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Effect of *ARS1* Mutations on Chromosome Stability in *Saccharomyces cerevisiae*

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We have used a set of deletion mutations in the *ARS1* element of *Saccharomyces cerevisiae* to measure their effect on chromosome stability. This work establishes the previously proposed existence of three domains in *ARS1*. Domain C, which we had previously inferred, but not proved, to be a part of *ARS1*, is now established. In addition, we show that increasingly large deletions of the domain have increasingly large effects, which was not realized before. Furthermore, we have provided the first positive evidence for the central importance of a 14-base-pair core sequence containing the *ARS* consensus element by showing that it has the ability to act as a replicator on a plasmid containing no other *ARS1* flanking sequence. The method of analyzing plasmid stability used in our study employs a novel and sensitive flow cytometry assay for β-galactosidase. We discuss ways in which flow cytometry, based on this assay, could be generalized beyond its particular application in this work to studying other aspects of the cell biology of yeast and higher cells. The actual flow cytometry method will be described in detail elsewhere.

DNAs that contain the *Saccharomyces cerevisiae* chromosomal segments designated *ARS*, an acronym for autonomously replicating sequence, transform *S. cerevisiae* at high frequency and can replicate extrachromosomally as plasmids. Because of this property, *ARS*s are thought to be chromosomal replicators (15), and a number of in vivo and in vitro studies have been carried out to characterize the elements within these segments of DNA that are relevant to their replication function. Evidence from several laboratories suggests that *ARS1* (27) consists of three domains (Fig. 1). There is good evidence that a core, domain A, consisting of a consensus sequence found at many other *ARS*s, is essential, since point mutations or deletions in this core eliminate *ARS* function (2, 4, 26). In addition, a region designated domain B that flanks the core on one side contributes to full *ARS* function, since deletions in this region decrease the stability of *ARS1* plasmids (4, 26, 31). There is indirect evidence that a third region, domain C, that flanks domain A on the side opposite B also contributes to the stability of *ARS1* plasmids (4), which is interesting since this is where replication bubbles are observed in vitro (3). Viewed thus, the minimum size of *ARS1* is about 300 base pairs (bp). *ARS2* has a minimum size of about 100 bp (30). Finally, the *ARS* at the *HO* gene has been thoroughly characterized by Kearsey (17); it consists of an essential 14-bp core and an additional 27 bp corresponding to domain B of *ARS1*. Point mutations in the core which also fall within the concensus sequence eliminate *HO ARS* function. It is not known whether the core is sufficient for replication at *HO*, but at *ARS1* it does not appear to be sufficient (4).

Further in vivo studies to define the contribution of the core and of each of the flanking domains, either by copy number analysis or by frequency of plasmid loss, yielded no information in addition to that summarized above because of the insensitivity of the assays employed to replication. The problem with the assays stems from the mitotic instability of the synthetic plasmids, which exhibit 30% loss of plasmid per cell per generation. In addition, there are a number of related properties, such as the tendency of the plasmids to integrate into the chromosome during growth, that complicate analyses. The instability is due, at least in part, to an abnormal, asymmetric segregation process demonstrated by the pedigree analysis of Murray and Szostak (21). One way to remedy the segregation problem is to add a centromere to the plasmid. Addition of a centromere to an *ARS*-containing plasmid increases its stability 30-fold, prevents integration, and reduces the copy number to one. The low copy number offers the additional advantage that a single failure to replicate results in complete plasmid loss from the cell after division. Thus, a single failure to replicate gives rise to a much greater, and therefore much more easily detectable, change in cellular behavior than the loss of a single plasmid from a cell containing the high-copy-number *ARS* plasmids.

Most important, the addition of a centromere to the plasmids being evaluated changes the nature of the questions being addressed in our studies. Previously, *ARS*s have been analyzed only from the point of view of replication function. The studies did not address the larger role of the sequences in chromosome stability. Addition of a centromere to the test plasmids thus makes them much more realistic models for chromosome function. (Recently, however, two reports have appeared that describe even more elaborate systems for such analysis [11, 18].)

The plasmid used in our studies contained *CEN4*. In addition, it contained the gene for *Escherichia coli* β-galactosidase for detecting plasmid-containing cells. A sensitive flow cytometry assay for the β-galactosidase encoded by the plasmid permits detection of the enzyme at the single-cell level. Using this vector and a set of *ARS1* deletion mutants described previously, we show the existence of domain C directly. In addition, we show that a 19-bp core is sufficient for replication of a segment of DNA if a centromere is present, although the replication is very inefficient in the absence of domains B and C.

