deletions to the left of the consensus, suggested that the additional sequences required for high-frequency transformation fall to the right of the *Bgl*II site. In fact, as we will show below, additional sequences on both sides of the consensus are necessary for the full ARS$^+$ phenotype. We have called the consensus sequence domain A.

Analysis of the contribution of sequences to the right of the *Bgl*II site by subcloning deletion mutations. We have shown that the consensus sequence is necessary but not sufficient for *ARS* function. To investigate the additional sequence required, we constructed plasmid YRpsb54, by inserting the 51-bp *Pst*I-*Eco*RI fragment of YRp09ΔR88 (nucleotides 582 to 627 of Sc4101) into the 4,700-bp *Eco*RI-*Pst*I fragment of YIp5 (44). The 51-bp *Pst*I-*Eco*RI fragment spans the *Bgl*II site; 26 bp are to the right of the *Bgl*II site, and 25 bp are to the left of the *Bgl*II site (Fig. 4). This plasmid, YRp09sb54, has the same phenotype as a second plasmid designated YRp09sb117, which we constructed by subcloning the *Eco*RI-*Pst*I fragment from YRp09ΔR8 (nucleotides 484 to 627 of Sc4101), which contains 117 bp to the left of the YRp17 *Bgl*II site and the same 26 bp to the right. Both plasmids are capable of high-frequency transformation, but transformants carrying these plasmids have a reduced growth rate and a doubling time in excess of 12 h, suggesting that the plasmids are less effective replicators than the parental ΔR8 and ΔR88 that contain 238 bp to the right of *Bgl*II (Fig. 5). Thus the rightward boundary of *ARS1* must be to the right of the *Pst*I site, as predicted by Stinchcomb et al. (41).

To further define this boundary of *ARS1* a series of deletions with *Bal*31 were constructed from the *Hin*dIII site of Sc4101 (Fig. 1) in plasmid YRp09ΔR8 (Fig. 2). The *Hin*dIII site is 211 bp to the right of the *Pst*I site and 237 bp to the right of the *Bgl*II site. A map of four of these deletion mutants, ranging from 77 to 201 bp, is shown in Fig. 5. Deletions of up to 120 bp are ARS$^+$. Two deletion mutants, −191 and −201 bp, designated H200 and H201, respectively, have a phenotype similar to the subclones YRp09sb54 and YRp09sb117, a high-frequency transformation but reduced replicative ability leading to a 12-h doubling time for the transformants, and an increased rate of loss of plasmid (>>30% per generation). Thus, at least 20 bp to the right of the *Pst*I site, and at most an additional 70 bp, are necessary for *ARS*$^+$ function. We have called this domain B (Fig. 4 and 5, nucleotide positions 601 to 647 or 697). This is a much larger region than was found by Kearsey at the *HO ARS* (23), where only 27 bp, at most, flanking the core on the right are relevant to *ARS* function.

