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Replicating Plasmids in *Schizosaccharomyces pombe*: Improvement of Symmetric Segregation by a New Genetic Element

WOLF-DIETRICH HEYER,[1]* MÁTYÁS SIPICZKI,[2] AND JÜRG KOHLI[1]

Institute of General Microbiology, University of Bern, CH-3012 Bern, Switzerland,[1] and Department of Genetics, Institute of Biology, University of Debrecen, H-4010 Debrecen, Hungary[2]

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We characterized a number of widely used yeast-*Escherichia coli* shuttle vectors in the fission yeast *Schizosaccharomyces pombe*. The 2μm vectors pDB248 and YEp13 showed high frequency of transformation, intermediate mitotic and low meiotic stability, and a low copy number in *S. pombe*, analogous to their behavior in [cir^0] strains of *Saccharomyces cerevisiae*. The *S. cerevisiae* integration vectors pLEU2 and pURA3 transformed *S. pombe* at very low frequencies but, surprisingly, in a nonintegrative fashion. Instead, they replicated autonomously, and they showed very high copy numbers (up to 150 copies per plasmid-containing cell). This could reflect a lack of sequence specificity for replication of plasmid DNA in *S. pombe*. pFL20, an *S. pombe ars* vector, and a series of plasmids derived from it were studied to analyze the unusually high stability of this plasmid. Mitotic stability and partitioning of the plasmids was measured by pedigree analysis of transformed *S. pombe* cells. An *S. pombe* DNA fragment (*stb*) was identified that stabilizes pFL20 by improvement of plasmid partitioning in mitosis and meiosis.

Many of the molecular genetic techniques which have revolutionized the study of biological problems in *Saccharomyces cerevisiae* have now been applied to the fission yeast *Schizosaccharomyces pombe*. Although some of the *S. cerevisiae* cloning vectors will transform *S. pombe* and be maintained fairly well, several reports have suggested that the two yeasts differ in plasmid behavior (14, 32, 33). To better understand this we performed a comparative analysis of the transforming ability and mitotic and meiotic segregation characteristics of several types of plasmids which replicate autonomously in *S. pombe*.

The first demonstration of high-frequency transformation of *S. pombe* was obtained with marker genes and plasmid vectors from *S. cerevisiae* (3). After this report, *S. pombe* gene banks were constructed employing different 2μm-based vectors, and genes were isolated by functional complementation of mutants (4–6, 31). In addition, *S. pombe* DNA fragments were isolated which confer high frequency of transformation to plasmids (3, 24, 32, 36) and thus resemble the *ARS* (autonomously replicating sequences) elements previously characterized in *S. cerevisiae* (34, 35). Integrative transformation based on recombination between homologous sequences was successfully applied to map the rDNA locus of *S. pombe* (36) and was found to occur with cloned DNA from the mating-type gene region with high efficiency (4). Gaillardin et al. (14) found frequent rearrangements including deletions and integration of plasmids that contain the entire *S. cerevisiae* 2μm DNA. An extensive analysis of plasmids carrying the *S. pombe ura1* gene and the associated *ars1* sequence has shown that these plasmids are established in *S. pombe* cells in a peculiar way. They assume a polymeric form by tandem amplification (32, 33).

Facing these disparate reports of varying plasmid behavior in *S. pombe* (14, 32, 33), we decided to characterize the most frequently used *S. pombe* vectors in more detail. We chose YEp13 (9) and pDB248 (3) for the *LEU2* marker system and as a control the *S. cerevisiae* integration vector pLEU2. These plasmids do not contain *S. pombe* DNA and can be used in both yeasts and in *Escherichia coli*. For the *URA3* marker system, the *S. pombe ars* vector pFL20 (24) and the *S. cerevisiae* integration vector pURA3 were chosen.

The results with these vectors demonstrate that high frequency of transformation (attribute of *ars* sequences) and effective replication capacity (measured as high copy number) are separable phenomena in *S. pombe*. In addition, an astonishing stability of the plasmid pFL20 was observed and led to the identification of a DNA fragment that enhances plasmid stability. Throughout this publication we use the purely operational definition for *ars* sequences: they confer high frequency of transformation to autonomously replicating circular plasmids. Implications of our data on plasmid replication and segregation are discussed.

MATERIALS AND METHODS

Strains and plasmids. The *S. pombe* and *E. coli* strains and the plasmids used in this study are described in Table 1. The gross structure of most plasmids is given in Fig. 1. pDB248 and YEp13 have two different 2μm DNA fragments, but on both plasmids the 2μm *ORI* region (8) and the *STB* locus (23) are present. In YEp13 no 2μm open reading frame remains intact, whereas in pDB248 the *REP2* gene is present. pLEU2 contains the 2.2-kilobase (kb) *Sal*I-*Xho*I *LEU2* fragment from *S. cerevisiae* inserted in the *Sal*I site of pBR322. pFL20 (24) carries two *S. pombe Eco*RI fragments that are termed *ars* (1.1 kb) and *stb* (1.3 kb). In pURA3-1.1 only the smaller (*ars*) and in pURA3-1.3 only the bigger (*stb*) fragment is present. pURA3 is pFL20 without *S. pombe* DNA, i.e., the 1.1-kb *Hin*dIII *URA3* fragment of *S. cerevisiae* inserted into the *Hin*dIII site of pBR322. pWH102 was constructed by replacement of the small *Pst*I-*Hin*dIII fragment of pLEU2 by the corresponding *Pst*I-*Hin*dIII fragment of pFL20. Thus, the 1.1-kb fragment (*ars*) has been transferred completely, and most (1.1 kb) of the 1.3-kb fragment (*stb*) has been transferred.

Genetic methods, transformation, and DNA hybridization. Standard genetic methods and media (MMA for minimal medium; YEA for full medium) for *S. pombe* are described in Gutz et al. (16). *S. pombe* was transformed as described

* Corresponding author.

