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Influence of a Replication Enhancer on the Hierarchy of Origin Efficiencies within a Cluster of DNA Replication Origins

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DNA replication origins in animal cells sometimes occur in clusters. Often one of the multiple origins within these clusters fires more frequently than the others. The reason for this hierarchy remains unknown. Similar origin clusters occur in the fission yeast, *Schizosaccharomyces pombe*. One such cluster is located near the *ura4* gene on chromosome III and contains three origins: *ars3002*, *ars3003*, and *ars3004*. In their natural chromosomal context (*ars3003* is about 2.5 kb upstream of *ars3002* and *ars3004* is adjacent to *ars3002* on the downstream side) their initiation frequencies display a striking hierarchy: *ars3002* ⩾ *ars3003* ⩾ *ars3004*. Here, we describe experiments that reveal a 400 bp replication enhancer within *ars3004*, adjacent to *ars3002*. The enhancer is essential for *ars3004* origin function in a plasmid, but even with the enhancer *ars3004* is an inefficient origin. The enhancer is not essential for *ars3002* plasmid origin activity, but dramatically stimulates this activity, converting *ars3002* from an inefficient plasmid origin to a very efficient one. It also stimulates the plasmid origin activity of *ars3001* and *ars3003* at all tested positions and orientations on both sides of each autonomously replicating sequence (ARS) element. If *ars3002* is redefined to include the enhancer, then the relative activities of the three ARS elements as single origins within separate plasmids or as origins when all three ARS elements are present in a single plasmid is the same as the chromosomal hierarchy. Thus, this replication enhancer defines the relative activities of the three origins in the *ura4* origin region. Similar enhancers may affect relative activities in the origin clusters of animal cells.

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Introduction

Although the DNA replication origins of animal cells are not as well characterized as those of yeasts, available evidence suggests that potential initiation sites frequently occur in clusters. The best characterized cluster consists of the amplification initiation sites distributed throughout the chorion gene region of *Drosophila melanogaster*. These amplification initiation sites are controlled by multiple, partially redundant *cis*-acting sequences which are dispersed throughout the region containing the initiation sites. One *cis*-acting sequence that is particularly important for amplification, ACE3, resembles an enhancer in that it functions relatively independently of position and orientation (Orr-Weaver *et al.*, 1989). Additional *cis*-acting sequences that contribute to amplification are termed AERs. Two-dimensional gel electrophoretic analyses of replication intermediates suggest that one of the initiation sites, which co-localizes with AER-d, is used more frequently than the others (Delidakis & Kafatos, 1989; Heck & Spradling, 1990). It is not yet known whether ACE3 or any of the AERs determines the relative efficiencies of the various origins in this cluster.

Origin clusters have also been described in the fungus fly, *Sciara coprophila*, and in mammalian cells. In the fungus fly cluster, replication initiates primarily within a stretch of 1 kb, but lower frequency initiation sites can be detected in a 6 kb zone surrounding the 1 kb primary initiation site (Liang *et al.*, 1993). In human ribosomal DNA,

Abbreviations used: ARS, autonomously replicating sequences; 2D, two-dimensional.

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initiation occurs most frequently a few kilobases upstream of the transcribed region, but lower frequency initiation events can be detected through most of the non-transcribed spacer (Little *et al*., 1993; Yoon *et al*., 1995). A 55 kb zone containing multiple initiation sites is located between the *DHFR* and *2BE2121* genes in CHO cells. Within this zone, some initiation sites fire much more frequently than others (Dijkwel *et al*., 1994; Kobayashi *et al*., 1998; Kalejta *et al*., 1998). Within the smaller (8 kb) initiation zone surrounding the human β-globin gene, some sites fire more frequently than others, and all of the initiation sites appear to be coordinately regulated (Aladjem *et al*., 1998).

Thus, the phenomenon of clusters of closely spaced origins is wide-spread in animal cells. What can be learned from yeasts about the relationships between origins in such clusters? In both the budding yeast, *Saccharomyces cerevisiae*, and the fission yeast, *Schizosaccharomyces pombe*, the replication efficiency of individual origins is reduced when other origins are brought into their proximity. This is called origin interference (Brewer & Fangman, 1993; Marahrens & Stillman, 1994; Dubey *et al*., 1994), and it is probably a consequence of the fact that when any single origin in a cluster fires, replication forks from that origin can move out and replicate neighboring origins, thereby inhibiting them from firing. Another type of relationship is revealed when one of two identical origins present at different locations in the same plasmid functions more efficiently than the other. This is called origin preference (Brewer & Fangman, 1994), and it is probably a consequence of the influence of flanking plasmid sequences on the activity of nearby origins.

In *S. cerevisiae*, flanking chromosomal sequences are also capable of influencing origin activity. Chromosomal sequences that can stimulate nearby origins regardless of orientation are called replication enhancers (Walker *et al*., 1990; Raychaudhuri *et al*., 1997). In some cases, replication enhancers have proved to be binding sites for transcription factors (Walker *et al*., 1990; Marahrens & Stillman, 1992; Fox *et al*., 1993), but in one case the transcriptional activation domain of the transcription factor is not required for replication enhancement (Wiltshire *et al*., 1997). In the cases tested so far, *S. cerevisiae* replication enhancers have proved to be origin-specific. That is, an enhancer capable of stimulating one replication origin may not be capable of stimulating a different replication origin (Fox *et al*., 1993; Raychaudhuri *et al*., 1997).

In *S. pombe*, there is a natural cluster of three replication origins near the *ura4* gene on chromosome III (Zhu *et al*., 1992a, 1994; Dubey *et al*., 1994). The *cis*-acting sequences that determine these three origins are also capable of specifying origins in plasmids and are therefore called autonomously replicating sequences or ARS elements. The three origins, which are named *ars3002, ars3003* and *ars3004* in the order of their discovery, fire with different efficiencies (*ars3002* > *ars3003* > *ars3004*), and they interfere with each other so that when *ars3002* is deleted, the *ars3003*-associated origin is strengthened, and when both *ars3002* and *ars3003* are deleted, the *ars3004*-associated origin is strengthened (Dubey *et al*., 1994). The organization of the *ura4* origin region resembles that of the closely spaced replication enhancing sequences in the chorion gene in *Drosophila* described earlier. Thus the *ura4* origin region could serve as a good model for understanding compound origins (as in *Drosophila*) or even broader initiation zones (as in mammals).

To better understand the *ura4* origins and their interactions, we have characterized the three ARS elements in the context of mono, bi, and tri-ARS plasmids. Our results led to the identification of a 400 bp stretch that is essential for *ars3004* and stimulates *ars3002*. It can also stimulate other ARS elements in an orientation-independent manner. This 400 bp replication enhancer dictates the hierarchy of origin usage in the *ura4* origin region.

Results

Each ARS element from the *ura4* origin region has relatively weak origin activity when it is the only ARS element in the plasmid

Our first goal was to characterize each of the ARS elements from the *ura4* origin region as an individual ARS element, free from interactions with the other ARS elements. For that purpose, we constructed the mono-ARS plasmids illustrated in Figure 1. The three restriction fragments containing *ars3002*, *ars3003*, and *ars3004* were cloned into two different vectors, YIp5 (Struhl *et al*., 1979) and pRS306 (Sikorski & Hieter, 1989). We then compared the ARS activities of the resulting mono-ARS plasmids.