Reconsideration of the contribution of sequences to the left

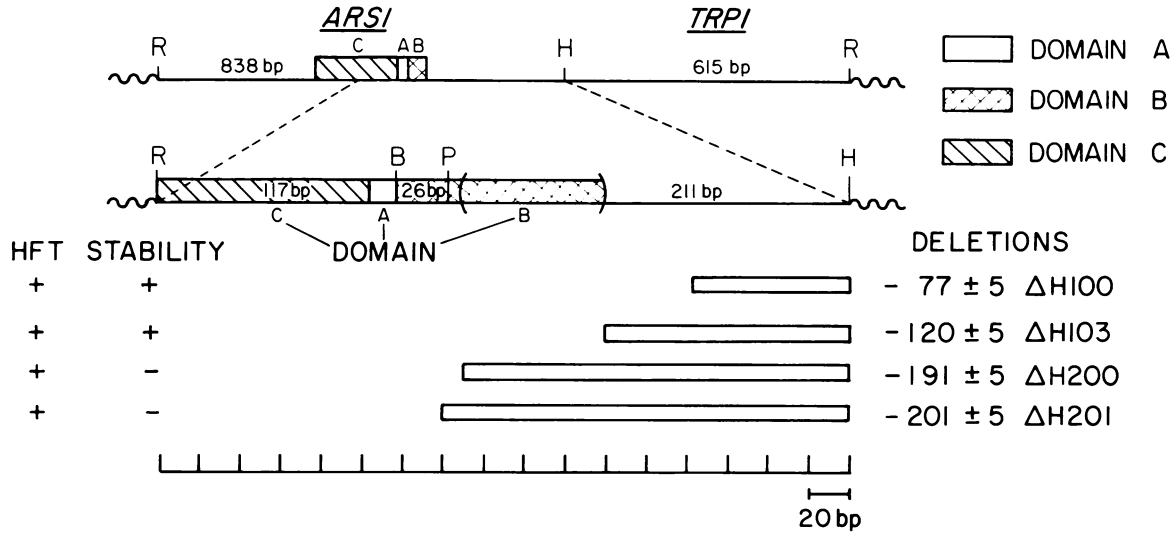


FIG. 5. Map of the *Bal*31 deletions at the *Hin*dIII site of YRp09ΔR8. Yeast plasmid YRp09ΔR8 (Fig. 2) was linearized at the *Hin*dIII site, and deletions were generated with *Bal*31. Shown are the size of the deletions as determined by restriction enzyme digestion and electrophoresis on polyacrylamide gels. All the DNA clones contain a synthetic *Hin*dIII site. HFT, High-frequency transformations; +, growth rate and rate of plasmid loss; −, slower growth rate and increased frequency of loss over YRp17. Domain A, open box; domain B, cross-hatched box; domain C, hatched box. Domain A is defined as the 11-bp consensus and includes position 597, the first nucleotide of the *Bgl*II restriction site. Domain B extends from position 598 to somewhere between 647 ± 5 and 707 ± 5. Domain C is defined as the region between positions 400 and 597.

of the consensus. Stinchcomb et al. (40) had previously shown that plasmid YRp522, which contains the complete 601-bp *Eco*RI-*Bgl*II fragment found in YRp17 (Fig. 1) but lacks all of domain B, gave high-frequency transformation of yeasts but was very unstable. We found that transformants carrying YRp522 had a doubling time of 7 h (compared with 2.5 h for YRp17) in the strains we used as hosts in these experiments. YRp522, however, is more stable than YRp09sb25, the subclone containing only the consensus sequence and almost no flanking yeast DNA, and is also more stable than YRp09sb117 and YRp09sb54, subclones we constructed and which contain only 117 and 19 bp to the left of *Bgl*II (Fig. 4) and, like YRp522, are missing domain B. These latter two plasmids give transformants with doubling times longer than 12 h. Thus, in the absence of domain B, sequences more than 117 bp to the left of the consensus sequence, which could be deleted with no adverse effect in the presence of domain B, apparently contribute to *ARS* function. These results define a third domain, the borders of which are somewhere between the consensus and the *Eco*RI site at position 1. We have designated this region domain C (Fig. 5). Interestingly, it is within this region, 200 bp to the left of the consensus sequence, that the center of the replication bubbles observed during in vitro replication are observed (5). Koshland and Hartwell (C. S. Newlon, *in* A. Rose and J. Harrison, ed., *The Yeasts*, vol. 3, in press) have also shown that deletions in domain C affect *ARS1* activity.

Functions of the various domains. Having gleaned an idea of the structural organization of *ARS1*, we would now like to understand the functions of each region. Although domain A is clearly required for high-frequency transformation, the contributions of domains B and C are not clear from the above analysis. In an attempt to obtain a better understanding of the function and interrelationship of the three domains, particularly domains B and C, we studied the copy numbers of the wild type and the deleted plasmids. Analysis