TABLE 1. Microorganisms and plasmids used in this study

Organism or plasmid	Genotype and other characteristics	Source or reference
Schizosaccharomyces pombe	h^-/h^+ *ura4-294*[a]	Bern collection
	h^-/h^+ *leu1-32*[a]	Bern collection
	h^+ *ura4-294 ade6-250*	Bern collection
	h^- 972	Bern collection
Escherichia coli[b]		
JA221	F⁻ *leuB6 trpE5 lacY hsdR hsdM⁺ recA1*	Clarke and Carbon (11)
BJ5183	F⁻ *recBC sbcB endo-1 gal met str thi bio hsd*	Losson and Lacroute (24)
Plasmid[c]		
pDB248	*LEU2* Amp^r Tet^r, 10.5 kb	Beach and Nurse (3)
YEp13	*LEU2* Amp^r Tet^r, 10.7 kb	Broach et al. (9)
pLEU2	*LEU2* Amp^r, 6.7 kb	A. Hinnen (CIBA-GEIGY, Basel)
pWH102	*LEU2* Amp^r, 8.6 kb	Constructed
pFL20	*URA3* Amp^r Tet^r, 7.9 kb	Losson and Lacroute (24)
pURA3-1.1	*URA3* Amp^r Tet^r, 6.6 kb	J. Boeke (Massachusetts Institute of Technology, Boston)
pURA3-1.3	*URA3* Amp^r Tet^r, 6.8 kb	Constructed
pURA3	*URA3* Amp^r Tet^r, 5.5 kb	Constructed
pcdc10-2	*LEU2 cdc10* Tet^r, 13.9 kb	Aves et al. (2)

[a] For both mutations rare revertants were found in the controls of transformation experiments.
[b] The genetic symbols follow the respective original author listed.
[c] Gross structure of most plasmids is given in Fig. 1, but for pcdc10-2 see Materials and Methods.

previously (3) with a few modifications (M. Sipiczki, W.-D. Heyer, and J. Kohli, Curr. Microbiol., in press). This protocol is based on transformation of spheroplasted cells. Alternatively, the lithium acetate protocol of Ito et al. (20) can be used (1×10^3 to 2×10^3 transformants per μg of pDB248 or pFL20). Recovery of plasmids from yeasts to *E. coli* was done exactly as described previously (5). Approximately 0.5 μg of DNA recovered from *S. pombe* was used to transform competent *E. coli* cells (BJ5183) which yielded about 5×10^5 transformants per μg of pBR322. About 2,000 transformants were obtained for the most easily recovered plasmids. Total DNA was prepared from transformed *S. pombe* cells grown under selective pressure (P. Nurse, manuscript in preparation). The cells were treated with Zymolyase 5000 for 30 min and lysed with 1% sodium dodecyl sulfate. Potassium acetate was added to a concentration of 1.3 M, forming a heavy precipitate. After centrifugation, the DNA was purified from the supernatant by repeated isopropanol or ethanol precipitation and by RNase digestion. It is vital to use Zymolyase instead of Novozym SP234 to render the DNA digestable with restriction endonucleases. Analysis of DNA by gel electrophoresis, transfer to nitrocellulose, and hybridization have been described previously (1).

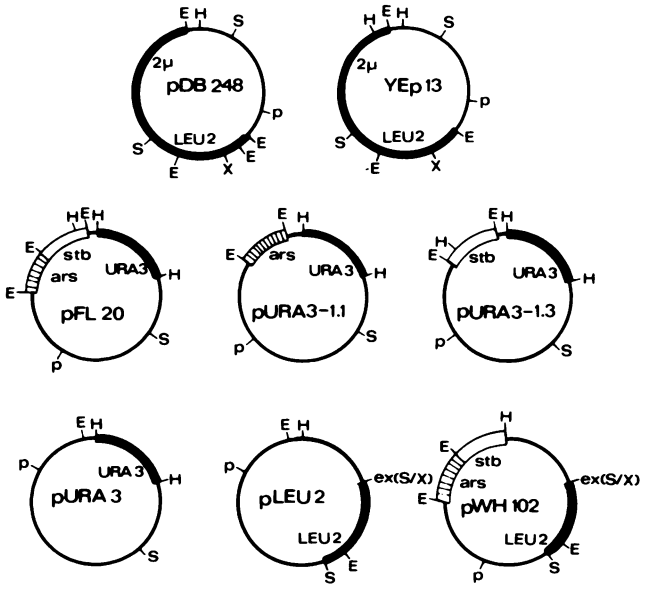

FIG. 1. Structures of plasmids used in this study. The maps are schematic and not to scale. The *Hin*dIII site of pBR322 is shown at the top of the map with the β-lactamase gene to its left. *S. pombe* DNA is indicated as follows: hatched boxes, *ars* element; open boxes, *stb* element or parts of it. *S. cerevisiae* DNA (2μm, *LEU2*, *URA3*) is drawn as a thick line, and pBR322 is shown as a thin line. Recognition sites of restriction endonucleases are abbreviated as follows: H, *Hin*dIII; E, *Eco*RI; S, *Sal*I; X, *Xho*I; P, *Pst*I.

Estimation of plasmid copy number in *S. pombe*. Total DNA was prepared from transformed *S. pombe* and digested with *Hin*dIII. Serial dilutions of the cleaved DNA were electrophoresed through a 0.8% agarose gel and transferred to nitrocellulose. The nick-translated plasmid pcdc10-2 was used as a hybridization probe. It is based on the vector pDB262 and carries the *S. pombe cdc10* gene on a 4.1-kb *Hin*dIII fragment (2). The *cdc10* band served as a single-copy gene standard, and with the same probe the different plasmid-borne fragments were detected in the same hybridization experiment due to the homologous sequences (pBR322, 2μm, and *LEU2*) in the probe and the plasmid to be tested. The structure of pDB262 was described previously (6); the vector does not contain *S. pombe* DNA and has no detectable homology to the *S. pombe* genome. The intensity of the hybridization signal from the vector-borne fragments was quantified by visual inspection of the dilution series in relation to the single-copy standard. The least-exposed autoradiogram was used to avoid nonproportional signal response of the film. The value obtained was corrected twice. First, the proportion of plasmid-containing cells in the culture for the DNA preparation was determined by plating a

suitable dilution on YEA plus leucine and uracil (YEA plus Leu-Ura) and replication of the colonies to MMA. This gave a significant correction for most plasmids (see Table 2), since a large fraction of cells is plasmidfree even under selective pressure. The second correction was introduced because of the different homology length of the probe to the chromosomal standard (4.1 kb) and to the different plasmids (3.6 to 9.5 kb). For most plasmids this correction was small. The copy number was estimated under the assumption that all DNA fragments were transferred to the filter with the same efficiency (agarose gels after transfer of the DNA to the filter showed no ethidium bromide-stainable material) and that hybridization is proportional to the amount of homology of the probe to the DNA fragments. The value given is the average copy number per plasmid-containing cell. For plasmids with more than 50 copies, the plasmid-derived fragments were clearly visible in the ethidium bromide-stained gel (see Fig. 5A). For some plasmids several independent estimations were made, and the results were closely similar.

Measurement of mitotic stability: replica plating assay. Transformed *S. pombe* cells were grown under selective conditions in liquid culture, and a suitable dilution was plated on YEA plus Leu-Ura plates. The colonies were then replicated to MMA, and the fraction of colonies capable of growth on MMA was determined. This gave the steady-state level of plasmid-containing cells under selective conditions. Cells from the same dilution used for plating were inoculated into YEA plus Leu-Ura and grown for 20 generations without selection. Then the fraction of cells growing on MMA was again determined by plating and replicas as described above. A total of 100 to 200 colonies were always tested. We assume that colonies that grew after replica plating onto selective medium arose from cells harboring the plasmid, while those that failed to grow arose from cells lacking the plasmid.