Measurement of ARS activity in *S. pombe* is not as straightforward as in *S. cerevisiae.* Transformation frequency alone is a relatively insensitive indicator of ARS activity, because even some vectors which contain an appropriate selectable marker can transform *S. pombe* cells, often with high frequency, but the transformant colonies produced by vectors are usually very small (Figure 2(b)). Plasmids containing weak ARS elements form colonies of heterogeneous size. Frequently the faster growing, bigger colonies contain rearranged forms of the plasmid, which are selected for their higher replication efficiency (Maundrell *et al*., 1985; Wright *et al*., 1986). Plasmids containing strong ARS elements generate transformants of large, uniform size that do not rearrange (Maundrell *et al*., 1988; Brun *et al*., 1995). Thus, only if colonies below a certain size are excluded from the count does transformation frequency become a useful measure of ARS activity. Measurement of mean colony size provides an alternative, complemen-

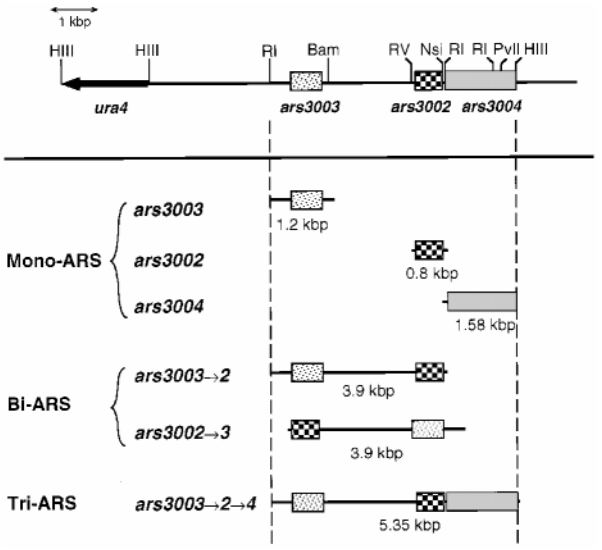

Figure 1. The *ura4* origin region and the studied restriction fragments. Top: The *ura4* origin region has three closely spaced ARS elements upstream of the *ura4* gene in chromosome III (Dubey *et al*., 1994). HIII, *Hin*dIII; RI, *Eco*RI; Bam, *Bam*HI; RV, *Eco*RV; Nsi, *Nsi*I; PvII, *Pvu*II. Bottom: For construction of mono-ARS plasmids, the 1.2 kb *Eco*RI-*Bam*HI fragment, 0.8 kb *Eco*RV-*Eco*RI fragment, and 1.58 kb *Nsi*I-*Hin*dIII fragment were chosen for *ars3003*, *ars3002*, and *ars3004*, respectively. The 3.9 kb *Eco*RI fragment containing both a*rs3003* and *ars3002* at each end was used for the bi-ARS plasmids, *ars3003* → *2* and *ars3002* → *3,* by insertion in both orientations. The intact 5.35 kb *Eco*RI-*Hin*dIII fragment was used for the tri-ARS plasmid. YIp5 and pRS306 vectors were used for construction of the mono and bi-ARS plasmids, but only YIp5 was used for the tri-ARS plasmid.

tary indication of ARS activity because, under selective conditions, colony growth rate is limited by plasmid replication efficiency (Kim & Huberman, 1998; Huberman, 1999).

As shown in Figure 2(a), all three ARS elements from the *ura4* origin region (Figure 2(a), b-h) are relatively weak, whether in YIp5 or pRS306 and whether measured by transformation frequency or colony size, compared to a previously characterized strong *S. pombe* ARS element, pYars2-2 (Figure 2(a), a; Wohlgemuth *et al*., 1994). When in YIp5, *ars3003* shows greater activity than *ars3002* or *ars3004*. All three ARS elements are more active in pRS306, in the order *ars3003* > *ars3002* > *ars3004*. This result appears to conflict with the observation that *ars3002* is the strongest origin in the chromosomal context (Dubey *et al*., 1994). An explanation for this apparent conflict will be provided below.

We have tested the possibility that a position effect may explain why the *ura4* ARS elements are weaker in YIp5 than in pRS306. We changed the insertion site for *ars3002* and *ars3003* in YIp5 from the *Bam*HI site to the *Pvu*II site on the opposite side of the vector. The resulting plasmids are called YIp5:3002opposite and YIp5:3003opposite. These plasmids displayed little or no difference in ARS activity (Figure 2(a), c and g; data not shown for YIp5:3003opposite), suggesting that the insertion position within YIp5 is insufficient to explain why *ars3002* and *ars3003* are weaker in YIp5 than in pRS306.

We also tested the possibility that the *S. cerevisiae URA3* gene, which was used as the selectable marker in both vectors, could not efficiently complement the *S. pombe ura4* gene defect when in YIp5. The 1.8 kb *Hin*dIII fragment containing the functional *ura4* gene (Grimm *et al*., 1988; Dubey *et al*., 1994) was substituted for the *URA3* gene in the YIp5 constructs containing *ars3002* and *ars3003*. Transformation results showed that neither of these could bring ARS activity up to the level of the pRS306 constructs (Figure 2(a), c and h; data not shown for YIp5:3003U4).

However, a difference between YIp5 and pRS306 was detected when they were transformed into *S. pombe* cells without ARS element insertions. The pRS306 vector produced detectable colonies whereas the YIp5 vector did not (Figure 2(b)). These results suggest that the vector effect shown above may be at least partially attributable to the ability of pRS306, but not YIp5, to replicate (albeit inefficiently) in *S. pombe* cells even when it does not contain an ARS element.

Both the pRS306 and YIp5 mono-ARS plasmids were unstable as monomers inside cells, regardless of vector. Southern blot analyses of these transformants showed that the plasmids were mostly rearranged to multimers or integrated into the chromosome (data not shown).

Multi-ARS plasmids replicate and segregate efficiently in mitotic cells

Previously our laboratory showed that the *ura4* origin region, as a whole, serves as a highly efficient chromosomal replication origin (Dubey *et al*., 1994). However, the data in Figure 2(a) show that none of the individual ARS elements from the *ura4* origin region has strong origin activity when it is the only origin in the plasmid. We suspected that the strong activity of the *ura4* origin region in the chromosome might be a result of interaction among those ARS elements. To test this possibility and to further study the relationships among these ARS elements, we constructed multi-ARS plasmids.

We chose *ars3003* and *ars3002* for construction of bi-ARS plasmids, because *ars3004* has very weak ARS activity in plasmids and origin activity in the chromosome. To maintain the same context as in the chromosome, the 3.9 kb *Eco*RI fragment (Figure 1), which contains *ars3003* at one end and *ars3002* at the other end, was inserted into YIp5 or pRS306. Constructs containing both orientations of the insert were obtained and used for transformation of *S. pombe* cells. All four constructs, YIp5:3003 → 2, YIp5:3002 → 3, pRS:3003 → 2, and pRS:3002 → 3, showed strong ARS activity, regard-