Besides the results pertaining to *ARS* function in *S.*

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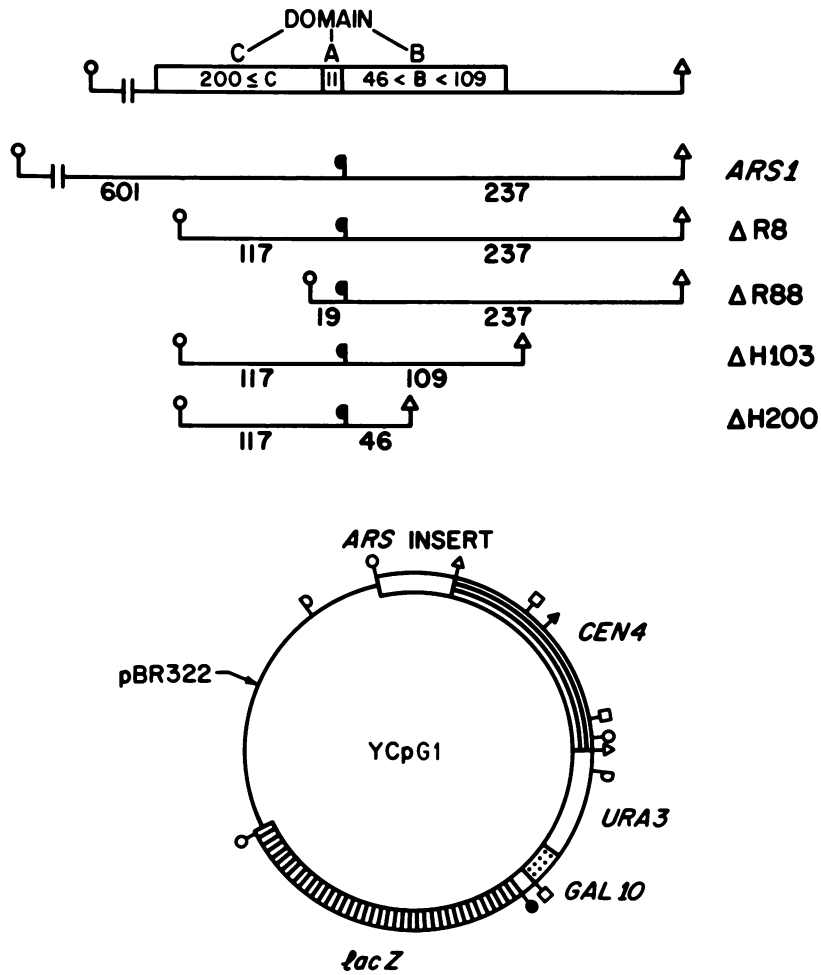

FIG. 1. Configuration of the plasmid vector used to measure *ARS* function. The configuration of *ARS1* as determined by previous analyses is shown on the top line (4). Domain A is the 11-bp consensus sequence. Domain B has a lower limit of 46 bp and an upper limit of 109 bp, as indicated by the numbers in the boxed area. Domain C has not been precisely mapped, but appears to be at least 200 bp long, its leftward boundary remaining to be determined (4). The linear restriction maps represent the *ARS* inserts used in this study. *ARS1* is fragment SC4101 of Stinchcomb et al. (27). The deletion mutants were constructed by deletion with Bal 31, as described previously (4). The segments shown at the top were combined with pLGSD5 and the *CEN4* fragment inserted to give YCpG1 plasmids containing the *ARS* deletions *ARS1*, R8, R88, H103, and H200, with the general structure shown at the bottom. The details of the constructions are given in the text. The *CEN4* fragment is the 2.5-kb *Hin*dIII-*Eco*RI segment described by Stinchcomb et al. (25) and does not contain *ARS4*. The 1.1-kb *URA3*, 0.365-kb *GAL10*, 3.5-kb *lacZ*, and 2-kb pBR322 sequences are from pLGSD5 (7). Restriction sites: ○, *Eco*RI; □, *Xho*I; ◐, *Bgl*II; ⓘ, *Pst*I; △, *Hin*dIII.

cerevisiae, one other aspect of the work needs introduction here because it is somewhat unfamiliar. In our laboratory, we have developed a flow cytometry method of analyzing yeast cells based on the detection of the bacterial enzyme β-galactosidase (24; unpublished data). Flow cytometry has been very useful for both analyzing and, in combination with a cell sorter, preparing higher cell types. Its use for microorganisms has, however, been limited by the lack of assays sensitive enough to detect smaller cells. Recently, modern microprocessors make the flow cytometry instrumentation far less costly and less demanding of special technical knowledge than was previously true, and this is what encouraged us to develop a method sensitive enough to detect yeast cells. In this current study of plasmid stability, we use the method to detect plasmid-containing cells and thus to measure plasmid stability. Although simpler methods of measuring plasmid stability have recently been published (11, 18), we present this application, namely, flow cytometry detection of plasmid-containing cells, as an illustration of a general method sensitive enough for analysis of other parameters of single yeast cells, and we suggest other applications of this assay.

MATERIALS AND METHODS

Strains and plasmids. *S. cerevisiae* D603, an a/α homozygous diploid, was constructed from strain YM603 (a *ura3-52 lys2-801 met his3 ade2-101 reg1-501*), obtained from M. Johnston, Washington University, St. Louis, Mo. *E. coli* MC1061 was used for DNA amplification and preparation. Plasmid pLGSD5 was a gift from L. Guarente, Massachusetts Institute of Technology, Cambridge (7). Plasmids YRp7 and YRp17 and their derivatives YRp09R8, YRp09R88, YRpH103, YRpH200, and YRpsb25 have been described previously (4, 27).

Materials. α-Naphthyl-β-D-galactopyranoside, pararosaniline hydrochloride, and amino acids were obtained from Sigma Chemical Co., St. Louis, Mo. Yeast nitrogen base without amino acids was from Difco Laboratories, Detroit, Mich. Restriction enzymes were purchased from

New England Biolabs, Beverly, Mass. Bacteriophage T4 DNA ligase was a generous gift from C. C. Richardson, Harvard University, Cambridge, Mass.

Media and growth of cells. *E. coli* was grown at 37°C in Vogel Bonner medium for DNA preparations or in L broth with added ampicillin (35 μg/ml) for plasmid selection. *S. cerevisiae* was grown at 30°C on SD minimal medium (22) supplemented as necessary and buffered with sodium citrate buffer (0.02 M, pH 4.5).

Growth rates. Cell growth was monitored by reading the optical density of the logarithmically growing cultures at 590 nm in 1-h intervals over a period of 8 h. Growth rates were determined by regression analysis. In all cases the regression coefficient was better then 0.995.