Measurement of mitotic stability: pedigree analysis. Since *S. pombe* divides symmetrically in mitosis no easy distinction is possible among the offspring cells. Therefore, we distinguish symmetric plasmid segregation (both daughters receive the plasmid) from asymmetric plasmid segregation (only one daughter receives the plasmid). A loopful of cells was streaked on a selective plate and incubated overnight at 30°C, and an individual cell was removed for micromanipulation. The plates were further incubated, and after each mitosis the products were separated for four to six divisions. After completion of micromanipulation the plates were incubated at 30°C. The cells that gave rise to colonies were assumed to harbor the plasmid, while those that failed to give rise to colonies were presumed to be plasmidfree. For lines of cells not giving rise to colonies, the number of divisions after plasmid loss was calculated from the number of cells produced after the asymmetric division giving rise to the plasmidfree cell line.

To demonstrate the viability of cells lacking the plasmid, some pedigrees were also performed on YEA plus Leu-Ura plates. The resulting colonies were replica plated to MMA. Those colonies that grew on MMA were assumed to harbor the plasmid, and those that failed to grow were assumed to have lost the plasmid. Both methods were reproducible and gave virtually identical results in pedigree analysis.

Measurement of plasmid transmission in meiosis. Transformed *S. pombe* cells were crossed to h^+ *ura4-294*, h^+ *ura4-294 ade6-250*, or h^+ *leu1-32* on synthetic sporulation medium (16) at very high titers (10^8 ml^{-1}) of the partners. These precautions were undertaken to leave the transformed partner under selective pressure and to prevent mitotic divisions before zygote formation so that plasmidfree cells should not accumulate. After incubation for 28 h at 25°C, tetrad analysis was performed on YEA plus Leu-Ura plates. The spore clones were replicated to MMA, and for those colonies that grew on MMA we assumed that the plasmid was present in the original spore. For those that failed to grow on MMA, we assumed that the plasmid was not present in the original spore.

TABLE 2. Transformation frequency, plasmid stability in mitosis, and copy number

Plasmid	No. of transformants per μg of DNA[a]	Mitotic stability[b] (% *LEU*$^+$ or *URA*$^+$)		Copy no. per plasmid-containing cell
		Under selection	After 20 generations without selection	
pDB248	2×10^4	41 ± 25	25, 3, 7	7–10
YEp13	3×10^3	31 ± 12	0, 1, 9	5–6
pLEU2	30	46[c] ± 7	2, 1	90
pWH102	2×10^4	54 ± 4	1	15
pFL20	2×10^4	95 ± 5	83, 80, 84	74–80
pURA3-1.1	2×10^4	34 ± 15	21, 9, 1	27
pURA3-1.3	20	33[c] ± 28	1	234
pURA3	20	57[c] ± 11	2	142

[a] Frequencies above 10^4 transformants per μg of DNA were not reached in every single experiment. For the non-*ars* plasmids (pLEU2, pURA3, pURA3-1.3), the spheroplast preparation was also transformed with an *ars* vector to check positively the transformation competence.

[b] Mitotic stability was assayed by the replica plating method (see Materials and Methods). Large fluctuations were observed when we checked 10 independant yeast transformants for each plasmid. Therefore, we chose one typical transformant for which the values are given in the table. Values of plasmid stability under selective conditions are the means ± standard deviation of 4 to 10 determinations. To measure the proportion of plasmid-containing cells after non selective growth, one to three independent experiments were made, and all values are given.

[c] Very often only a small sector was *LEU*$^+$ or *URA*$^+$ after replication from YEA to MMA. The doubling time of these transformants under selective conditions was longer than for *ars* or 2μm plasmids, although precise measurements were not made.

RESULTS

Plasmids in *S. pombe*: non-*ars* plasmids can replicate in *S. pombe*. The *S. cerevisiae LEU2* gene complements the *S. pombe leu1-32* mutation, and the vectors pDB248 and YEp13 can efficiently transform *S. pombe* (Table 2). Transformation frequencies were consistently lower with YEp13 than with pDB248, and YEp13 may be less stable in *S. pombe*. These differences may be due to the different 2μm DNA fragments present or due to the different lengths of the *LEU2* DNA fragment (Fig. 1).

To our astonishment we found *S. pombe* transformants with pLEU2, a plasmid which bears no homology to the *S. pombe* genome and carries no *ars* sequence. The rare transformants showed the typical instability of *ars* plasmids (46% *LEU*$^+$ under selection). We wondered whether this was due to autonomous replication of the plasmid in *S. pombe* or integration. We analyzed DNA from pLEU2-transformed cells (Fig. 2) and could not detect supercoiled monomer plasmid species. With rare exceptions no supercoiled monomers were detected in *S. pombe* DNA preparations for any of the plasmids tested. Instead, a heavy hybridization signal came from the bulk of undigested chromosomal DNA and from plasmid species running even more slowly (Fig. 2, lane 2). This represents, most likely, polymeric forms of the plasmid. Although the probe contains chromosomal DNA, the strength of the signal, as compared