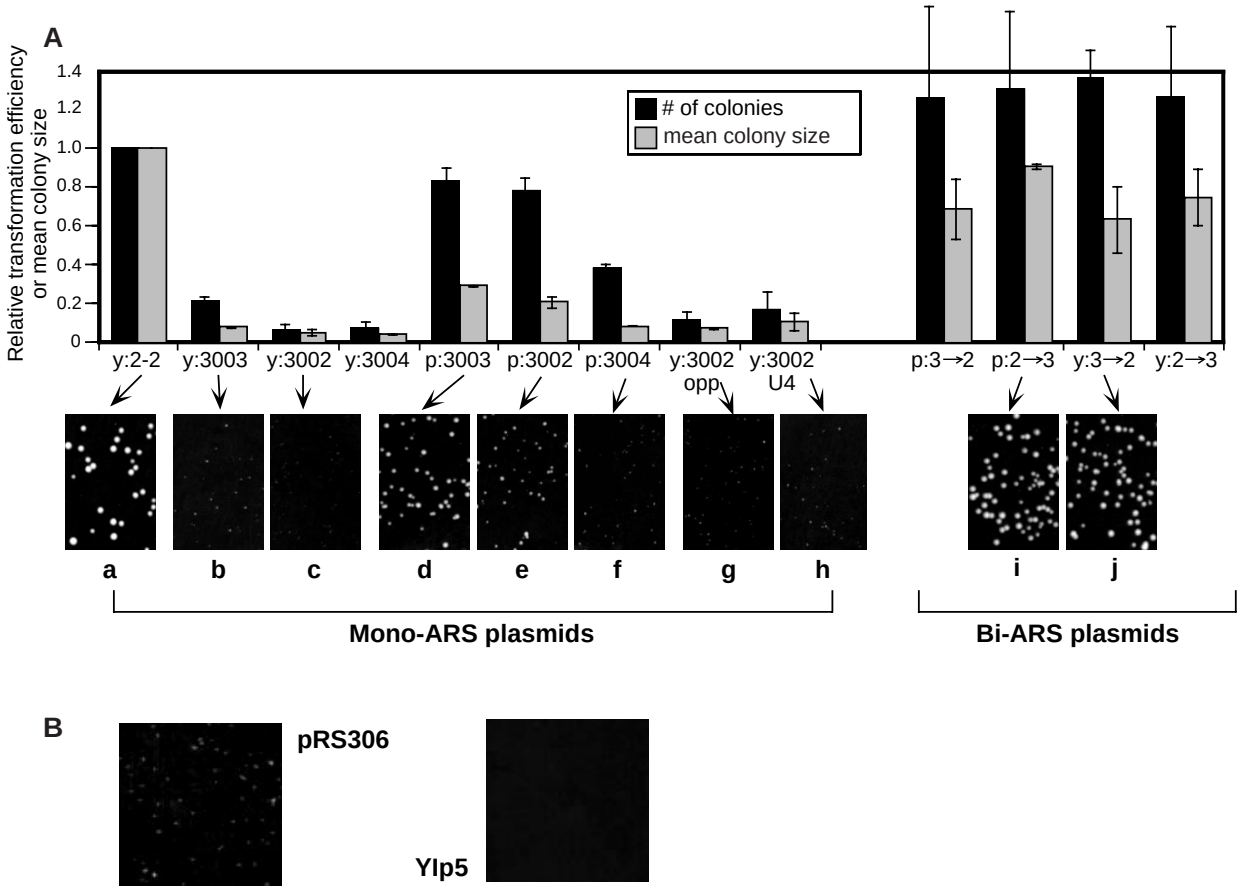

Figure 2. Activities of mono and bi-ARS plasmids. (a) Transformations with the indicated plasmids were carried out in parallel using the same batch of competent *S. pombe* cells. The results shown are an average of two independent experiments, and the error bars show the data range. All values are relative to the results obtained with YIp5: *ars2-2*, which had been characterized as a strong *S. pombe* ARS element (Wohlgemuth *et al.*, 1994). The images in the insets show portions of the Petri plates after five days at 30 °C and provide a visual impression of relative colony size and heterogeneity. y, YIp5; p, pRS306; U4, *ura4*; opp, opposite; 2-2, *ars2-2;* 3 → 2, *ars3003* → *2*; 2 → 3, *ars3002* → *3*. (b) Either pRS306 or YIp5 vector alone was transferred into *S. pombe* cells to test basal transformation frequency. Pictures were taken at the same magnification after eight days at 30 °C. Tenfold more cells were used per plate than the plates in (a) to aid in the detection of the rarer transformant colonies.

less of vector or orientation (Figure 2(a), i and j; image data not shown for pRS:3003 → 2 and YIp5:3002 → 3).

The structures of the plasmids inside transformed *S. pombe* cells were tested by Southern blot analysis. The plasmids were found to remain stable as monomers even after ⩾20 generations (Table 1). This fact allowed us to test plasmid retention in selective medium and plasmid loss rate in non-selective medium (Table 1). We also measured the average copy numbers of these plasmids inside cells. A Southern blot bearing restricted total DNA (genomic + plasmid) from each of the transformants was prepared and hybridized with a probe containing sequences common to the genomic and plasmid DNAs. The signals were quantified with a phosphorimager, and the average copy number per cell was calculated (Table 1). The low copy number, low loss rate and high plasmid retention are comparable to those of a strong ARS plasmid bearing a centromere sequence to maintain stable segregation in *S. cerevisiae* (Clarke & Carbon, 1980; Dani & Zakian, 1983). Although it is possible that some sequence elements located within the 3.9 kb fragment serve a centromere-like function, it is also possible that the symmetric division mode of *S. pombe* partially obviates the need for a centromere to ensure equal probability of segregation of daughter plasmid molecules to the two daughter cells.

In order to obtain a plasmid containing all three ARS elements of the *ura4* origin region, we ligated the 5.35 kb *Eco*RI-*Hin*dIII fragment (Figure 1) into the YIp5 vector. Despite many efforts, we never succeeded in cloning this construct in bacterial cells. Instead, *S. pombe* cells were transformed directly by the ligation mixture, and then transformants were screened by Southern blot analysis to identify colonies bearing plasmids with the anticipated tri-ARS structure. We found that the tri-ARS plasmids were stable as monomers and were maintained at low copy numbers (Table 1).

In summary, the results presented in Figure 2 and Table 1 show that each of the ARS elements

Table 1. Plasmid retention under selection, loss rate and copy number of multi-ARS plasmids

Constructs		% Cells containing plasmid[a]	% Loss per generation[b]	Average copy number per cell	Plasmid structure[c]
Bi-ARS	YIp5:3003→2	76 ± 7 ($n = 3$)	4.2 ± 0.5 ($n = 2$)	2.0 ± 0.3 ($n = 2$)	Monomers[d]
	YIp5:3002→3	67 ± 6 ($n = 3$)	1.7 ± 0.8 ($n = 2$)	3.6 ± 0.5 ($n = 2$)	
Tri-ARS	YIp5:3003 → 2 → 4	ND	ND	2.8 ± 0.2 ($n = 2$)	Monomers

n, number of experiments; ND, not determined.
[a] The percentage of plasmid-containing cells in a logarithmically growing culture in selective medium. Average values of three independent experiments are shown with standard deviation.
[b] The percentage plasmid loss per generation in non-selective medium. Average values of two independent experiments are shown with data range.
[c] Plasmid structure inside the cells checked by Southern blot analysis. Transformant cells were grown for $\geqslant$20 generations in liquid selective medium before DNA isolation for Southern blot analysis.
[d] Un-rearranged monomer plasmids.

from the *ura4* origin region is relatively weak by itself. In contrast, bi-ARS plasmids containing both *ars3003* and *ars3002* replicate efficiently and appear to segregate surprisingly well, even at low copy numbers. Thus, it appears that two or more ARS elements of the *ura4* origin region are required to obtain strong origin activity.

ars3002 and *ars3003* are used as origins with similar frequency in the bi-ARS plasmids

We employed neutral/neutral (N/N) two-dimensional (2D) gel analysis of origin efficiency (Brewer & Fangman, 1987) to compare each of the *ura4* origins in a plasmid context with the corresponding origin in the chromosomal context (Dubey *et al*., 1994). Because there is no difference in ARS activity between the bi-ARS constructs in YIp5 or in pRS306 (Figure 2(a), i and j), and more appropriately spaced restriction enzyme sites are available in YIp5, only the YIp5 constructs were analyzed by 2D gels.

S. pombe cells transformed by the mono-ARS plasmids in YIp5 grew too slowly and the plasmids were too unstable to permit their use for 2D gel analysis. In contrast, the cells transformed by the bi-ARS plasmids grew to a large colony size, and their doubling time in liquid minimal medium (~three hours/generation) was similar to that of wild-type cells. To check if the DNA preparations used for 2D gel analysis contained primarily monomeric plasmid, Southern blotting was used to test each DNA preparation before 2D gel electrophoresis. In every case, monomeric plasmid structure was confirmed.