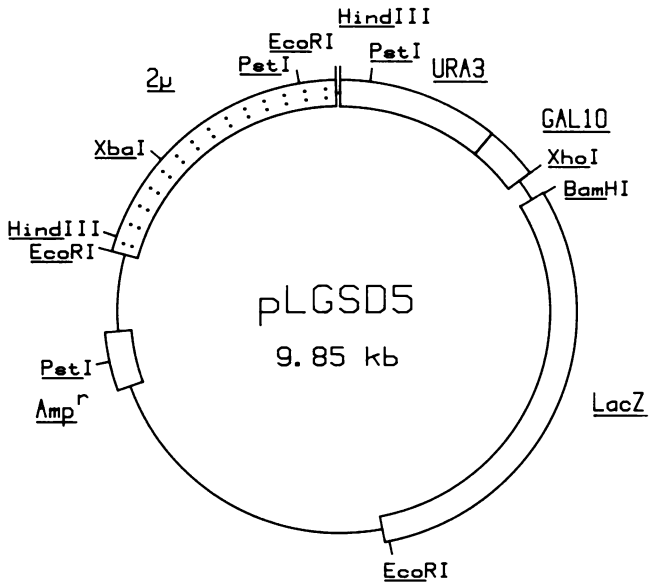


FIG. 6. Construction of β-galactosidase carrying plasmids for copy number determination. Plasmid pLGSD5 contains the 2.2-kb *Eco*RI fragment from the 2μm plasmid (B form), the 1.1-kb yeast *URA3* fragment, a 2.9-kb fragment containing the *E. coli lacZ* gene linked to the yeast *GAL10* promoter, the origin of replication, and the β-lactamase gene of pBR322 (15). The 2μm fragment of pLGSD5 were replaced, as described in the text, by the *Eco*RI-*Hin*dIII fragment either from YRp7 containing the complete *ARS1* region or from YRp09ΔR8, YRp09ΔR88, ΔH103, and ΔH200 containing the deletions around the *ARS1* region described in Fig. 2 and 5. All resulting plasmids, pLGARS1, pLGR8, pLGR88, pLGH103, and pLGH200, carry the *ARS1* fragment as the *Eco*RI-*Hin*dIII fragments of Fig. 2 and 5, and are in the same orientation. Finally a plasmid, pLG2ARS1, was constructed that carried two copies of *ARS1*.

of copy number mutants has been invaluable in analyzing *E. coli* plasmid replication mutants.

We carried out blot-hybridization analysis to compare amounts of plasmid DNA in strains carrying plasmids having deletions in either domains C or B as described above. Surprisingly, copy number was the same for all plasmids, whether or not they were deleted (data not shown because Fig. 8 below demonstrates the same point). This is different from the findings of Stinchcomb et al. (40), who reported a fourfold lower copy number in a lambda plasmid containing the 638-bp *Eco*RI-*Pst*I fragment of YRp17 (i.e., domains C, A, and 26 bp of B) relative to lambda carrying *ARS1*. The discrepancy could be due to the difference in the constructions or in the method used in the two studies to determine copy number. Both methods depend on there being no differential recovery of linear, chromosomal DNA and plasmid DNA in the analysis.

To corroborate general trends in copy number analysis, a different type of copy number determination was carried out. Just as β-galactosidase fusions have been used to study the strengths of promoters, we used β-galactosidase as a marker enzyme for determining plasmid copy number. *ARS1* and the deletions R8, R88, H103, and H200 (Fig. 2 and 5) were subcloned into the 2μm vector pLGSD5 (15; Fig. 6). This latter plasmid contains the *E. coli lacZ* gene fused to the yeast *GAL10* promoter such that β-galactosidase can be induced by galactose in the medium. For our studies, the 2μm sequences were completely replaced by the *ARS1* DNAs, as described in the legend to Fig. 6 and above. All resulting plasmids show stabilities similar to the parental YRp derivative, as estimated by the doubling time of transformants (Fig. 7A); that is, all plasmids show growth rates similar to the parental *ARS1*, except the domain B mutant H200, which has increased generation times. β-Galactosidase levels were determined in strains transformed with each plasmid and grown in medium containing galactose in addition to glucose (Fig. 8A). The average copy number was calculated by dividing the activity found in cultures of the plasmid-containing cells by that obtained in the culture of D603i. Specific activities (units per cell [$\times 10^{-9}$]) were 45, 89, 309, 295, 34, 31, 24, 30, and 24 for i, 2i, pLGSD5, SD5/2, ARS1, R8, R88, H103, and H200, respectively. The number obtained by comparing D603i (i) and D6032i (2i) agrees well with the expected copy number of 2 for the latter strain. The copy number per plasmid-containing cell (Fig. 8A) was determined by dividing the average copy number by the percent cells containing plasmid. This was 86, 56, 3.8, 3.5, 2.0, 3.3, and 2.9% for pLGSD5, pLGSD5/2, pLGARS1, pLGR8, pLGR88, pLGH103, and pLGH200. Note that plasmid 2ARS1 fortuitously integrated into the chromosome in this experiment. When not integrated, the copy number is equal to that of *ARS1*. In summary, we find that the copy numbers for all *ARS* plasmids, deleted or not, are similar, 20 to 30, and deleted plasmids do not have low copy numbers. No correction has been made for the fact that cells that have lost the plasmid may contain β-galactosidase and *URA3* gene product activity for several generations after plasmid loss, which introduces some error into the calculation. *GAL4* levels do not appear to be limiting, at least up to a copy number of 30 (also see reference 15). These assumptions do