Transformation. *E. coli* cells were transformed as previously described (4). For *S. cerevisiae* transformations, the lithium acetate procedure of Ito et al. (14) was modified as described by Kuo and Campbell (19).

DNA preparation, modification, and analysis. Large-scale DNA preparations from *E. coli* were made as described by Celniker et al. (4). Small-scale DNA preparations from *E. coli* were obtained from cultures grown in 5 ml of selective L broth. The culture was sedimented by centrifugation (10 min, 5,000 rpm) and suspended in 0.4 ml of water. After the addition of 0.04 ml of lysozyme solution (10 mg/ml in 0.25 M EDTA, pH 7.5), the suspension was incubated at 0°C for 15 min. DNA was purified by extraction twice with equal volumes of phenol and once with chloroform-isoamyl alcohol (24:1) and by precipitation with ethanol. The dried preparation was dissolved in 50 μl of 0.01 M Tris-hydrochloride (pH 7.6)–1 mM EDTA buffer.

DNA restriction, dephosphorylation, and ligation procedures were standard and are described by Maniatis et al. (20). Gel electrophoresis was carried out in 0.8% agarose gels containing TAE buffer (40 mM Tris-hydrochloride pH 7.5, 10 mM sodium acetate, 2 mM EDTA). DNA was visualized by staining with ethidium bromide.

Flow cytometry assay of β-galactosidase. For analysis in the flow cytometer, cells were grown first in 5 ml of SD medium. An inoculum of 0.5 ml of this culture was used to start a 50-ml culture in a 250-ml Erlenmeyer flask. At an A_{590} of 0.2, 5 ml of galactose (20%, wt/vol) was added to induce β-galactosidase synthesis. In standard assays the culture was harvested 300 min after the addition of inducer. We then used a modification of the staining procedure described previously (24). *S. cerevisiae* cells were harvested and permeabilized with isopropanol as described previously (24). The cells were stained at 37°C in a 3-ml final volume containing 1 ml of buffer (0.2 M sodium phosphate, 10 mM $MgCl_2$, pH 7.0), 0.1 ml of hexazonium pararosaniline (solution A), and 0.1 ml of α-naphthyl-β-D-galactopyranoside (5 mg/ml in water). Solution A was prepared immediately before the assay by mixing 0.4 ml of pararosaniline hydrochloride (20 mg/ml in 2 N HCl) with 0.3 ml of sodium nitrite (5%, wt/vol), neutralizing with 0.3 ml of 1 N NaOH, and diluting with water (1:4). The staining reaction (37°C, 2 min) was started by adding 0.1 ml of the permeabilized cell suspension and stopped by adding 10 ml of water (0°C). After centrifugation, the cells were suspended in 1 ml of water and analyzed in the flow cytometer (Cytofluorograf 50 H; Ortho Instruments) at a flow rate of approximately 1,000 cells per s. The light source was the 514.5-nm wavelength of an argon-ion laser (model 95; Laxel Corp., Palo Alto, Calif.) with a light-stabilized beam power of 1 W. Fluorescence signals, collected by a Zeiss lens (Epiplan LD 40/0.60, D = 1.5), passing a 690-nm cutoff filter (no. 65.1385; Rolyn Optics, Pasadena, Calif.) and detected by a high-sensitivity photomultiplier tube, and small-angle light scatter signals were processed with the Cytomic 12 data acquisition unit (Kratel GmbH, Stuttgart, Federal Republic of Germany).

Description of plasmids. The goal of the construction was to add a *CEN* element to the plasmids pLGARS1, pLGR8, pLGR88, pLGH103, and pLGH200, the construction of which has been described previously (4). The unique *Hin*dIII site in all these plasmids was chosen as the insertion point for the *CEN* element. The source of the *CEN* element was YSp19, a derivative of YIp5 in which the pBR322 *Eco*RI-*Bam*HI fragment is replaced by a 3.55-kilobase (kb) *Eco*RI-*Bam*HI fragment containing *CEN4* (25; D. Stinchcomb, personal communication). This fragment does not contain *ARS4*. The *CEN4* fragment was inserted into all the plasmids by the same scheme. Linear YSp19 DNA was isolated from a gel after *Eco*RI digestion. The 3.6-kb *Pst*I-*Eco*RI fragment from pBR322 was isolated from a gel and treated with alkaline phosphatase. The isolated fragments were ligated and cut with *Hin*dIII. The resulting fragments (one of them contained *CEN4* on a 2.5-kb *Hin*dIII piece) were ligated with each of the series of *Hin*dIII-restricted pLG plasmids carrying the different *ARS* fragments. To avoid a high transformation frequency of undesired plasmids, the ligation mixture was digested with *Sal*I before transformation in *E. coli*. Transformants were screened by digesting plasmids with *Xho*I. YCpG2, the plasmid containing the 3.55-kb *Bam*HI-*Eco*RI *CEN4* and the 25-bp *ARS1* fragment, was constructed in a similar way starting with pLGR88 and YSp19. The 3.5-bp *CEN4* fragment was linked to the 29-bp *Eco*RI-*Hin*dIII fragment from pBR322, and this sequence was inserted in the *Bgl*II- and *Hin*dIII-restricted plasmid pLGR88.

RESULTS

Design of the vector for analyzing *ARS* function. Up until now, *ARS* function has been studied in chimeric plasmids containing sequences that allow selection in both *E. coli* and *S. cerevisiae*, that allow replication in *E. coli*, and that will replicate autonomously in *S. cerevisiae* when an *ARS* is inserted. *ARS* function was determined by the ability of a plasmid to transform an auxotrophic yeast to prototrophy by some selectable marker on the plasmid. Because of the mitotic instability of *ARS* plasmids, it was not possible to use plasmid stability to measure *ARS* strength. To improve the assay for *ARS* strength, we added two features to the parental vector, a centromere to provide additional mitotic stability and a marker enzyme that allows sensitive discrimination on a single-cell basis between plasmid-containing and plasmid-free cells.