The two bi-ARS constructs, YIp5:3003 → 2 and YIp5:3002 → 3, have the same insert, the 3.9 kb *Eco*RI fragment (Figure 1), but in opposite orientations. For each construct, two restriction fragments, centered on either *ars3002* or *ars3003,* were subjected to N/N 2D gel analysis. Upon probing with plasmid sequences within the fragments (to prevent potentially confusing signals from the chromosomal fragments), a full bubble arc (due to origin firing; see the example in Figure 3(b)) and a complete Y arc (due to passive replication of the restriction fragment by forks from the other origin; see the example in Figure 3(b)) were detected from both fragments in both plasmids (Figure 3).

Judging by the bubble to Y ratios, both origins appeared similarly efficient. However, the data in Figure 3 suggest that the ARS element located on the right side of the vector (Figure 3(b) and (d)), nearer to the selectable marker, fires at slightly higher frequency (higher bubble to Y ratio) for both orientations. This slightly different origin usage frequency between the right and left side of the plasmid is obviously caused not by the difference between the two ARS elements but by a plasmid context effect. We conclude that there is no clear origin preference between *ars3002* and *ars3003* in the bi-ARS plasmids.

Simultaneous firing of both ARS elements in a bi-ARS plasmid is not detectable

In the fragments studied in Figure 3(a) and (c), we also detected faint termination signals (arrows in Figure 3(a) and (c)), which come from double Y-shaped molecules containing converging replication forks (Brewer & Fangman, 1987; Zhu *et al*., 1992b). These termination signals could be explained in either of two ways. First, it is possible that initiation could occur at both ARS elements in the same molecule. In this case, double Y replication intermediates would be produced from the regions between the two ARS elements. Alternatively, initiation may take place in only one of the two ARS elements. In this case, termination signals should be detected only in the larger region between ARS elements. The fact that the other analyzed fragments (Figure 3(b) and (d)) do not display clear termination signals tends to argue against the first possibility, because all of the fragments studied in Figure 3 share the smaller region between ARS elements. To answer this question more clearly, the 6.25 kb *Pst*I fragment centered on the shorter region between ARS elements was subjected to 2D gel analysis. These large *Pst*I fragments from both bi-ARS plasmids yielded weak bubble and strong late Y arcs, but no detectable termination signals (Figure 4(a) and (b)). This result clearly indicates that in most or all cases only one ARS element fires in each cell cycle, excluding the first possibility.

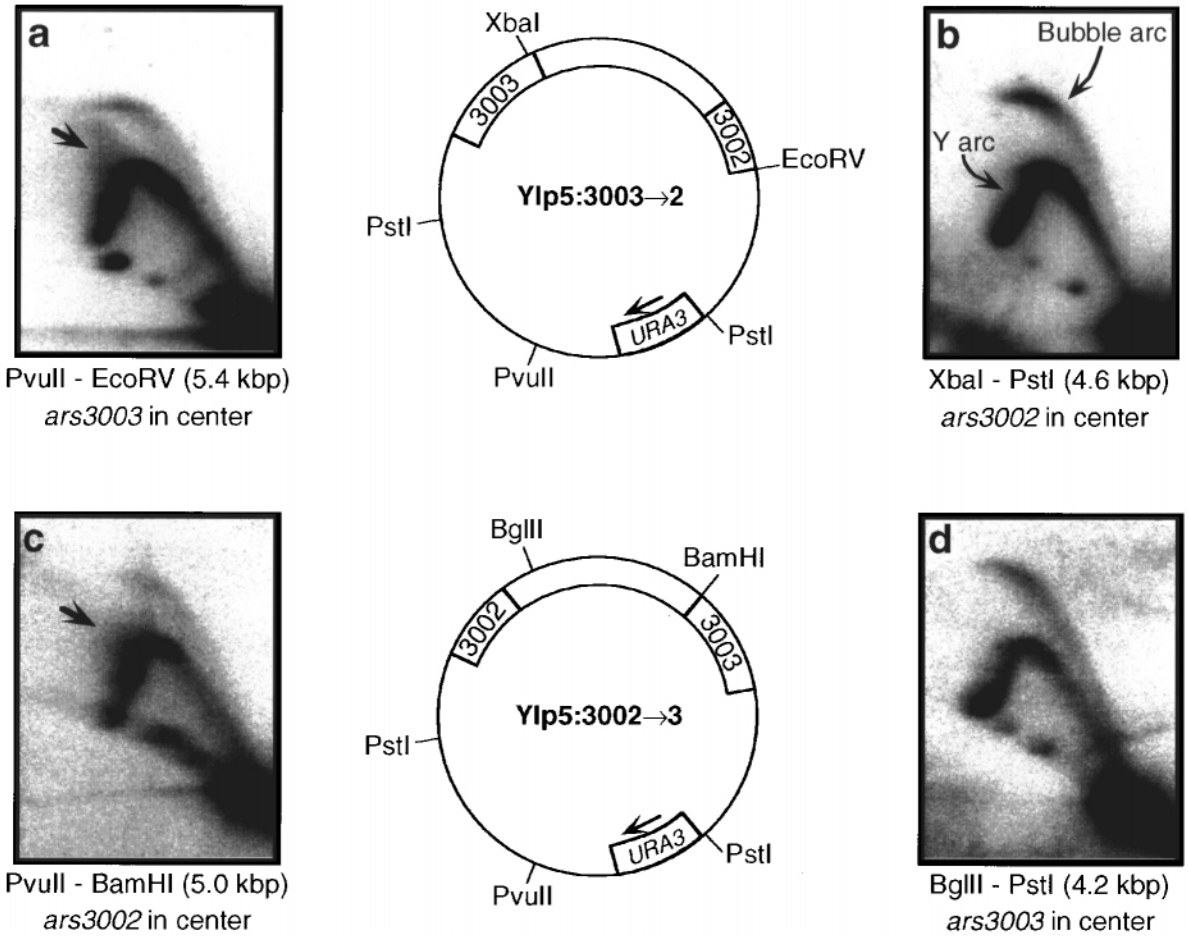

Figure 3. Similar origin function of *ars3002* and *ars3003* in bi-ARS plasmids. For each construct, YIp5:3003 → 2 or YIp5:3002 → 3, two restriction fragments were analyzed by neutral/neutral 2D gel analysis. Each fragment contains *ars3003* or *ars3002* in its center, as indicated. 3003, *ars3003*; 3002, *ars3002*.

The thickness of the late Y signal was reproducible. Two factors may contribute to this thickness. First, heavier signal in the later portion of the Y arc is expected for a fragment in which an origin is located asymmetrically (Brewer & Fangman, 1987). Second, the signal in the later portion of the Y arc may be enhanced due to possible slow movement of the replication fork emanating from the active origin when it encounters the complex of proteins bound to the inactive origin.

Termination occurs primarily in a broad region about 180° away from the initiation region

To test the second possibility, that termination events are concentrated in the larger region between ARS elements, the short, 3.15 kb *Pst*I fragments were also analyzed by 2D gel electrophoresis (Figure 4(c) and (d)). In addition to the Y arcs, fainter signals suggestive of termination at many positions throughout these 3.15 kb fragments were evident (Zhu *et al.*, 1992a). This result, therefore, suggests that the relatively faint termination signals detected in Figure 3(a) and (c) originate from the 1.5 kb region between the *Pst*I and *Pvu*II sites, which is a part of the termination region.

The chromosomal hierarchy of origin function is reproduced in the tri-ARS plasmid

As reported earlier, there is a clear hierarchy of initiation frequency among the three ARS elements at the *ura4* origin region in the chromosome (*ars3002* > *ars3003* > *ars3004*; Dubey *et al.*, 1994). It is striking that in contrast there is no distinguishable origin preference between *ars3002* and *ars3003* in the bi-ARS plasmids. We attempted to find the cause of this difference between plasmid and chromosomal replication. First, we examined the possibility that the vector sequence may have some effect on the bi-ARS construct, such as changing the free energy required for unwinding near *ars3003* so that *ars3003* could be used more easily than in the chromosome. To test this possibility, the free energy for unwinding of the bi-ARS plasmids was calculated by the THERMODYN program (window sizes = 300, 600, and 900, data not shown; Natale *et al.*, 1992). We found that in either orientation the vector sequence does not influence the ease of unwinding of either *ars3002* or *ars3003* (data not shown). Thus, it seems unlikely that this is the case.