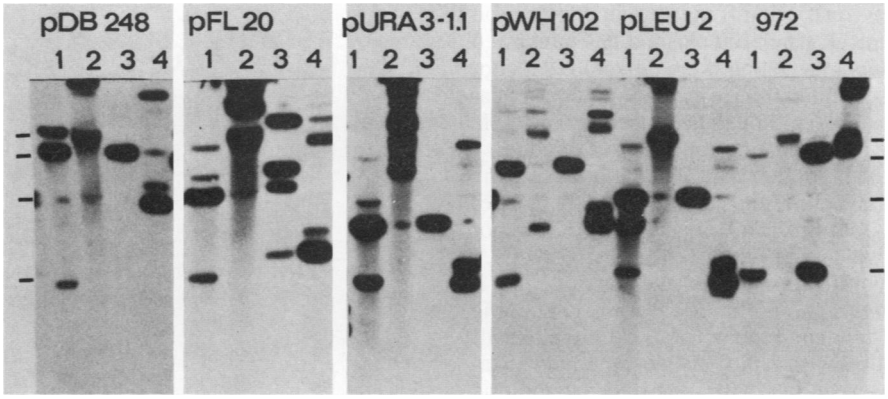

FIG. 2. State and structure of plasmid DNA in *S. pombe*. The figure is a composite of different autoradiograms. As a size marker the positions of λ *Hin*dIII bands (23.6, 9.6, 6.6, and 4.3 kb) are given. The DNA was run on 0.8% agarose gels and transferred to nitrocellulose filters. The hybridization probe was nick-translated pcdc10-2. For each plasmid total DNA isolated from a yeast transformant was analyzed (lanes 1 and 2). As a control the original plasmid preparation from *E. coli* was analyzed (lanes 3 and 4). The DNA in lanes 1 and 3 was digested with *Hin*dIII (lane 3 of pFL20 shows partial digestion products in addition to the expected 6.6-kb band). The DNA in lanes 2 and 4 was undigested. The upper band in lane 1 of pDB248 most likely represents a rearranged plasmid species, since the digestion of the genomic DNA with *Hin*dIII was complete. Lanes 1 and 2 for 972 show the hybridization of pcdc10-2 probe to DNA from an untransformed wild-type strain. Two fragments of 4.1 kb (strong hybridization [= *cdc10* gene]) and 12 kb (weak hybridization) are recognized by the probe in the *Hin*dIII digest of lane 1 and are visible in all lanes 1. Lanes 3 and 4 for 972 display the results with the pcdc10-2 plasmid itself. The position of plasmid DNA-derived hybridization was confirmed by reprobing the blots with pBR322, a plasmid-specific probe (data not shown; see text). The lower part of the autoradiograms is not shown because there were no additional hybridization signals.

with that in the lane with the untransformed 972 control, clearly shows the presence of DNA sequences added by the transformation event (equal amounts of DNA were loaded). This was confirmed by reprobing the blots shown in Fig. 2 with pBR322, a plasmid-specific hybridization probe (data not shown). Nevertheless, free pLEU2 plasmids exist in *S. pombe*, because transformation of *E. coli* with undigested total DNA preparations of pLEU2 transformants yielded about 500 transformants. From all *S. pombe* transformants checked *E. coli* colonies were obtained with the original pLEU2 plasmid, as judged from restriction analysis. Yet 20% of the secondary *E. coli* transformants tested carried a rearranged pLEU2 plasmid. Thus, rearrangement of pLEU2 can occur in *S. pombe* as has been observed for many other plasmids (see below and Fig. 2 where a shorter version of pLEU2 is also visible in the *Hin*dIII digest of lane 1). The high-molecular-weight band seen in lane 1 is the same as in the untransformed control (972) and therefore does not represent a junction fragment. In conclusion, the data on plasmid stability (Table 2) and transfer back to *E. coli* indicate that pLEU2 may replicate autonomously in *S. pombe*.

The same analysis was applied to YEp13 (data not shown) and pDB248 (Fig. 2), which are assumed to replicate autonomously (3, 5, 31). When total DNA from transformants with these plasmids was digested with *Hin*dIII, the original fragment pattern was found to be present in all cases. Additional hybridization bands are visible in digests with YEp13 (see Fig. 6) and pDB248 (Fig. 2). In an analysis of undigested DNA from pDB248 transformants (Fig. 2, lane 2), two plasmid-specific hybridization signals were detected: one comigrating with the bulk of chromosomal DNA and one clearly migrating slower, most likely representing polymeric forms of the plasmid. Rearrangements of YEp13 occurred less frequently than those of pLEU2. Again, unrearranged plasmids could be recovered into *E. coli* from all *S. pombe* transformants analyzed. This indicates that the additional fragments visible in Fig. 6 derive from independent plasmids, not from composite plasmids containing the original and rearranged sequences. Transfer of plasmids from *S. pombe* back to the bacterium again yielded large numbers of *E. coli* transformants (500 to 2,000). Among them the deletion plasmids had often lost an antibiotic resistance. pDB248 could also be shuttled back to *E. coli* with high frequency, and no rearranged plasmids were detected in these secondary *E. coli* transformants, although these clearly exist in *S. pombe* (Fig. 2).

The *S. cerevisiae URA3* gene in pFL20 and in the plasmids derived from it complements the *S. pombe ura4-294* mutation. In contrast to the 2μm vectors described before, pFL20 is an *S. pombe ars* vector. pFL20 transformants show an exceptionally high stability for an *ars* plasmid (Table 2), and experiments analyzing this property are described below. Here we concentrate on the structure of pFL20 and its derivatives (Fig. 1) in *S. pombe* and after transfer back to *E. coli*. The overall behavior of these plasmids varied considerably. The hybridization patterns of undigested DNA from transformants with pFL20 (Fig. 2), pURA3-1.3 (data not shown), and pURA3 (data not shown) resembled very much the behavior of pLEU2 and pDB248 described above: there is diffuse hybridization in the region of bulk chromosomal DNA and also above chromosomal DNA which most likely represents polymeric forms. The additional bands in the *Hin*dIII digest for pFL20 (Fig. 2, lane 1) do not represent junction fragments: the upper band corresponds to the upper band in 972 (lane 1) (untransformed control) and the second upper band (7.9 kb) results from incomplete digestion. The plasmids pURA3 and pURA3-1.3 serve again as non-*ars* controls for pFL20. Like pLEU2, these plasmids were able to transform *S. pombe* at a low frequency (Table 2), and they showed a mitotic stability comparable with the 2μm vectors. pURA3 and pURA3-1.3 were easily shuttled back to *E. coli* (1,000 to 2,000 transformants). The analysis of 32 secondary *E. coli* transformants with undigested DNA from each of eight independent yeast pURA3 transformants revealed only one rearranged plasmid. With pURA3-1.3 no rearrangement was found at all. Southern blots of total transformant DNA, cleaved with an enzyme that does not cut the plasmid,

should give rise to new restriction fragments in comparison with an undigested control, when the plasmid has integrated into the genome. No such new bands were detected (data not shown). This is another indication for autonomous replication of the non-*ars* plasmids, although an extremely unstable integration of amplified DNA cannot be totally excluded.

The plasmids pFL20 and pURA3-1.1 which both carry the same *ars* sequence behave differently as indicated by blots of undigested yeast transformant DNA (Fig. 2). Shorter exposure of the filter shown in Fig. 2 showed that pURA3-1.1 displays a ladder of hybridization bands. We conclude that it propagates in *S. pombe* in multimeric forms containing different numbers of tandem repeats of the unit plasmid. Upon complete digestion with *Hin*dIII, the same hybridization pattern was observed as with digested monomeric plasmid obtained from *E. coli* (Fig. 2). Such tandem amplification of plasmids containing *S. pombe* DNA has been studied in detail by Sakaguchi and Yamamoto (32). They found also that such highly multimeric plasmids cannot be shuttled back to *E. coli*. Correspondingly, undigested DNA from pURA3-1.1 yeast transformants yielded only very few *E. coli* transformants (5), while pFL20 gave high frequencies of secondary *E. coli* transformants (1,500). The analysis of the recovered plasmids showed a very infrequent occurrence of rearrangements for both pURA3-1.1 and pFL20. The analysis of secondary *E. coli* transformants cannot faithfully describe the state of plasmids in *S. pombe*. But we take the high (or low) frequency of secondary transformants as reasonable evidence for the presence (or absence) of monomeric plasmids in *S. pombe*.