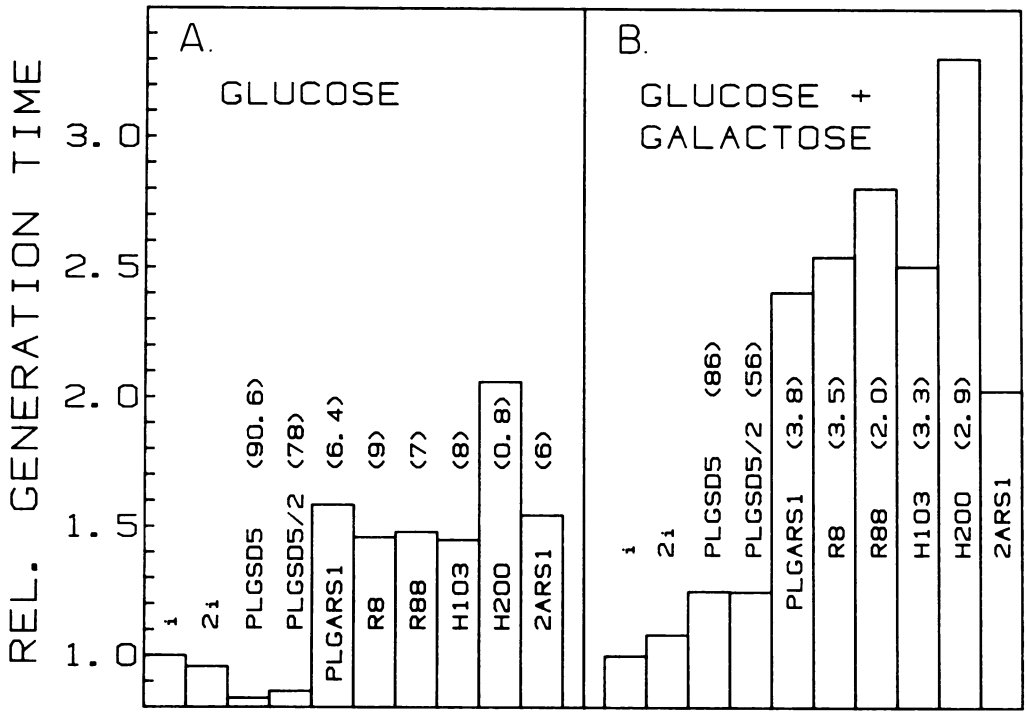


FIG. 7. Growth rate of strains carrying β-galactosidase plasmids. Derivatives of plasmid pLGSD5 containing the entire *ARS1*, designated *ARS1*, and deletions R8, R88, H103, and H200 (see Fig. 2, 5, and 6) were introduced into strain D603, a *ura3-52* strain that does not carry out glucose repression (*reg1*). pLGSD5 and pLGSD5/2 are the 2μm-containing parental plasmids, the latter plasmid being a dimer. Growth rates of various plasmid-containing strains are expressed relative to the growth rate of strain D603i that was isolated after spontaneous integration of plasmid pLG2ARS1. D603i and D6032i, designated i and 2i in the figure, contain one and two copies of integrated plasmid DNA, respectively. Numbers within parentheses refer to the percent plasmid-containing cells in the culture. Strain D603 is [cir$^+$], accounting for the greater stabilities of pLGSD5 and pLGSD5/2 relative to the *ARS1* plasmids.