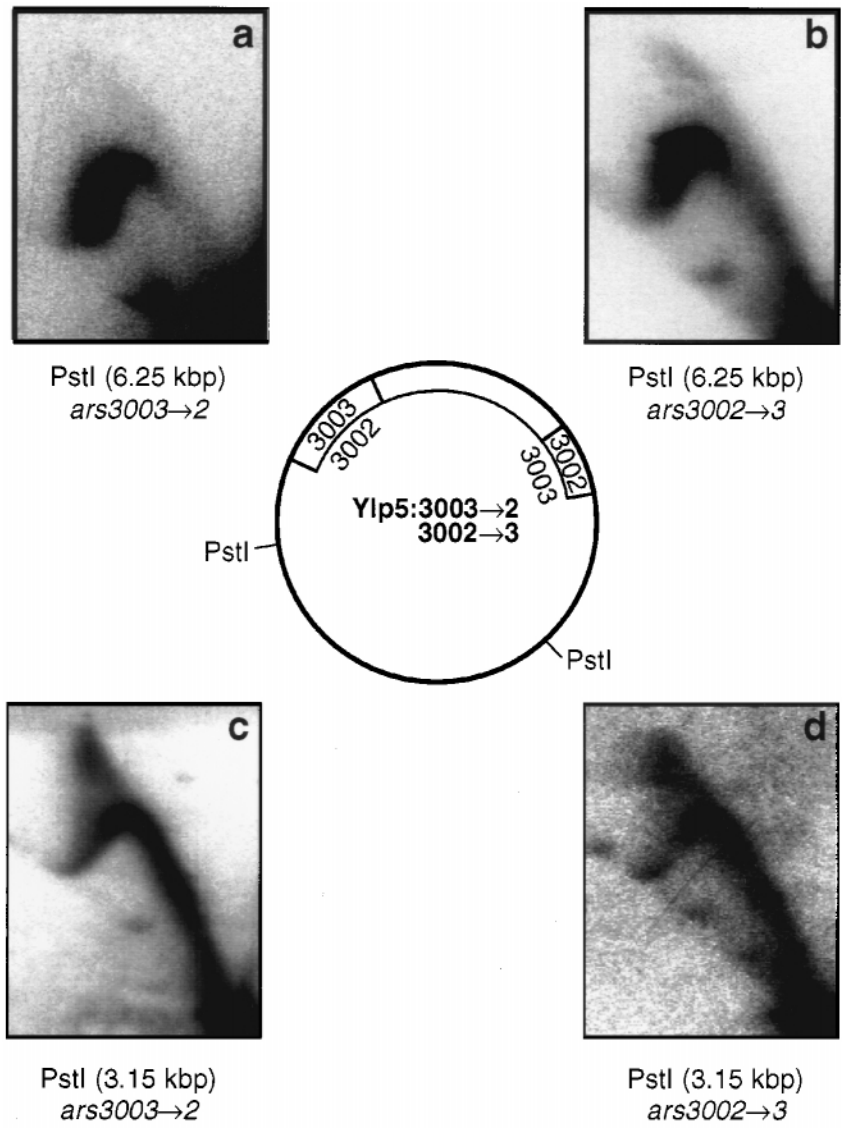

Figure 4. Both ARS elements do not fire in the same plasmid, and termination occurs across from the ARS elements. See the legend to Figure 3 for details.

Secondly, we considered the possibility that there may be some elements in the flanking chromosomal region containing *ars3004* which activate *ars3002* function, but which are missing in the bi-ARS construct. To test this possibility, we used 2D gel electrophoresis to analyze a tri-ARS plasmid which contains a restriction fragment of chromosomal DNA with all three ARS elements of the *ura4* origin region (Figure 1, 5.35 kb *Eco*RI-*Hin*dIII). As shown in Figure 5(b), a fragment of the tri-ARS plasmid containing *ars3002* in its center generated a strong bubble signal. A fragment with *ars3003* in its center (Figure 5(a)) showed a weak bubble arc and a strong Y arc, and no detectable bubble signal was produced by a fragment containing *ars3004* in its center (Figure 5(c)). Thus, the origins in the tri-ARS plasmid fire with a hierarchy of frequencies, *ars3002* > *ars3003* > *ars3004*, similar to that of the *ura4* origin region in the chromosome (Dubey *et al*., 1994). Thus, a sequence or sequences within the 1.45 kb region containing *ars3004* appears important for stimulating the origin activity of *ars3002*.

A DNA stretch of at least 1 kb is required for minimal *ars3004* activity

The next question was, what is responsible for the stimulation of *ars3002* activity in the tri-ARS plasmid? Is it *ars3004* itself or a special sequence element which is located within the 1.45 kb fragment? Since *ars3004* had been only roughly defined as the 1.45 kb *Eco*RI-*Hin*dIII fragment (Figure 1; Dubey *et al*., 1994), and not sequenced, it was necessary to sequence the fragment and localize *ars3004* more precisely within it. The nucleotide sequence (GenBank accession number: AF040271) proved to be somewhat A + T-rich (66 % compared to 69-75 % for other *S. pombe* ARS elements; Maundrell *et al*., 1988). We searched the sequence for close matches to the *S. pombe* ARS element consensus motifs and critical sequences previously detected by others (Maundrell *et al*., 1988; Clyne &