The plasmids used in the current study are derived from pLGARS1, which was described previously (4). The important feature of pLGARS1 is that it carries the *E. coli lacZ* gene, the marker we used to allow single-cell detection of plasmids. There are several reasons for this choice of marker. First, *S. cerevisiae* does not encode β-galactosidase activity, and thus there is no background activity in plasmid-free cells. Second, the β-galactosidase gene has been cloned in *S. cerevisiae* and placed under the control of a hybrid promoter that consists of parts of the yeast *GAL10* and *CYC1* promoters by Guarente and colleagues (7) so that the enzyme is inducible after the addition of galactose to the growth medium. Rapid induction is achieved through use of an *S. cerevisiae reg1* mutant host, which does not carry out glucose repression (M. Johnston, personal communication). Inducibility is important because only cells that have a

plasmid at the time galactose is added can produce β-galactosidase, minimizing the contribution to the results of cells that continue to contain enzyme after losing the plasmid. Murray and Szostak (21) showed that the *HIS3* gene product persisted for up to five generations after the loss of a CEN^+ plasmid carrying the gene. The third reason for choosing this marker is that we have developed a rapid and sensitive flow cytometry method for assaying β-galactosidase (24; this paper). This assay allows detection of β-galactosidase produced by a single cell containing a single copy of the *lacZ* gene, that is, a cell with a plasmid copy number (defined as the number of plasmids per cell) of one.

Plasmid pLGARS1 has a copy number of 20 per cell. In a steady-state population under selective conditions, only 5 to 8% of the cells contain a plasmid, and the plasmid is lost at a rate of about 30% per cell per generation in nonselective medium. We added the 2.5-kb *Eco*RI-*Hin*dIII *CEN4* sequence (25) to this plasmid to obtain YCpG1 (Fig. 1). This *CEN* plasmid has a copy number of one and a frequency of plasmid loss of 5% per cell per generation. It is important to note that the centromere segment does not contain *ARS4* (25).

Finally, we had previously constructed a set of deletion mutations in *ARS1* and studied their effect on replication (4), but many questions remained. What is the role of domain C? Why does the function of domain C appear only in the absence of domain B? What is the role of the consensus sequence? If a centromere is present to provide stability, is the consensus sequence alone, without domains B and C, sufficient for replication? The *ARS* fragments from an appropriate subset of these mutants were used to assay *ARS* function in the new plasmid background to answer these questions.

The configuration of a set of plasmids containing each of these segments is shown in Fig. 1. The DNAs were constructed as described above. All the *ARS* fragments had *Eco*RI and *Hin*dIII termini and were in the same orientation, as shown in the figure.

Flow cytometry determination of plasmid-carrying cells. The flow cytometry method of determining plasmid-containing cells is based on detection of the enzyme β-galactosidase, which is encoded on the plasmid. Previously, we showed that plasmid stability could, in principle, be measured with a flow cytometer by demonstrating that when cells containing plasmids encoding β-galactosidase activity are mixed with cells free of plasmids, both populations could be distinguished with the flow cytometer (24). However, using the instrumentation, plasmid, and reagents described at that time, we were not able to develop an assay sensitive enough to measure a dynamic population of cells carrying an unstable plasmid. The realization of such a method of measuring plasmid stability came through use of the new plasmids described above and also the identification of a new substrate that can be employed at higher concentrations than that used previously, allowing a greater rate and extent of reaction. Permeabilized cells are exposed to the fluorogenic substrate α-naphthyl-β-D-galactopyranoside and the azo-coupling reagent hexazonium pararosaniline. Cleavage of the substrate by β-galactosidase results in production of α-naphthol. The azo-coupling reagent immediately reacts with naphthol to form an insoluble product that fluoresces in the far red region. Because of its insolubility, the fluorescent product remains associated with the cell containing the enzyme and cannot diffuse to plasmid-free cells. A similar approach was used by Field and Schekman (6) to localize phosphatase activity to individual yeast cells.

Cells that contain plasmids constitutively expressing β-galactosidase retain a sufficient amount of enzyme after plasmid loss to be scored as plasmid-positive cells and cause significant error in the measurement we wished to make (data not shown). Therefore, the ability to grow cells in the absence of expression and to induce the enzyme as closely as possible to the time of measurement is crucial to the flow cytometry assay. In summary, the protocol for determining which cells carry plasmids comprises four steps. (i) The cell sample is grown to logarithmic phase in medium containing glucose, and activity is then induced by the addition of galactose. (ii) The cells are permeabilized. (iii) The staining reagents are added. (iv) The fluorescence of 10^5 cells is measured in the flow cytometer.