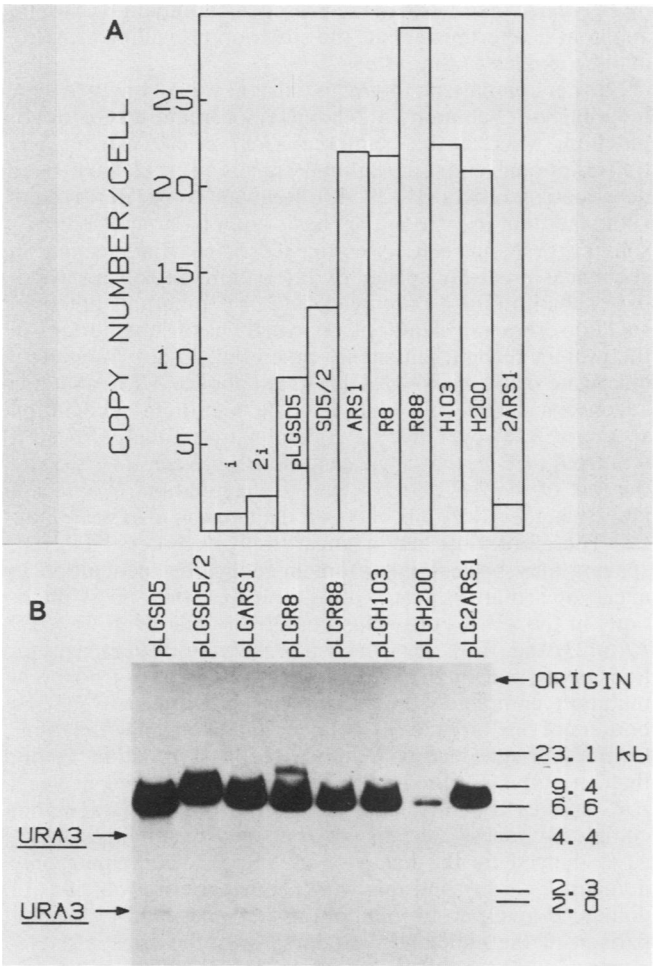


FIG. 8. (A) Copy number of deletion mutants. Cultures of strain D603 transformed with designated plasmids (50 ml) were grown in SD minimal medium lacking uracil and containing glucose and galactose (2% each) at 30°C. Doubling times were determined, and when cells reached logarithmic growth, they were plated on medium containing or lacking uracil to determine the fraction of plasmid-containing cells. A sample was taken for the determination of β-galactosidase activity, and copy number was determined as described in the text. (B) Copy number as determined by Southern blotting. Cells were grown to the logarithmic phase of growth in medium containing only glucose and harvested, and DNA was prepared as described in the text. DNA was digested with *Hin*dIII. Whereas *Hin*dIII cleaves outside of the *URA3* gene, the *ura3-52* allele in D603 contains a TY1 insertion, and digestion with *Hin*dIII gives rise to two fragments hybridizing to a *URA3* probe. *Hin*dIII cleaves most of the plasmids used in this study once. pLGSD5 and pLG2ARS1 give two fragments with *Hin*dIII, but only one is homologous to the probe. The DNA was run on an agarose gel and blotted to nitrocellulose. A nick-translated, ^{32}P-labeled DNA fragment (10^6 cpm; 1.5 kb) containing only *URA3* and *GAL10* sequences, the *Hin*dIII-*Xho*I fragment shown in Fig. 6, was added. After hybridization and autoradiography, radioactivity in individual bands was determined by densitometer analysis. (Longer exposures were used to estimate the amount of radioactivity in the *URA3* bands.) Calculation of copy number is presented in the text.

not, in any case, affect our conclusions about relative copy numbers.