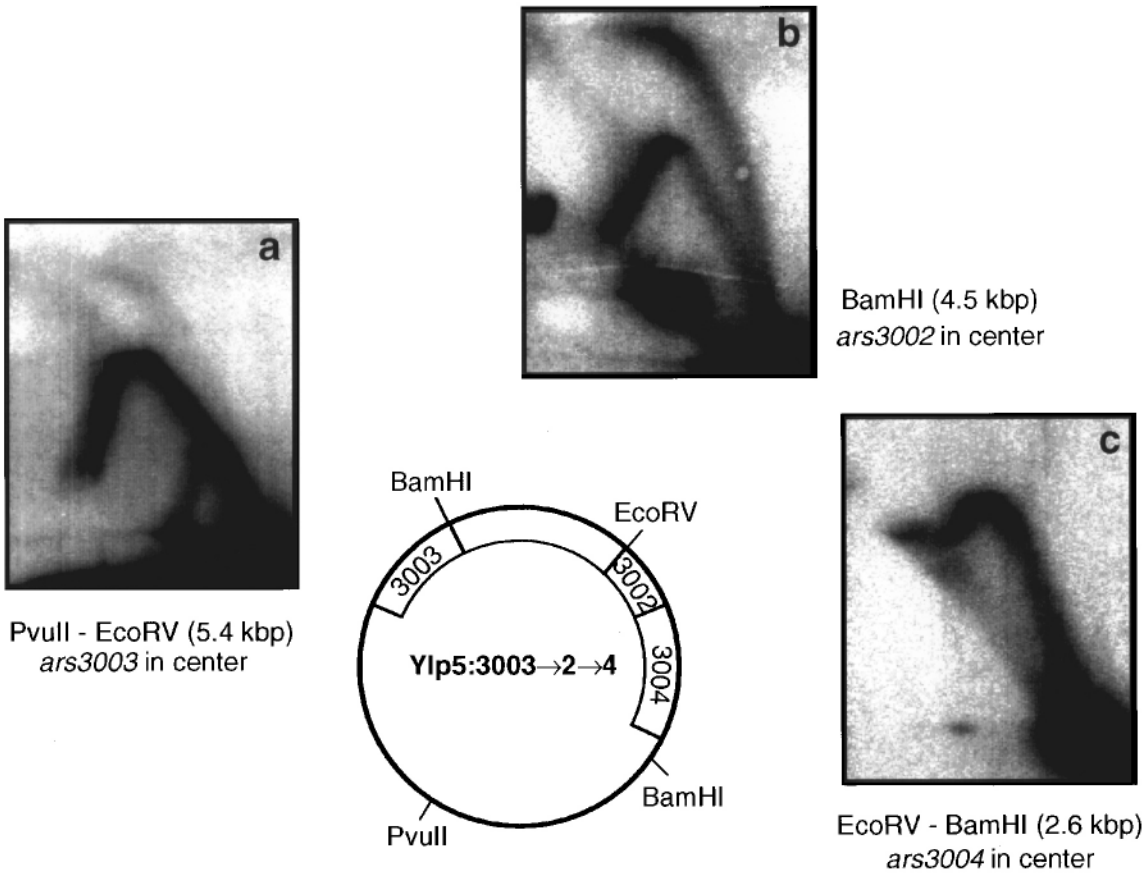

Figure 5. The chromosomal hierarchy of origin function is reproduced in the tri-ARS plasmid. The complete 5.35 kb *Eco*RI-*Hind*III fragment (see Figure 1) was inserted into the YIp5 vector and cloned in *S. pombe* cells. Three fragments, centered on (a) *ars3003*, (b) *ars3002*, and (c) *ars3004*, were studied by neutral/neutral 2D gel analysis. The DNAs for the 5.4 kb *Pvu*II-*Eco*RV and 4.5 kb *Bam*HI fragments were prepared without BND-cellulose enrichment.

Kelly, 1995) and by our laboratory (Zhu *et al*., 1994; Dubey *et al*., 1996; Kim & Huberman, 1998). Some matches were found, mostly concentrated within the 1 kb *Eco*RI-*Eco*RI fragment (Figure 6, top), but none of the matches was perfect. Based on the sequence search, we constructed a series of deletions, either from one side or from both sides of the fragment. As shown in Figure 6 (bottom), most of the transformation activity of the 1.58 kb *Nsi*I-*Hin*dIII fragment is maintained within the 1 kb *Eco*RI fragment, but the size of colonies is reduced. None of the further deletions, even a deletion as small as 100 bp from the right side of the *Eco*RI fragment (Figure 6(h)), showed ARS activity comparable to that of the 1.58 kb *Nsi*I-*Hin*dIII or 1 kb *Eco*RI fragments. These results suggest that a fragment of at least 1 kb is required for significant *ars3004* activity.

The 400 bp *Eco*RI-*Hin*cII fragment harbors the *ars3002*-stimulating effect

Since an additional ARS element can influence the activity of a pre-existing ARS element (Figures 2-4), it was necessary to determine whether the *ars3002*-stimulating effect requires functional *ars3004* or is the result of a portion of *ars3004* that lacks ARS activity by itself. For this purpose, we compared the stimulatory effects on *ars3002* of the 1.15 kb *Eco*RI-*Pvu*II fragment including the 1 kb *Eco*RI fragment (which has ARS activity; Figure 6(c)) and the 400 bp *Eco*RI-*Hin*cII fragment. These were placed at the right side of *ars3002*, as in the natural context. As shown in Figure 7, addition of the 1.15 kb fragment to *ars3002* greatly increased ARS activity (compare (a) with (b)). Interestingly, the 400 bp fragment, which does not have ARS activity by itself (Figure 6(f)), also stimulated *ars3002* (Figure 7(c)). Since the 400 bp DNA stretch is A + T-rich (72 %), we then tested the possibility that the stimulating effect is simply a result of its A + T-richness. To do this, we chose a 400 bp DNA stretch from the region downstream of the *S. cerevisiae GAL7* gene that is also 72 % A + T-rich as a control. We could not detect any stimulation by this control fragment (Figure 7(d)). These results suggest that the *ars3004* stimulatory effect is not simply the consequence of addition of one more ARS element and is not simply due to A + T-richness but requires a specific sequence element(s) within the 400 bp *Eco*RI-*Hin*cII fragment.