In summary, the results described here and the data by Yamamoto and co-workers (32) suggest that most autonomously replicating plasmids in *S. pombe* assume a polymeric form. The degree of multimerization is subject to wide variation when different plasmids are compared. It can reach an extent at which direct reestablishment of a plasmid in *E. coli* is no longer possible (32). The absence of supercoiled monomers in *S. pombe* transformant DNA could be due to a nicking activity present during DNA isolation. That supercoiled monomers can be obtained is shown in Fig. 2 for the plasmid pWH102. Considerable differences between the investigated plasmids also exist concerning the frequency of structural aberrations. In this collection of vectors that carry no *S. pombe* DNA or only small pieces of the *S. pombe* genome, we mostly observed deletions in plasmids, but some were rearranged only very rarely (e.g., pURA3 and related plasmids). In addition, we observed that pURA3-1.3 always exists in *E. coli* BJ5183 as a mixture of monomers and multimers despite attempts to obtain a bacterial strain carrying only monomers. All *S. pombe* transformations with pURA3-1.3 were thus carried out with this mixture of multimers and monomers.

High stability of pFL20 is due to the presence of the *S. pombe* 1.3-kb *Eco*RI fragment. The plasmid pFL20 transformed *S. pombe* very efficiently and exhibited an exceptionally high stability in yeasts (Table 2). pFL20 carries two *S. pombe Eco*RI fragments, and we asked whether just one or both are needed for *ars* activity. Both fragments were recloned separately (Fig. 1), and the resulting plasmids were named pURA3-1.1 (contains the 1.1-kb *Eco*RI fragment) and pURA3-1.3 (contains the 1.3-kb *Eco*RI fragment). From the transformation data (Table 2), we conclude that the 1.1-kb fragment confers full *ars* activity while the 1.3-kb fragment does not at all.

A difference is apparent between pFL20 and pURA3-1.1 in mitotic stability (Table 2). When the 1.3-kb fragment was missing the unusual high stability of pFL20 was lost, and the pURA3-1.1 plasmid then resembled the 2μm vectors. While the 1.1-kb fragment conferred the *ars* activity, the 1.3-kb fragment promoted stability and is therefore named *stb* element. The *stb* element alone (in a plasmid without *ars*) seemed to have no visible effect on stability as concluded from the data with pURA3-1.3 (Table 2).

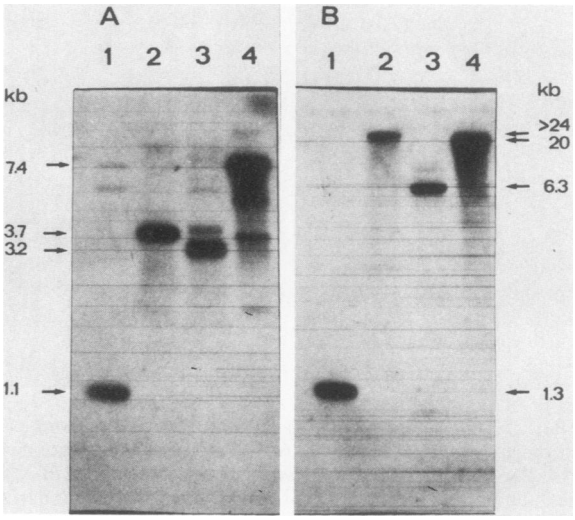

FIG. 3. Organization of the *ars* and *stb* fragments of pFL20 in the *S. pombe* genome. DNA from wild-type *S. pombe* was digested with *Eco*RI (lane 1), *Bam*HI (lane 2), *Hin*dIII (lane 3), and *Pst*I (lane 4), run on a 0.8% agarose gel, and transferred to a nitrocellulose filter. (A) Hybridization pattern with nick-translated pURA3-1.1; (B) hybridization pattern with nick-translated pURA3-1.3 to the DNA of the same filter after washing off the first probe. Both hybridization patterns are unique. No common bands were detected. Also, the vector pURA3 does not hybridize to the *S. pombe* genome. The size is given for the most prominent bands. As size markers, *Hin*dIII-digested λ DNA was used.

For a confirmation of the data just described and the exclusion of an influence of the *URA3* sequence on transformation frequency and stability, we constructed pWH102 (see Materials and Methods and Fig. 1). It combines the *ars* element and most of the *stb* element of pFL20 with the *LEU2* marker system. pWH102 transformed at high frequency, and on selective medium it was of intermediate stability between pURA3-1.1 and pFL20 (Table 2). This was confirmed by pedigree analysis of pWH102-transformed cells (see below). The partial loss of stability may be due to the terminal deletion of 200 base pairs from the *stb* fragment or to the different marker (*LEU2* instead of *URA3*) present on pWH102. But the latter explanation is unlikely because *URA3* did not have an impact on plasmid stability, as concluded from the fact that pURA3-1.1 is a very unstable plasmid (Table 2).

We wondered whether the *ars* and *stb* fragments of pFL20 are adjacent in the genome of *S. pombe* and analyzed wild-type DNA by genomic blot hybridization (Fig. 3). Wild-type DNA was restricted with various enzymes, and the filter was first hybridized with the pURA3-1.1 probe (Fig. 3A), and then the label was washed off, and the same filter was rehybridized with the pURA3-1.3 probe (Fig. 3B). The hybridization patterns for the two probes are completely different and show that the two fragments are not adjacent in the *S. pombe* genome. Instead, they were coligated during the construction of pFL20, which is easily understood from the original protocol for the isolation of pFL20 (24). In

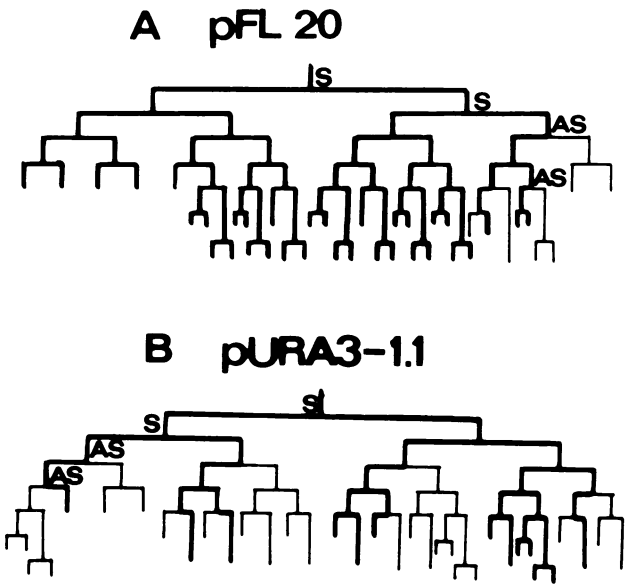

FIG. 4. Pedigree analysis of *S. pombe* cells carrying plasmids. After each mitotic division the daughter cells were separated on the agar surface and finally left to develop colonies. The colonies were checked for the presence or absence of the plasmid in the cells (see Materials and Methods). Two typical examples of actual pedigrees from pFL20 (A) and pURA3-1.1 (B) are shown. S indicates a symmetric plasmid segregation; AS shows an asymmetric segregation. The heavy contours denote the lines of plasmid inheritance.

addition to one main hybridization signal, the pURA3-1.1 probe showed two additional faint bands (Fig. 3A). This indicates that part of the 1.1-kb *ars* fragment is repetitive in the genome.