Determination of *ARS* strength. Cells carrying the plasmids described in Fig. 1 were grown on selective medium, and the fraction of plasmid-carrying cells in each population was determined on the flow cytometer or by direct plating on selective and nonselective media. Typical data obtained on the flow cytometer for the *ARS1* plasmid are shown in Fig. 2A. The diagram represents a two-parameter histogram in which each cell type is characterized by fluorescence intensity, which is related to plasmid content, and light scatter intensity, which is related to cell size. The vertical axis shows the frequency at which each cell type occurs within the population. Cells without a plasmid show a nonspecific background fluorescence of low intensity, and cells with a plasmid show a distribution of higher fluorescence values. The number of plasmid-free cells increased as the extent of the deletion in the *ARS1* element increased (Fig. 2A through E). The fraction of plasmid-free cells was determined with integration routines, which are built-in features of the data acquisition and evaluation unit used (16). The total number of cells in each histogram was calculated by integrating the cell number over the whole area defined by the fluorescence and light scatter axes. The number of plasmid-free cells was obtained by setting a trace in the fluorescence-light scatter plane around the plasmid-free cell population and integrating the cell number over the area defined by this trace. The limits of this trace were determined in the histogram representing the population containing the *ARS1* plasmid (Fig. 2A), since there the plasmid-free cell population was small and significantly separated from the plasmid-containing cell population. However, no corrections were made for the partially overlapping populations. We wish to point out that the reproduction of a three-dimensional histogram in two dimensions suffers and that the boundaries are much clearer if the trace is observed and drawn directly on the data acquisition unit, which allows a three-dimensional impression. We also calibrated our measurements with plasmid-free cells and cells containing one or two copies of the plasmids integrated in the chromosome to ensure that overlap was minimized.

The results of these calculations and the overall growth rates of cultures of cells containing the various plasmids are summarized in Table 1. The first novel observation is that deletions in domain C definitely had an effect. There was a graded decrease in stability (expressed in terms of overall growth rates and fraction of plasmid-containing cells) as the size of the deletion in domain C increased (compare deletions *ARS1*, R8, and R88). The result with domain C was particularly noteworthy since plasmids containing the R8 and R88 deletions but no centromere could not be distinguished by transformation frequency, growth rate, or fraction of plasmid-containing cells from a plasmid containing the complete *ARS1* fragment (4, 17). The effect of domain C

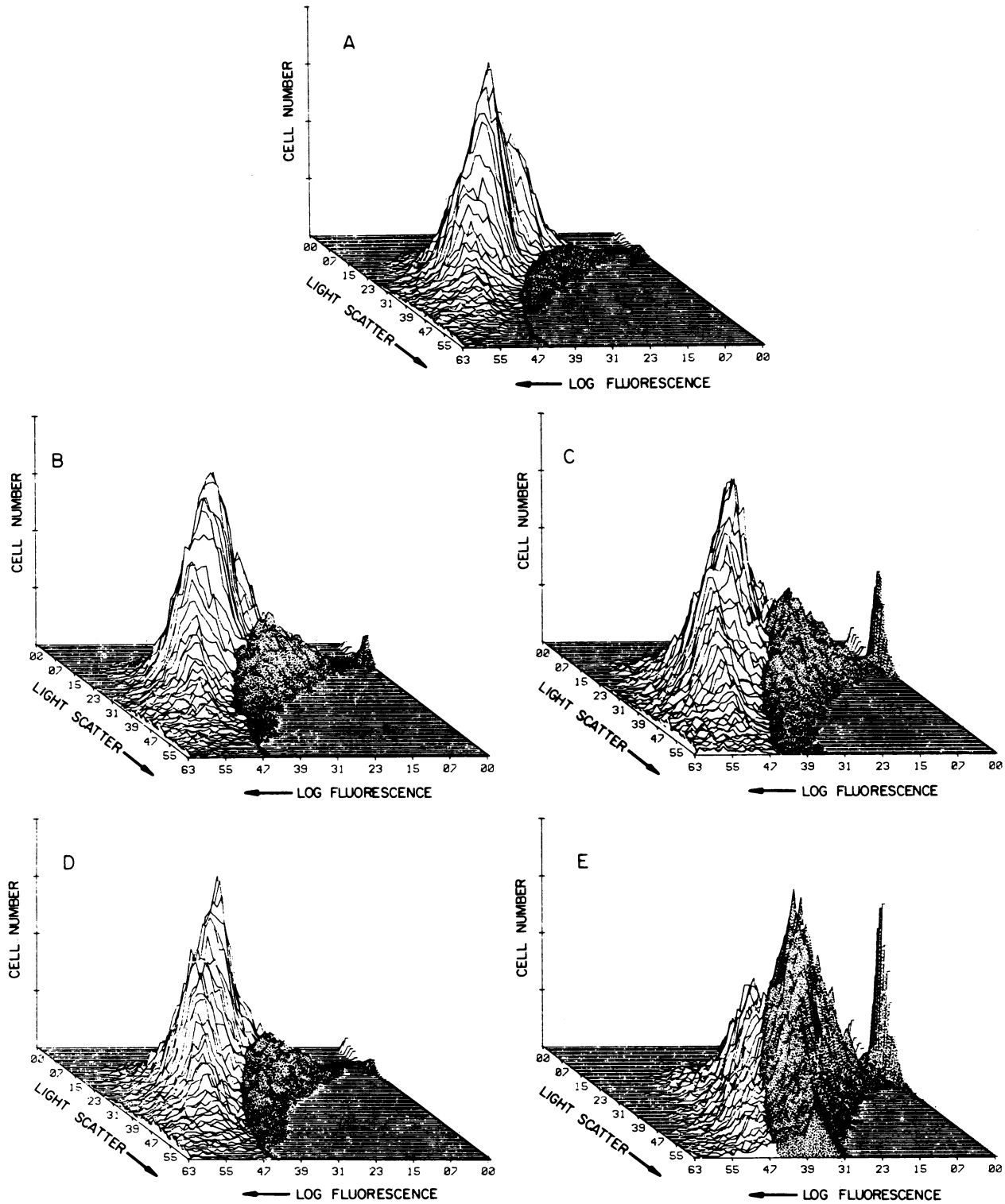

FIG. 2. Frequency distribution of plasmid-free and plasmid-containing cells as determined by flow cytometry. Cells were grown and prepared for assay of β-galactosidase as described in the text. Plasmids are referred to by the *ARS* deletion insert (Fig. 1). Each panel is a two-parameter histogram of the frequency distribution of measured single-cell fluorescence (x axis) and cell size (y axis). As an example of how to read this in the histogram, consider the x column labeled 1 where we see the size distribution of cells with identical fluorescence; similarly, in the y line 1, we see the fluorescence distribution of cells with identical size, and so on. (For definition of column and line see Kachel et al. [16].) The fluorescence axis (x) is represented on a logarithmic scale to allow ready visual discrimination between plasmid-free and plasmid-containing cells. Shaded areas are plasmid-free cells (see the text). A, *ARS1*; B, R8; C, R88; D, H103; E, H200.