Copy number was also determined in cultures grown on glucose such that β-galactosidase is not expressed, since from Fig. 7 it is clear that expression of β-galactosidase alters the growth rate of plasmid-containing cells and might have an effect on the copy number. We used the Southern blotting technique as described above to quantitate plasmid levels. The average copy number was determined as the amount of plasmid DNA divided by the sum of the amount of the two *URA3* bands and the *GAL10* band. Copy number per cell was calculated by dividing the average copy number by percent plasmid-containing cells. The percent plasmid-containing cells was 97% for pLGSD5, 80% for pLGSD/2, 11% for pLGARS1, 9% for pLGR8, 5.5% for pLGR88, 12% for pLGH103, notably only 0.8% for pLGH200 the unstable mutant, and 11% for pLG2ARS1. Calculated copy numbers for these plasmids are 11, 21, 100, 100, 200, 200, 100, 600, and 100. Each plasmid has a higher absolute copy number when cells are grown under these conditions than when cells are grown in the presence of galactose (Fig. 7A), but relative numbers are consistent with the enzymatic method of copy number determination. This is consistent with the higher growth rate of the cells grown on glucose alone, seen by comparing Fig. 7A and B. We again conclude that the deletion mutants, whether they have a doubling time of 2.5 or 12 h, all have high copy numbers similar to that of the wild type. In fact, the least stable plasmid, H200, has an even higher copy number per cell than does the wild type.

To explain these findings we propose that the instabilities of the mutant plasmids are due to segregation defects in addition to (or rather than) replication defects. Although plasmids like 2μm segregate equal amounts of DNA into the mother and the bud (symmetric segregation), Murray and Szostak (33) recently showed that 50% of cells containing *ARS* plasmids segregate asymmetrically, with the entire plasmid content segregating 19:1 into the mother instead of the bud. The deletion mutations may increase the asymmetry. Clearly, a more unambiguous assay will be required to measure replication alone. One can envision such assays using centromeres to stabilize plasmids in combination with our β-galactosidase assays or using in vitro assays based on soluble replication systems as have been used in bacterial studies (see below).

DISCUSSION

Stinchcomb et al. (40) distinguished two functional domains within *ARS1* and first proposed the importance of the consensus sequence that was later more completely defined by Broach et al. (2). We have further characterized the sequences necessary for *ARS1* function in *S. cerevisiae* in vivo and have revised the description of the sequence to include three domains. The information necessary for stable autonomous replication resides in a 124-bp region. The boundaries of this region are 15 bp to the left of the *Bgl*II site, domain A, and 109 bp to the right of the *Bgl*II site, domain B. Additional sequences are necessary in the absence of domain B. These fall within domain C, which extends at least 200 bp to the left of domain A. Domain A appears to be the only absolutely essential region, since deletions of domains B or C destabilize the plasmids but do not abolish replication, as do deletions or point mutations in domain A.

A potentially useful outcome of our mutagenesis is that a plasmid was generated that contains the consensus sequence bearing a single point mutation. First, because this abolishes *ARS* function, it suggests that the consensus sequence is essential for high-frequency transformation of yeasts (see below for alternative interpretations of these mutations).

Furthermore, it offers the possibility of a genetic approach to isolating mutants in putative proteins that might recognize or bind to or mediate their action through the consensus sequence. One could simply select for suppressors of the point mutation that allow the plasmids to multiply again. This genetic approach would form a perfect complement to a biochemical approach to isolating these protein components of the replication apparatus.

From results obtained in this and other systems, a working model for the functional organization of *ARS1* can be proposed. In this scheme, domain A, the consensus sequence, would be the recognition site for a DNA binding protein with a key role in replication, analogous to the dnaA protein of *E. coli*. The dnaA protein binds to a 9-bp recognition sequence within *oriC* and participates directly in the initiation of DNA synthesis (13). In support of our proposal, a high-molecular-weight replication complex has been shown to bind in the region of the consensus sequence by electron microscopy (22). In addition to its role in replication, the yeast consensus sequence seems to have a role in regulating histone biosynthesis and the transcriptional state of *HML* and *HMR* loci (43). Similarly, the recognition site for dnaA protein is found in the promoter region of the dnaA gene, perhaps accounting for the observed autoregulation of dnaA protein synthesis (see reference 13). Thus the dnaA protein may be bifunctional, having a role in both transcription and replication. Although the consensus sequences found near *HML*, *HMR*, and the H2b gene could mediate their effect through replication and alteration of the state of the chromosome, they could also be directly important as recognition signals in the transcriptional processes they affect.