Pedigree analysis of transformed *S. pombe* cells. The measurement of mitotic stability in a population of cells (Table 2) showed a large fluctuation between various primary transformants (see the standard deviation values). Even multiple assaying of one transformant gave considerable scattering of data. Therefore, the results in Table 2 are of restricted value, and we decided to assess plasmid segregation by pedigree analysis. Two examples of pedigrees are shown in Fig. 4 for pFL20 and pURA3-1.1, which lacks the *stb* element present in pFL20. Since *S. pombe* divides symmetrically in mitosis

TABLE 3. Pedigree analysis: mitotic segregation of plasmids in *S. pombe*

Plasmid	Feature	No. of divisions analyzed[a]	No. of plasmid segregations[b]		Segregation frequency[c]	No. of post-plasmid-loss divisions
			AS	S		
pDB248	2μm	134	43	91	0.32	4
YEp13	2μm	89	40	49	0.45	5
pFL20	*ars stb*	211	27	184	0.13	2
pURA3-1.1	*ars*	53	22	31	0.42	5
pWH102	*ars stb*[d]	80	24	56	0.30	3

[a] Total number of cell divisions analyzed where plasmid segregation was observed. Data are pooled from experiments performed on MMA or YEA plus ura-leu plates (see Material and Methods).
[b] AS, Asymmetric segregation; S, symmetric segregation (see also Fig. 4).
[c] The segregation frequency is defined by the division of the number of asymmetric segregations by the total number of plasmid segregations (28).
[d] In this plasmid a short terminal fragment of *stb* is missing.

TABLE 4. Meiotic segregation pattern of URA3$^+$ or LEU2$^+$ phenotype in crosses between a transformed strain and an untransformed strain[a]

Transformed by:	No. of asci containing prototrophic:auxotrophic spores					% Prototrophic spores	Total tetrads analyzed
	4:0	3:1	2:2	1:3	0:4		
pDB248	0	0	1	4	19	6	24
YEp13	0	0	0	0	22	0	22
pFL20[b]	3	2	6	19	45	16	75
pURA3-1.1	0	0	0	1	24	1	25

[a] Crosses were performed as described in Materials and Methods. Mating type always segregated 2 h^-:2 h^+. The viability of the progeny was normal.
[b] Data are pooled from crosses against h^+ *ura4-294* (31 tetrads) and against h^+ *ura4-294 ade6-250* (44 tetrads). *ade6* is genetically linked to the centromere of chromosome III.

only asymmetric (AS) and symmetric (S) plasmid divisions are distinguished. The data in Table 3 show clearly that the stability of pFL20 is due to better symmetric segregation in mitosis and that this feature is conferred to pFL20 by the *stb* element, which is missing in pURA3-1.1. The segregation frequency of pWH102 (0.30) is intermediate between the values for pURA3-1.1 (0.42) and pFL20 (0.13). This indicates that the 200 base pairs of the *stb* element which were deleted in the construction of pWH102 may be required for its proper function. The comparison of YEp13 and pDB248 confirms the lower stability of YEp13 as determined by population analysis (Table 2).

Pedigrees performed on selective (MMA) and full (YEA plus Leu-Ura) media gave virtually identical data and were therefore pooled in Table 3. This argues against an influence of selection for the plasmid-borne marker on the segregation behavior. The pedigrees also demonstrate why not every cell grown under selective conditions contains a plasmid. From the number of cells produced after plasmid loss the number of cell divisions after plasmid loss was calculated (Table 3). It was shown by pedigree analysis on full medium that essentially all division products are viable. This ability of the cell to divide a couple of times after plasmid loss on minimal medium is not due to leakiness of the *ura4-294* and *leu1-32* mutations. Strains with these mutations were completely blocked and did not divide at all when transferred from supplemented medium to MMA. We rather believe that this ability to divide after plasmid loss reflects the persistence of the plasmid-coded gene product. The *URA3* gene product is known to be very persistent (7). The number of divisions after plasmid loss and the segregation frequency influenced the steady-state level of plasmid-containing cells under selective conditions (compare Tables 2 and 3).

Plasmid transmission in meiosis. One obvious idea regarding the unusual stability of pFL20 is that it contains a DNA fragment from a centromere region of an *S. pombe* chromosome. If there exists an analogy between *S. cerevisiae* (13) and *S. pombe*, centromere-carrying plasmids should be transmitted through meiosis and segregate in a 2:2 fashion in appropriate crosses. To test this hypothesis and to study plasmid transmission through *S. pombe* meiosis in general, we performed crosses of transformed *S. pombe* strains with the untransformed parental mutant strains. The meiotic segregation pattern of the plasmid-borne marker was followed. In such crosses only a very few spores received the plasmids (Table 4). However, pFL20 transformants had a significantly higher fraction of prototrophic spores (16%). Still, only 6 tetrads showed 2:2 segregation, of 30 tetrads with transmission of pFL20 to spores. In a cross against a