TABLE 1. Stability of plasmids containing *ARS* deletions

ARS deletion	Generation time (h)	Plasmid-containing cells[a]		% Loss per generation
		FC	Plate	
ARS1	2.3	94	82	5
R8	2.6	86	67	15
R88	2.9	72	68	23
H103	2.6	82	73	15
H200	3.5	25	17	37

[a] FC, Flow cytometry. The values reported here were obtained in the experiment shown in Fig. 2. The standard deviation from up to four independent experiments was less than ±2% except for H200 (±12%). Plate, Plating cells on selective and nonselective media. Shown are results obtained with the same cultures used for the experiment shown in Fig. 2. Mean values from three different experiments had standard deviations of less than ±10%.

on *ARS1* could only be seen indirectly in competition experiments or in the absence of domain B. Koshland et al. (18) were the first to report a requirement for domain C in *CEN* plasmids, and our result confirms their finding in a different system. The second observation from these data is that, as expected from studies on plasmids not containing a *CEN*, there was an additional decrease in stability as deletions in domain B increased (note differences between plasmids H103 and H200).

The advantage of the flow cytometry assay was demonstrated by comparing its results with those obtained by direct plating for numbers of plasmid-containing and plasmid-free cells on selective medium. Although the same trends were apparent, the differences in stability were small and only reproducible when the determination was carried out in the statistically significant manner provided by the flow cytometer.

In a cell population growing on selective medium, the fraction of plasmid-containing cells reaches a steady state that is determined by the stability of the plasmid and the growth properties of the plasmid-containing and plasmid-free cells (1, 13, 21). Consequently, differences in stability between plasmids should be manifested quantitatively in differences in the fraction of plasmid-containing cells and hence in the overall growth rate of the culture. Both of these quantities can be easily measured. We expressed plasmid stability as percent loss per generation for each of the plasmids investigated (Table 1). To calculate this value, we used the measured growth rates of cultures containing the different plasmids and equation 3 (see Appendix). The value obtained for *ARS1* was very close to that obtained by Hieter et al. (11) by a very different method.

A 19-bp sequence is sufficient for weak *ARS* function. We had previously shown that a 19-bp sequence containing the 11-bp consensus sequence was not sufficient to allow autonomous replication of a plasmid lacking a centromere, although abortive transformants were observed (4). The 25-bp *Eco*RI-*Bgl*II fragment of plasmid YRpsb25 (4), which contained the 19-bp sequence and six nucleotides derived from the *Eco*RI linker, was inserted into a plasmid called YCpG2, identical to those described above except that it contained the 3.55-kb *Eco*RI-*Bam*HI *CEN4* fragment (25). As in the other plasmids, this *CEN4* segment did not contain *ARS4*, and the additional sequences of *CEN4* were included only for convenience in the construction (Fig. 3). Surprisingly, with this plasmid, transformants were observed at the same frequency as for the *ARS1*-containing plasmid. The transformants grew more slowly into colonies, but clearly were not abortive transformants, since they could be

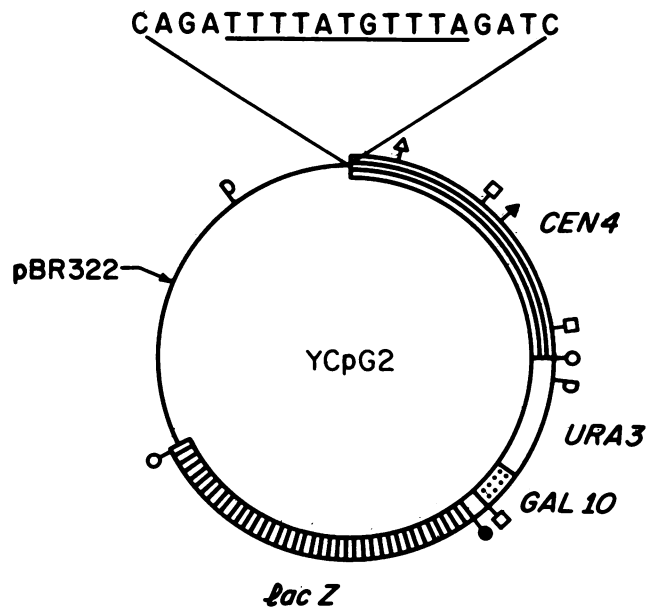

FIG. 3. Plasmid containing a minimal *ARS*. The plasmid is identical to the YCpG1 plasmids shown in Fig. 1, except that the *CEN4* element is the 3.55-kb *Eco*RI-*Bam*HI fragment (25). Construction is described in the text. The *ARS* insert is derived from plasmid YRpsb25, which was constructed previously (4). Symbols for restriction enzyme sites are defined in the legend to Fig. 1.

restreaked onto agar and they grew in liquid medium. The generation time of a strain carrying this mutant plasmid was greater than 15 h, compared with 2.3 for those carrying plasmids with the complete *ARS1* segment and 3.5 h for those carrying the mutant plasmid H200, which had significant deletions of both domains B and C. In spite of the slow growth rate and hence the presumably inefficient replication, it is striking that only 19 bp was sufficient to signal that a piece of DNA was to replicate in *S. cerevisiae*. Even in procaryotes, much larger sequences are thought to be required.