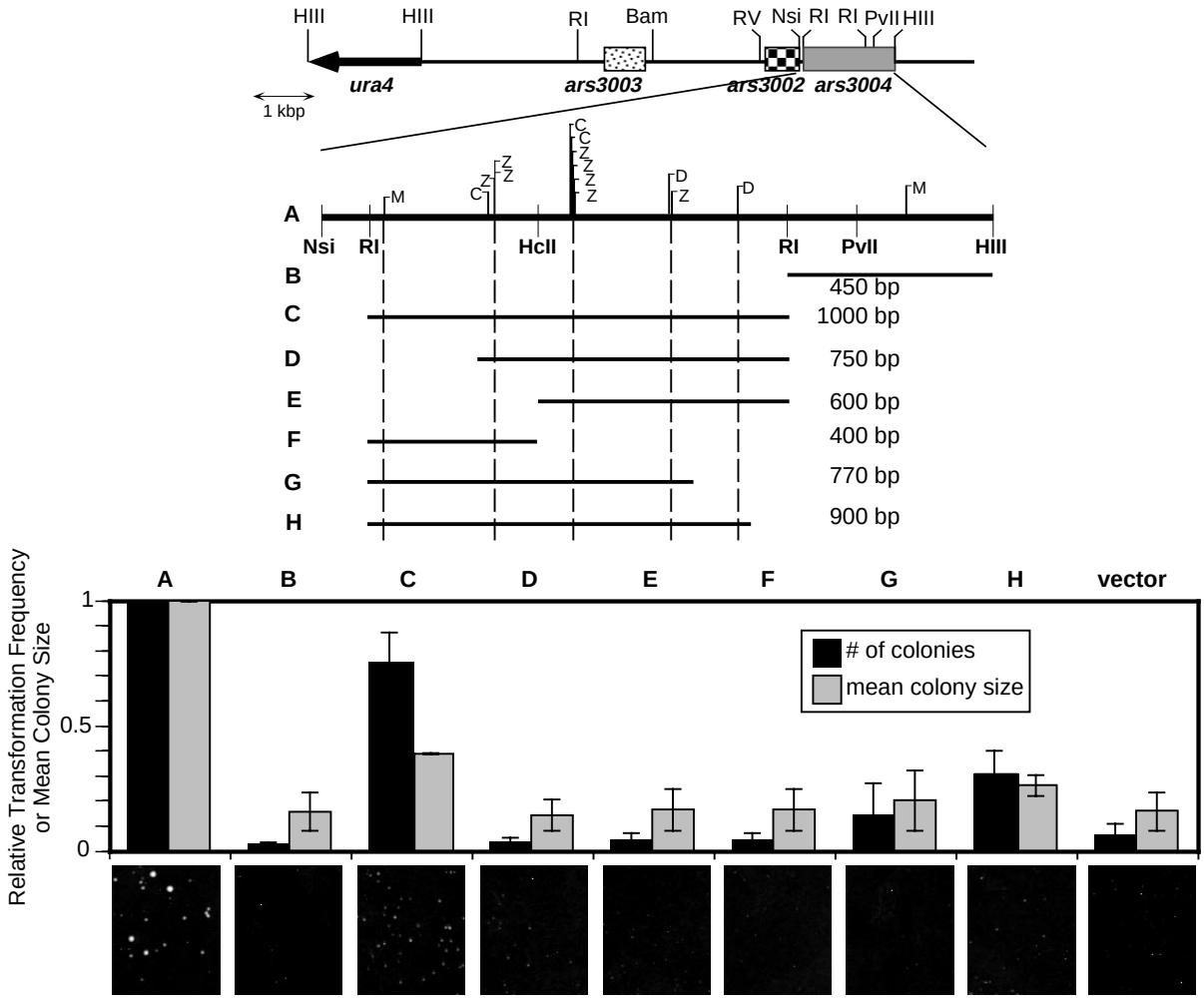

Figure 6. Localization of *ars3004*. The 1.58 kb *Nsi*I-*Hin*dIII fragment, defined previously as *ars3004* (Dubey *et al*., 1994), is shown. The positions of close matches to previously proposed *S. pombe* ARS consensus motifs are labeled M, Z, C, or D; M, WRTTTATTTAW, W = A/T, R = A/G (Maundrell *et al*., 1988); Z, WWTTWTWTTWTT (Zhu *et al*., 1994); C, TTGTATTTTAATTTGTATTTTTTGTAATTT (Clyne & Kelly, 1995); and D, WTWTWTTTYTTTTTWTTTTA, Y = T/C (Dubey *et al*., 1996; Kim & Huberman, 1998). One mismatch was allowed for M and Z, ten for C, and four mismatches were allowed for D. Based on the search, different external deletions (top) were made to help define a boundary for *ars3004* and were tested for ARS activity (bottom). All values are relative to the results obtained with the 1.58 kb *Nsi*I-*Hin*dIII *ars3004*. Images of colonies are all at the same magnification. HIII, *Hin*dIII; RI, *Eco*RI; Bam, *Bam*HI; RV, *Eco*RV; Nsi, *Nsi*I; PvII, *Pvu*II; HcII, *Hinc*II.

The 400 bp DNA stretch stimulates other ARS elements

Since the 400 bp sequence greatly stimulates but is not an essential part of *ars3002* (see Figure 3(b) and (c); replication initiation occurs at *ars3002* without the 400 bp stretch), we suspected that this sequence may function as a replication enhancer. To test whether the 400 bp stretch can stimulate other ARS elements in an orientation-independent manner, we cloned it adjacent to two other ARS elements, *ars3003* (which is one of the ARS elements in the *ura4* origin region) and *ars3001* (which is located in the non-transcribed spacer region in the ribosomal DNA repeat; Sanchez *et al*., 1998; Kim & Huberman, 1998). The transformation results (Figure 8) indicate that the 400 bp stretch stimulates the ARS activity of both *ars3003* and *ars3001* whether it is placed upstream or downstream of the ARS element and regardless of orientation, and it affects *ars3003* even when placed about 600 bp upstream of the ARS element. Although stimulation of *ars3003* was somewhat position-dependent (Figure 8; 3003La,b > 3003Ra,b), these results suggest that the 400 bp DNA stretch is an enhancer for *S. pombe* ARS elements.

Revision of the boundaries of *ars3002* to include the 400 bp stretch leads to a hierarchy of ARS activities consistent with chromosomal origin activities in the *ura4* origin region

The transformation results presented in Figure 9 show that, when the boundaries of *ars3002* are broadened to include the 400 bp enhancer, a hierarchy of ARS activities consistent with the results of 2D gel analyses in the chromosome (Dubey *et al*.,

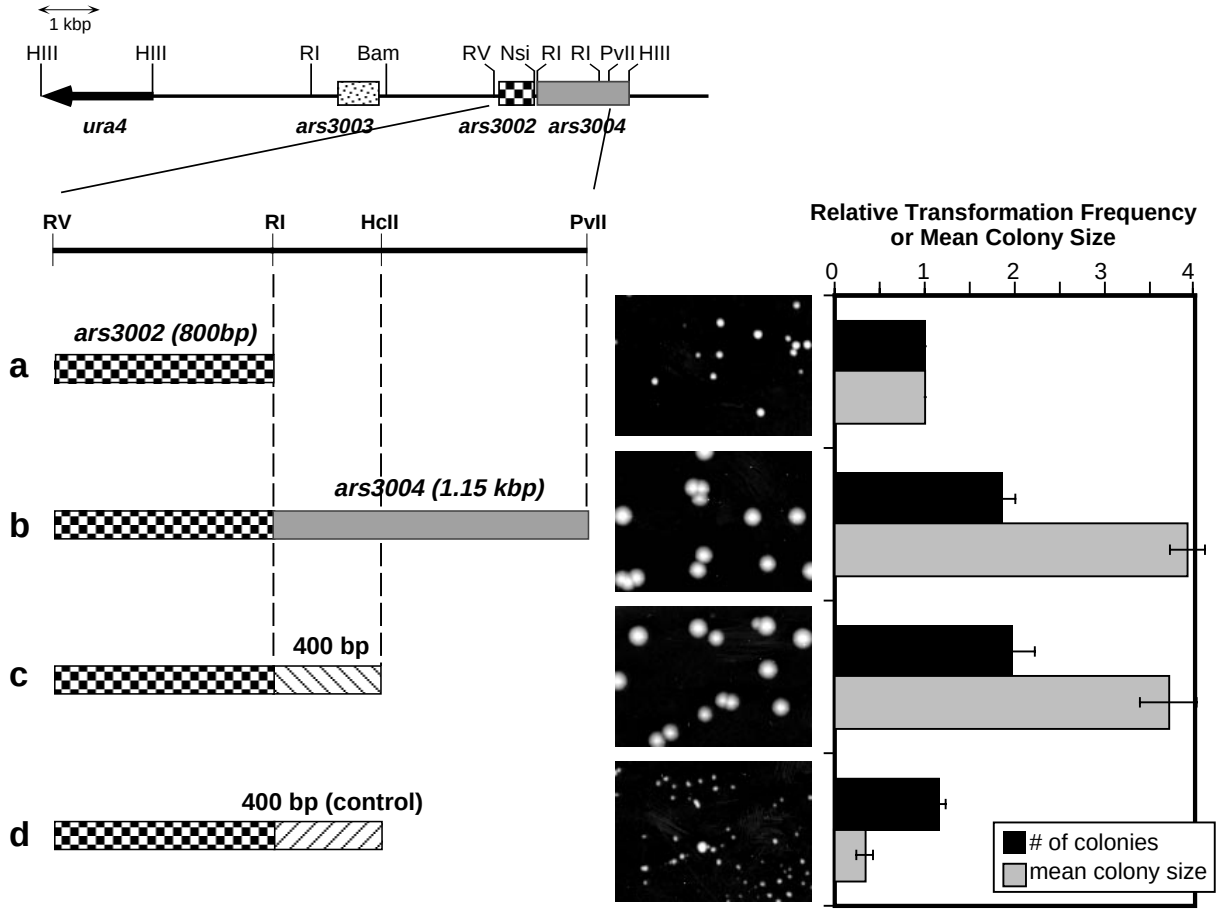

Figure 7. Localization of an *ars3002*-stimulating sequence within *ars3004*. The stimulatory effect on *ars3002* activity of the indicated restriction fragments was tested by transformation frequency and colony size measurement. All values are relative to those of *ars3002*. The stimulatory effect of the 1.15 kb fragment is equaled by that of the 400 bp fragment. A 400 bp control (72 % A + T) fragment from the *S. cerevisiae* gal7 gene was also tested. Images of colonies are at the same magnification.

1994) and in the tri-ARS plasmid (Figure 5) is obtained. The revised *ars3002* extends for 1.2 kb between the *Eco*RV and *Hin*cII sites. Since the same 400 bp stretch is essential for *ars3004*, the boundaries of *ars3002* and *ars3004* overlap by 400 bp.

Discussion

We have characterized the replication activity of plasmids containing one, two, or all three of the ARS elements of the *ura4* origin region in *S. pombe*. When it was the only ARS element in the plasmid, each of these ARS elements was weak compared to other known *S. pombe* ARS elements (Figure 2(a), a-h and data not shown for another strong ARS element, pYars2-1 (previously pYAM6); Caddle & Calos, 1994). The activity of the monomeric ARS elements was dependent on the vector environment. pRS306 offered better support for the weak ARS elements than did YIp5.