Domain B, which shows little if any homology between different *ARS*s, could contain sequences for specific regulation, such as activation of different replication origins at different times during each S phase. Since domain B apparently stabilizes the plasmids but does not affect their copy number, an additional function might be to provide a site for nuclear attachment, either to the chromosome or to the nuclear matrix or membrane. Both physical and genetic evidence suggest that the endogenous plasmid of yeast, the 2μm circle, is associated with chromosomes during portions of the cell cycle (36, 47). Murray and Szostak (33) have demonstrated that circular *ARS* plasmids segregate asymmetrically, suggesting that they do not diffuse freely in the nucleus. It has been proposed that the 2μm *REP* loci form a nuclear attachment system and that the 2μm plasmid is stable because this system somehow ensures detachment for proper segregation as necessary (24). If domain B has some attachment function, domain B deletions might be expected to function normally in the presence of a centromere on the plasmid or with the 2μm *REP* loci present in *cis*. Preliminary evidence from L. Hartwell suggests that this is in fact the case (personal communication).

A role for domain C is indicated by in vitro replication studies that show that the replication bubbles initiate in this region (5), suggesting that, although domains A and B may be necessary for replisome assembly and for regulation, DNA synthesis may actually initiate in domain C. Since domain C has a less significant role in stability, this might seem at first unreasonable. However, there is precedent for this type of organization in that replication initiates in vitro outside the minimal sequence required for *oriC* function in *E. coli* (46) and any adjacent sequence will serve as an initiation site of bidirectional replication, as long as the minimal sequence is intact. Since domain C can be replaced by *E. coli* plasmid DNA in the presence of domain B, with no apparent deleterious effect, the situation prevailing at *ARS1* may be similar to that at *oriC*.

Further comparison to *oriC* is a useful way to analyze *ARS* function. *oriC* contains a 245-bp region required for origin function, whereas the complete *ARS1* is ca. 300 bp. The transcriptional and translational signals present have been described by others (19, 39, 48). Relevant to our new results is the fact that in vitro mutagenesis and taxonomic comparisons suggest that *oriC* is composed of protein recognition sequences precisely spaced by intervening regions in which base substitutions do not inactivate *oriC* functions but even small insertions or deletions do. Single base substitutions in the protein recognition sites weaken but do not completely inactivate *oriC* (13, 46). As discussed above, *ARS1* contains a consensus sequence that may be a protein recognition sequence. However, we have demonstrated that *ARS1* differs from *oriC* in that small insertions between this site and the rest of the *ARS* do not lead to inactivation. We do not know whether deletions between the two can also be tolerated. Therefore, just as in eucaryotic promoters (14, 30), spacing may be less important in replicator recognition in eucaryotes than in procaryotes. Furthermore, point mutations in the yeast consensus completely inactivate the *HO ARS* (23) and, from our work with reservations in interpretation mentioned in the text, also *ARS1*, whereas no point mutation completely inactivates *oriC*. Unlike *ARS1*, the borders of *oriC* are clearly defined, and even small deletions from either side lead to inactivation of *ori* function, rather than the type of modulation of function observed in yeast *ARS* studies. For instance, deletion of domain B does not completely inactivate plasmids but only destabilizes them, as evidenced by the behavior of YRp522, containing only domains C and A (40; this work). Furthermore, portions of domain B give partial function, as demonstrated by a comparison of the subclone containing only the consensus sequence (sb25) with the subclone containing 26 bp of domain B in addition (sb54). YRpsb25 does not give high-frequency transformation at all, whereas sb54 does, although the sb54 plasmids are very unstable. Finally, deletion of domain C shows no effect at all under certain conditions. However, we have added centromere sequences to plasmids containing the domain C deletion, and these are far less efficient replicators than the corresponding *ARS1-CENIV* control plasmids (F. Srienc and J. L. Campbell, manuscript in preparation). Furthermore, cotransformation with YRp17 and YRp09ΔR88 does give rise to only YRp17 clones, suggesting, but not proving, that YRp17 does replicate or is transmitted more efficiently than the deletions (data not shown).

Our most surprising finding was that stable and unstable plasmids have similar copy numbers. Changes in stability without changes in copy number have been observed in other yeast plasmids. Kikuchi (24) has shown that mutations in the *REP* loci of 2μm plasmids can cause reductions in stability but not in copy number.