DISCUSSION

Most in vivo tests for *ARS* strength are based on measurement of plasmid stability—the ability of cells in culture to maintain the plasmid during growth. Differences in *ARS* strength result in differences in the probability of loss of a plasmid from a single cell. Thus, any plasmid-carrying cell culture is actually a heterogeneous cell population consisting of both plasmid-free and plasmid-containing cells in ratios related to the strength of the *ARS* element on the plasmid. One way that the probability of plasmid loss can be measured directly is at the single-cell level by pedigree analysis of plasmid inheritance (21). Another way is to analyze the heterogeneity of an entire cell culture by determining the kinetic parameters of the growing cell population. By using the rules governing the dynamic behavior of the population, such as those described in the Appendix, quantitative information can be obtained about the stability of a particular plasmid. We have followed the latter rationale to investigate the functional structure of *ARS1*. Flow cytometry determination of single-cell levels of a plasmid-encoded enzyme was the method chosen to quantitate one of the kinetic parameters—plasmid-free and plasmid-containing cells—in cultures containing *ARS1* deletion mutants.

Originally, the *ARS1* region was isolated on a 1.4-kb

*Eco*RI fragment carrying, in addition, the *TRP1* gene (27). The region mediating *ARS* activity was delimited to an 850-bp *Eco*RI-*Hin*dIII fragment of the original isolate. Removal of a further 200 bp from the *Hin*dIII site drastically diminished *ARS* stability, but did not reduce transformation frequency. Thus, Stinchcomb et al. (26) proposed that *ARS1* consists of two domains, one that mediates high frequency of transformation and one required for stable replication. The region required for high frequency of transformation was within a 600-bp fragment and was not further defined. We carried out a more detailed mapping of the functional structure of the *ARS1* element, confirmed the earlier proposal for *ARS* organization, and in fact defined three regions within *ARS1*, which we called domains A, B, and C (see reference 4 for a full discussion). On the basis of the latter work, the core of the *ARS1* element, a sequence of 11 bp that is a member of a consensus sequence present in all *ARS* regions characterized so far (2), is defined as domain A. This region seems to be absolutely necessary for high-frequency transformation, since single point mutations in this region abolish *ARS* activity completely (4, 17). In the current study we have shown that a minimal sequence of 19 bp, the 11-bp consensus sequence and 4 bp flanking it on each side, is both necessary and sufficient for *ARS* activity. This *ARS* activity is weak and can only be observed in the presence of a *CEN* element on the same plasmid (compare reference 4). Analyzing the *ARS* at the HO gene by using unilateral deletions, Kearsey (17) inferred that a core region of 14 bp was sufficient for replication. Comparing this core with our minimal *ARS*, we found only 1 bp conserved with respect to this flanking region, and it may represent the only additional requirement for *ARS* activity in our minimal segment.

Domains B and C adjoin the consensus sequence on both sides. The findings of Stinchcomb et al. (26) were extended by deletion analysis to define the borders of domain B, somewhere between 46 and 109 bp from the consensus sequence (4). This finding was confirmed by our current study (compare plasmids H103 and H200; Fig. 1 and 2). Indirect evidence was also obtained for domain C by showing that deletions of domain C were deleterious in plasmids that also contained deletions of domain B, although in plasmids with an intact domain B deletions of domain C had no effect at all (4). In the current work, we have provided direct evidence for domain C, in that deletions R8 and R88 did destabilize the vector used in these studies. Koshland et al. (18) were the first to show this. Our results, however, not only substantiate the existence of domain C, but also show that there is a gradual decrease in plasmid stability in the plasmids containing deletions *ARS1*, R8, and R88, suggesting that domain C also has a substructure. It is not clear what function domain C has. However, one potentially interesting point here is that the region designated *ARS1'* by Stinchcomb et al. (26) was deleted in the domain C deletions R8 and R88. This sequence corresponded in all but 2 bp to the consensus sequence, so that one could speculate that it is relevant to *ARS* activity.

Our current picture of *ARS* organization is consistent with the view that a core sequence is absolutely required for initiation of a replication event and that flanking regions only increase the probability that such an event happens during the limits of S phase. The core sequence could be viewed as a recognition site for a protein like the *dnaA* protein of *E. coli*, which prepares the adjacent sequences for assembly of the replisome after binding. The flanking sequence might determine the probability of an event by having a greater or lesser affinity for certain DNA-binding proteins of the components of the replisome. These could be the proteins responsible for the creation of the replication bubble, which was mapped to domain C in in vitro replication studies (3). Thus, the increasing instability of the mutants we describe could be due simply to a decreased probability for the replication event to take place during the limited time provided by the S phase. Such an organization could also explain the timing of activation of different replicons during the S phase, as observed by Fangman et al. (5).