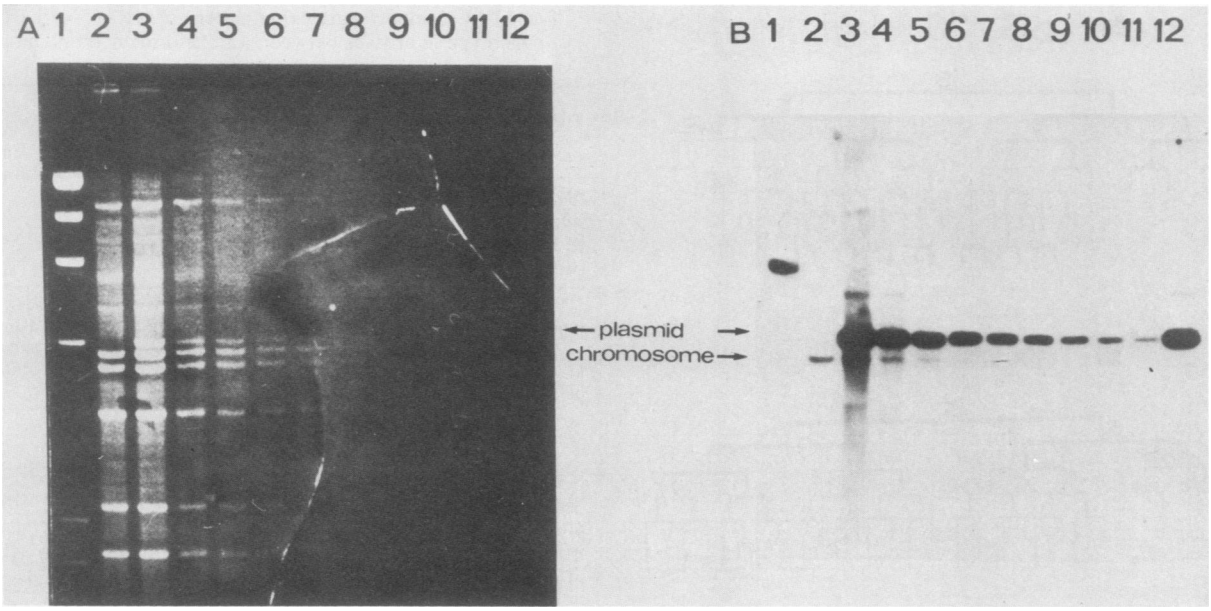

FIG. 5. Copy number estimation for plasmid pURA3-1.3 in *S. pombe*. DNA was prepared from the strain h^- *ura4-294* transformed with pURA3-1.3, digested with *Hin*dIII, and run on a 0.8% agarose gel. The DNA was transferred to a nitrocellulose filter and hybridized with nick-translated pcdc10-2. For details on the copy number calculations see Materials and Methods. (A) Ethidium bromide-stained agarose gel; (B) hybridization pattern of the probe to the DNA bound on the filter from the gel shown in panel A. Lanes: 1, size markers, *Hin*dIII-digested λ DNA; 2, DNA from untransformed *S. pombe*; 3 to 11, serial dilutions of the DNA from pURA3-1.3-transformed *S. pombe* (lane 3, undiluted; lane 4, one-third of the DNA loaded in lane 3; lane 5, one-sixth of the DNA loaded in lane 3; and so on to lane 11, 1/384th); 12, original pURA3-1.3 digested with *Hin*dIII.

strain with an *ade6* mutation, a centromere-linked marker, the two tetrads segregating 2:2 for the plasmid marker (*URA3*) and for the *ade6* marker were both tetratypes. The results indicate that the mechanism of plasmid stabilization for pFL20 is different from that for centromere-stabilized plasmids in *S. cerevisiae*.

Plasmid copy number in *S. pombe*. The copy number of plasmids in *S. pombe* was estimated from blots as shown in Fig. 5 and 6. In Fig. 5 an example for the non-*ars* plasmid pURA3-1.3 is given. The plasmid-derived band is clearly visible in the ethidium bromide-stained gel (Fig. 5A, lane 3 versus lane 2). The quantitation from the autoradiogram (Fig. 5B) yields a copy number of about 230 copies per plasmid-containing cell. In Fig. 6 an example for a low-copy-number vector, YEp13, is given. In this transformant several plasmid species coexist: the original YEp13 and two other plasmids with deletions. Only the original YEp13 could be recovered into *E. coli*. This shows that the three plasmid species did not exist as a composite in the yeast transformant. The inability to recover the rearranged plasmids may be explained with deletion of sequences essential for replication or selection in *E. coli*. The copy numbers determined for the different plasmids are given in Table 2. Several features arise from these data. (i) There is no correlation between high plasmid copy number and high stability. (ii) Non-*ars* plasmids replicate at a higher copy number than comparable *ars* plasmids. (iii) The 2μm vectors pDB248 and YEp13 replicate at a low copy number. (iv) Plasmids with the *URA3* marker tend to have a higher copy number than comparable plasmids with the *LEU2* marker. This correlates with poor expression of the *URA3* gene of pFL20 in *S. pombe* (24). In *S. cerevisiae* it has been found that plasmids replicate at higher copy number when they carry the *LEU2* gene on a special fragment resulting in poor expression of *LEU2* (12).

DISCUSSION

Behavior of autonomous replicating plasmids in *S. pombe*. The first class of vectors, the 2μm-based pDB248 and YEp13, showed characteristics similar to the ones they show in the [cir^0] strains of *S. cerevisiae* that contain no 2μm DNA (8, 21, 28). These plasmids are completely foreign to *S. pombe* in the sense that they contain no DNA from *S. pombe* and they do not cross-hybridize. Transformants were unstable due to asymmetric segregation of the plasmids to daugh-

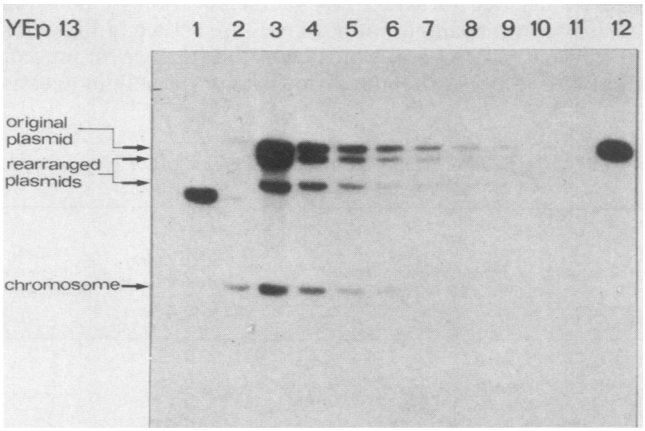

FIG. 6. Copy number estimation of plasmid YEp13 in *S. pombe*. DNA was prepared from a yeast transformant, digested with *Hin*dIII, and run on a 0.8% agarose gel. The DNA was transferred to nitrocellulose and hybridized to nick-translated pcdc10-2. The hybridization pattern of the probe to the DNA on the filter is shown. Details are given in the legend to Fig. 5. Lanes: 1, size marker; 2, untransformed control; 3 to 11, serial dilutions of DNA from transformed *S. pombe*; 12, original plasmid cleaved with *Hin*dIII.

ter cells. Transmission of 2μm vectors in *S. pombe* meiosis was very inefficient, as in *S. cerevisiae* [cir^0] strains (8). The copy number of the two plasmids in *S. pombe* was rather low (5 to 10), again as in *S. cerevisiae* (21). YEp13 was more prone to rearrangements (mostly deletions) than pDB248. Both vectors have been used successfully for the cloning of *S. pombe* genes by complementation of mutations (5, 31).