In vivo analyses of *ARS* function are difficult since chromosome transmission is measured—not just replication but also segregation—is measured. Various DNA sequences have been shown to increase the fidelity of transmission of chromosomes in yeasts: centromeres, telomeres, and the 2μm *REP* or *FLP* loci or both (43). The studies presented in this paper are an attempt to study *ARS* function in the absence of these, to establish a background for in vitro replication studies on the plasmids. Insofar as our studies have defined domains A, B, and C they have been useful.

However, they do not allow us to decide whether the instability we observe in our mutants is due to plasmid loss, rather than to a replication defect. In addition to their usefulness in in vitro studies, these deletion mutants will be useful in in vivo systems that distinguish segregation from replication. Such systems have been developed in the laboratories of R. Davis and of L. Hartwell (C. S. Newlon, in press) with plasmids that contain centromeres in addition to *ARS*s.

Comparison of *ARS1* and the *ARS* at the *HO* gene. Recently, another *ARS*, that near the yeast gene *HO*, has been studied by methods similar to those used here (23). Similarities and differences, some a matter of definition and some of fact, are apparent. First, the consensus sequence is essential at both sites, although at *HO* it is TTTTAATATTTT, differing at the fifth nucleotide from the consensus at 12 other *ARS*s. However, just as at *ARS1* the consensus is not sufficient in the *HO ARS*. This was demonstrated by the fact that deletions of bases next to the consensus at *HO* lead to a completely ARS$^-$ phenotype. Second, as at *ARS1*, deletions to the left of the *ARS* (domain C) do not affect *ARS* function as long as sequences normally present at the right are intact. Perhaps effects of domain C deletions at *HO* would show up in the absence of domain B, or in the presence of a centromere as we have found at *ARS1*, but such studies have not yet been reported. Third, a much smaller region to the right of the consensus than that at *ARS1* is essential at *HO*. Only 27 bp to the right of the consensus are necessary for the full ARS$^+$ phenotype at *HO*, whereas 47 to 109 bp (domain B) are necessary at *ARS1*. Fourth, we originally proposed the extent of domain A as only the 11 bp of the consensus, even though we had only deleted up to within four bases to its right since these bases are not conserved at other *ARS*s and we did not think it likely they were essential. However, the mutations of Kearsey actually show that deletion of one of these four nucleotides, at least at *HO*, gives an ARS$^-$ phenotype. This leaves open the possibility that the core *ARS* may actually be larger than the consensus sequence alone at *ARS1*, as well. Since these 4 bp have not been deleted at *ARS1*, an uncertainty remains as to their essentiality at *ARS1*. It is therefore not possible to conclude whether the point mutants in the consensus that arise as a result of our S1 deletions inactivate *ARS1* because of the loss of essential nucleotides, because of a change in spacing between domains A and B, or because of the point mutation in the consensus. Kearsey elegantly shows that two point mutations in the consensus sequence do inactivate the *HO ARS*, so it is possible that it is the point mutations that are effective at *ARS1*. Even if these additional nucleotides do turn out to be essential at *ARS1*, they are not sufficient at *ARS1*, since in the subclone sb25, we have cloned a fragment that contains the sequence in question in addition to the consensus and yet the plasmid is ARS$^-$, giving only abortive transformants. Therefore, on their own, these additional nucleotides do not make the core functional at *ARS1*. In interpreting the results of deletions that come to within a few nucleotides of the consensus sequence, it should be kept in mind that others have noted an ARS$^-$ phenotype for the 601-bp *Eco*RI-*Bgl*II fragment (domains C + A), which we and Stinchcomb et al. (40) find is ARS$^+$. The point is that the context of the *ARS* fragment in the clones is different between the ARS$^-$ and the ARS$^+$ cases and, as pointed out by Kearsey in his studies, sometimes deletions appear ARS$^-$ that clearly are ARS$^+$ in another context (23). Thus, the effect of DNA, to which deletion mutations are fused, clouds precise definition of the *ARS* boundaries. Unfortunately, none of the point mutations at *HO* fall in the 3 bp outside the consensus sequence. Thus, precise delineation of the structural features of the ARS awaits purification of the proteins that recognize and interact with these elements or more sensitive assays, for instance, in the presence of a centromere.

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