The flow cytometry method of determining plasmid stability is not the only one for which the plasmid vector YCpG1, described in this paper, is suitable. Tests that monitor sectoring of yeast colonies have recently been developed in two other laboratories and can be used to characterize the segregation of plasmid-free clones from plasmid-containing cells on indicator plates (Hieter and Davis, personal communication; Koshland and Hartwell, personal communication). Such assays have the advantage that they can measure plasmids that are more stable than *ARS1*, which our method cannot do well (Fig. 2A). The flow cytometer, however, is more sensitive for measuring less stable plasmids. Furthermore, our plasmid, YCpG1, could also be used for sectoring assays. In principle, any substrate for β-galactosidase that gives colored or fluorescent products can be added to agar plates. Segregation or loss of the plasmid results in the inability of the cells to cleave the substrate, and such cells can be observed as a colored or colorless colony or sector, depending on the dye chosen, when plasmid-containing cells are grown on such plates. In practice, a compound must be employed that can be added at concentrations high enough that entry into cells can occur by diffusion. Cells containing the YCpG plasmids produce blue colonies on minimal plates containing glucose, galactose, and Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), whereas cells without a plasmid produce white colonies (data not shown). Other indicators that might potentially work with *S. cerevisiae* are *o*-nitrophenyl-β-D-galactopyranoside, 4-methylumbelliferyl-β-D-galactopyranoside, and reagents that can couple to azo dyes, such as were used in the current study. α-Naphthol and the azo-coupling reagent used in our studies produce a dark red color sufficiently intense to be visible in single cells (data not shown) and can be used after colonies are permeabilized with isopropanol. Although protocols based on permeabilization are sufficient for analytical purposes, since treatment might kill all of the cells, they might not be useful in selecting nuclear mutants deficient in plasmid maintenance by screening for colonies with increased (or decreased) sectoring, as can be done with the two systems mentioned above.

The flow cytometry test used in our study was suitable since the data can be obtained rapidly and are statistically reliable. Qualitative differences in plasmid stability are immediately obvious in the histograms obtained, and the method gives quantitative information statistically superior to that obtained by just plating the cells on selective and nonselective media or by replica plating. In our study, the flow cytometry method was only used for rapid detection of plasmid-containing and plasmid-free cells, based on the presence or lack of β-galactosidase. In addition to this rather rough classification, the data can give much more detailed information about the cell population. In particular, information about the cell cycle position of individual cells can be derived either by analyzing the light-scattering intensity, a measure of cell size which in turn is a function of the cell cycle position, or by staining and measuring the DNA content of single cells, which gives a more precise classification of cells into the different cell cycle phases (G1, S,

G2+M) (23). Quantification of the β-galactosidase content on the single-cell level as a function of cell cycle position provides a new, rapid, nonperturbative method of investigating partitioning and periodic expression of an enzyme within the cell cycle, as was observed, for instance, by other methods for the histones, thymidylate synthetase, and galactokinase of *S. cerevisiae* (8, 10, 28). Furthermore, the β-galactosidase assay used here is not limited to yeast but is also applicable to mammalian cells. Another application might be in studying protein transport phenomena. Transport could be measured with fluorescence-labeled antibody, for instance, which gives a more direct correlation between the signal measured and the amount of protein present than the enzyme assay used here. Finally, Koshland (personal communication) has suggested that the method could be used directly for looking at mechanisms that allow certain proteins to segregate asymmetrically during cell division, such as the *KAR* gene product. Although flow cytometry is not yet widely used, such applications might be worth looking into, given the unique ability of the method to measure yeast cells for a single gene and gene product as illustrated in this report.

APPENDIX

Relationship between growth rates, fraction of plasmid-containing cells, and plasmid stability. In Table 1, the stability of plasmids containing the *CEN4* fragment was expressed as rate of loss per generation. This value can be obtained from the experimentally determined overall growth rates according to a model describing the growth of genetically unstable cultures (1, 13, 21; J. H. Seo and J. E. Bailey, Biotechnol. Bioeng., in press). This model describes the growth of plasmid containing cells as

$$P_t = P_o e^{\mu_+(1-s)t} \quad (1)$$

where P is the overall number of plasmid-containing cells, μ_+ is the specific growth rate of the plasmid-containing cells, s is plasmid stability expressed as rate of loss per generation, and t is time. The fraction of plasmid-containing cells (f^+) can be expressed as $f^+ = P/(P + F)$, with F representing the number of plasmid-free cells. Intuition suggests that after a sufficient number of generations of growth on selective medium, the fraction f^+ will approach a constant or steady-state value. This same conclusion also follows from the population growth models mentioned above. Evaluated for conditions corresponding to those in experiments presented in this paper, these models indicate that 15 to 20 generations are sufficient to approach closely a constant value of f^+, and this result is consistent with the results of the cultivation and sampling protocols used in our measurements. Under conditions when f^+ is constant, the number of plasmid-containing cells (P) as well as the number of plasmid-free cells (F) must increase with the same overall specific growth rate (μ_o); which is the experimentally observed specific growth rate for the unstable recombinant culture at steady-state conditions. Thus, one can also describe the increase of plasmid-containing cells as

$$P_t = P_o e^{\mu_o t} \quad (2)$$

Comparing equations 1 and 2 gives

$$\mu_o = \mu_+ (1 - s) \text{ or } s = 1 - (\mu_o/\mu_+) \quad (3)$$

The growth rate of the culture containing plasmid YCpG1 with the *ARS1* deletion, corrected for the fraction of plasmid-containing cells (95%), gives a very close estimation of the correct specific growth rate (μ_+) of the plasmid-containing cells. Since only 5% of the cells in this culture lacked a plasmid and since the effective growth rate of these cells was expected to be significantly less then μ_+, it may be reasonably assumed that plasmid-free cells contribute insignificantly to overall growth. With this value and the experimentally determined overall specific growth rates (μ_o) for various recombinants, the corresponding plasmid stability s can be calculated through equation 3. These considerations are exactly true only when the growth rate of the plasmid-containing cells is not changed if the *ARS* function on the plasmid fails to work properly, i.e., a normally replicating cell with two plasmid copies after passing the S phase should have approximately the same growth rate as a cell in which replication fails, yielding just one plasmid copy per cell.

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