The second group of plasmids (pFL20, pURA3-1.1, and pWH102) all carry a specific *ars* element from the *S. pombe* genome. Their behavior was again similar to that of *ars* plasmids of *S. cerevisiae*. They transformed at high frequencies and were unstable, with the notable exception of pFL20 (see below). Their copy number varied between 15 and 80 as compared with 10 to 200 for different *ars* plasmids in *S. cerevisiae* (19, 39). In both yeasts only a fraction of cells grown under selective conditions contain an *ars* plasmid (19, 34, 39). A number of *ars* elements from *S. pombe* have been characterized, and only a part of them also function as *ars* in *S. cerevisiae* (25). The *ars* consensus sequence for *S. pombe* differs from that for *S. cerevisiae* (K. Maundrell, personal communication). pLEU2, pURA3, and M13 have no sequences that match this consensus sequence, consistent with their non-*ars* behavior in *S. pombe*. Most autonomously replicating plasmids assumed a partial polymeric form in *S. pombe*. Especially, pURA3-1.1 produced polymers with various numbers of repeat units. Such tandem amplification of plasmids in *S. pombe* has been studied in detail by Yamamoto and co-workers (32, 33).

The third class of autonomously replicating plasmids comprises pLEU2, pURA3, and pURA3-1.3. The former two contain no sequences that cross-hybridize with the *S. pombe* genome and are *S. cerevisiae* integration vectors (18). These vectors transformed *S. pombe* at low frequency, but the resulting transformants were almost as stable as transformants with 2μm and *ars* plasmids. They were maintained at surprisingly high copy numbers, and stable integration into the *S. pombe* genome was not observed.

From the data presented here it appears that *ars* vectors (pFL20, pURA3-1.1, pWH102) are less prone to rearrangements in *S. pombe* than 2μm and non-*ars* plasmids. For the latter two categories some transformants were obtained with two (pDB248, pLEU2) or three (YEp13) coexisting plasmid species in *S. pombe*: the original plasmid and rearranged species. From all these transformants only the original plasmid could be recovered into *E. coli* and not a copolymer of the original and the rearranged plasmid. This argues against the existence of composite plasmids in *S. pombe*, although this possibility cannot be fully excluded. Our conclusion that all the plasmids studied are replicating autonomously is based on the low stability of the transformants (exception, pFL20), the absence of simple integrations (as judged from genomic Southern blots), and the observation that the plasmids can easily be shuttled back to *E. coli*. The direct demonstration of supercoiled monomeric plasmid in undigested total DNA isolated from *S. pombe* was achieved only for the vector pWH102. Other authors have said that isolation of supercoiled plasmids from *S. pombe* is difficult (32, 38). In genomic blots of uncut DNA most plasmid hybridization was associated with the bulk of chromosomal DNA. This might be related to the frequently observed tandem amplification of plasmids in *S. pombe* (32), but our data show that plasmids can persist as monomers. Recently, a report has been published that states that plasmid rearrangements also occur in *S. cerevisiae* (10). But it seems that rearrangements (tandem duplication, deletion) are more frequent in *S. pombe*, and very similar observations were made in *Neurospora crassa* (15). We expect that introduction of additional sequences into the vectors (e.g., for cloning experiments) may alter plasmid behavior in *S. pombe*.

Role of *ars* sequences in *S. pombe*: establishment of plasmids. The identification of chromosomal DNA fragments that endow autonomous replication and high frequency of transformation to *S. cerevisiae* plasmids (34, 35) led to the concept that *ars* elements may be origins for chromosomal replication. An *ars* consensus sequence was derived for *S. cerevisiae* which is essential for *ars* function but not sufficient (22). The two functions have been assigned physically to different domains (22, 34, 27; see discussion in reference 27). One domain is responsible for high frequency of transformation, and the other is necessary for efficient autonomous replication. The situation in *S. pombe* is different. We showed that the bacterial plasmid pBR322 carrying different marker genes is able to replicate in *S. pombe* to yield high copy numbers. The transformation frequency with such plasmids was low (Table 2). Similar results have been obtained with bacteriophage M13 carrying a yeast marker gene (Nurse, personal communication). It thus seems that *S. pombe* lacks specific DNA sequence requirements for plasmid replication. The same observation has been made for *Xenopus* eggs (26).

The integration of *S. pombe ars* sequences or of the heterologous 2μm origin into the above plasmids led to high frequency of transformation and reduction of copy number in *S. pombe*. The stability of transformants was not significantly improved (Table 2). This induces us to conclude that the major role of *ars* elements in *S. pombe* lies in the establishment of plasmids in the host cell. That the uptake of plasmids into the cell only rarely leads to their prolonged maintenance has been demonstrated for *S. cerevisiae* (30). Transformation under nonselective conditions and early monitoring for plasmid presence (transient expression of a marker gene) showed that up to 4% of regenerated protoplasts had taken up the plasmid. Transformation under selection conditions yielded much lower frequencies (2%). We propose that *S. pombe ars* elements may improve this process of plasmid establishment and thus promote high frequency of transformation.

Improvement of symmetric segregation by a new element. In contrast to all other plasmids, pFL20 yields rather stable transformants. This stability increase could be attributed to the 1.3-kb *Eco*RI fragment of pFL20, which was termed *stb*. The *stb* element increases the frequency of symmetric plasmid segregation in mitosis and also improves the rate of plasmid transmission in meiosis. These properties are only visible in conjunction with the *ars* element present in pFL20, but in the *S. pombe* genome the *stb* element is not linked with the *ars* sequence in question. The *stb* element is a single-copy genomic sequence and was mapped after targeted integrative transformation (29) to the right (= longer) arm of chromosome III (17), completely unlinked to the respective centromere (data not shown).

In *S. cerevisiae* circular plasmids are stabilized by centromeric DNA (13). In comparison, the *S. pombe stb* carrying plasmid pFL20 segregated still an order of magnitude more frequently in asymmetric fashion during mitosis than the *CEN* plasmids in *S. cerevisiae* (28). Also, transmission through meiosis did not resemble the behavior of *CEN* plasmids (13). In any case, the *S. cerevisiae* assay for centromeric sequences (13) does not seem to be applicable to *S. pombe* (L. Clarke, B. R. Fishel, H. Amstutz, and J. Carbon, J. Cell. Biochem. Suppl., in press; M. Yanagida,

personal communication). Finally, the *stb* element did not reduce the copy number close to unity (as *CEN* plasmids do); it actually seemed to increase copy number as concluded from the comparison of pFL20 with pURA3-1.1 (Table 2). All these arguments together with the mapping data exclude the possibility that the *stb* sequence is part of a centromere. It is unlikely that the increased copy number of pFL20 is the cause for its high stability, because from the data in Table 2 and in *S. cerevisiae* (19, 39) a correlation between high copy number and high stability is not evident. Stability of plasmids in *E. coli* has been shown to depend on a *cis*-acting locus called *par* for partitioning (37). In the *S. cerevisiae* 2μm plasmid a very similar element, called *STB*, has been characterized (23). Both *cis* elements are located adjacent to the origins of replication of these plasmids. In contrast, the vector pFL20 is artificial for *S. pombe*, and the *stb* element is of chromosomal origin. Some preliminary data indicate that *stb* is also acting in *cis* rather than in *trans*.

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