However, the combination of *ars3002* and *ars3003* in bi-ARS plasmids produced strong ARS activity and overcame the vector effect. Bi-ARS plasmids replicated and segregated efficiently and were maintained as stable monomers inside cells (Figure 2 and Table 1). Similar enhancement of ARS activity by combination of weak ARS elements has also been shown in other studies. In *S. cerevisiae*, a DNA sequence that contains a few mismatches to the *S. cerevisiae* ARS consensus sequence and has no ARS activity achieves strong ARS activity when simply reiterated 11 times (Fangman *et al*., 1989). In mutant cells with defects in initiation of DNA replication, the loss rate of plasmids containing multiple origins is significantly reduced (Hogan & Koshland, 1992). In *S. pombe,* a plasmid, pDblet (Brun *et al*., 1995), achieved strong ARS activity by duplication of a 600 bp sequence from *ars3002*. Thus it may be that simple multiplication of a weak ARS element is sufficient to promote origin function. This idea is also supported by the fact that the weak mono-ARS plasmids in this study tended to become multimerized inside cells when plasmid structure was studied by Southern blotting (data not shown). Even though the bi-ARS plasmids described here are not duplications of a single ARS element, it seems likely that similar enhancement of ARS activity could be generated by placing two non-identical ARS elements into the same plasmid.

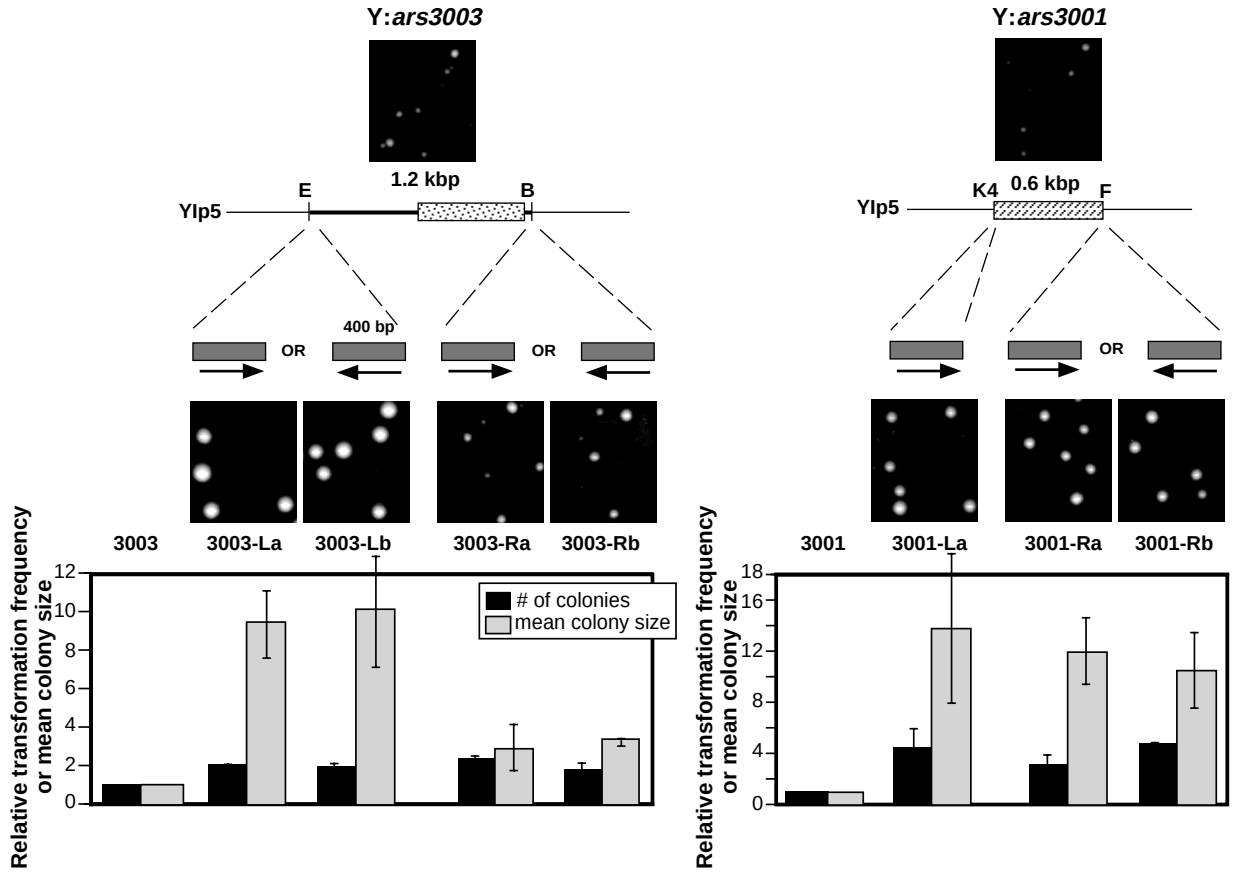

Figure 8. The 400 bp fragment stimulates other ARS elements in an orientation-independent manner. To test the effects of the 400 bp fragment on other ARS elements, the 400 bp fragment (gray box) was moved to the left or right side of *ars3003* or *ars3001* in both orientations (only one orientation could be cloned for the left side of *ars3001*). The 1.2 kb *ars3003*-containing *Eco*RI-*Bam*HI fragment is from the *ura4* origin region (see Figure 1), and the 573 bp *ars3001* fragment, defined by exonuclease III deletion point K4 and a *Fok*I restriction site, is the ARS element in the rRNA gene repeat (Kim & Huberman, 1998). The thick line indicates DNA sequences from the *ura4* origin region, and the thin line indicates vector sequences. The orientations of the fragment insertions are marked by arrows. The measurements of ARS activity (transformation frequency and colony size) were relative to those of *ars3003* or *ars3001.* Colonies were imaged at the same magnification in the upper (before stimulation) and lower (after stimulation) panels. Y, YIp5; E, *Eco*RI; B, *Bam*HI; F, *Fok*I; K4, an exonuclease III deletion end point (Kim & Huberman, 1998).

Additional information was obtained when 2D gel analysis was applied to the bi-ARS plasmids. First we found a slight dependence of origin efficiency on ARS position (relative bubble to Y ratio of Figure 3(a) and (c) less than that of Figure 3(b) and (d)). This type of origin bias has been observed in *S. cerevisiae* by Brewer & Fangman (1994). They studied two copies of *ARS1* in the same plasmid and found that the ARS element closest to the *URA3* gene initiated more frequently. The 3′ two-thirds of the *URA3* gene was identified as responsible for the origin bias. Although our 2D gel results also showed that the ARS element located closest to the *URA3* gene had slightly better initiation frequency, the relative orientation and distance of the ARS elements to the *URA3* gene in our study were different from their study. Thus further investigation is required to find out if the sequence element within the *URA3* gene that affects *S. cerevisiae* ARS elements also affects *S. pombe* ARS elements. Since we detected origin bias in both orientations (that is, *ars3002* > *ars3003* in Yip5:3003 → 2, and *ars3003* > *ars3002* in Yip5:3002 → 3), this bias is obviously not dependent on the ARS element *per se*. These bi-ARS results are consistent with the fact that the mono-ARS activities of *ars3003* and *ars3002* were not very different from each other (see below).

We could not detect any termination signal from the small region between the two ARS elements in the bi-ARS plasmid, indicating the absence of simultaneous initiation (Figure 4(a)). Thus it appears that in the bi-ARS context, only one ARS element is selected as an origin in each cell cycle, and initiation at the other origin is suppressed by ''origin interference''. Origin interference between closely located ARS elements has previously been reported for *S. cerevisiae* ARS elements, both in the plasmid (Brewer & Fangman, 1994) and in the chromosomal context (Brewer & Fangman, 1993; Marahrens & Stillman, 1994), and for *S. pombe* ARS elements in their natural chromosomal

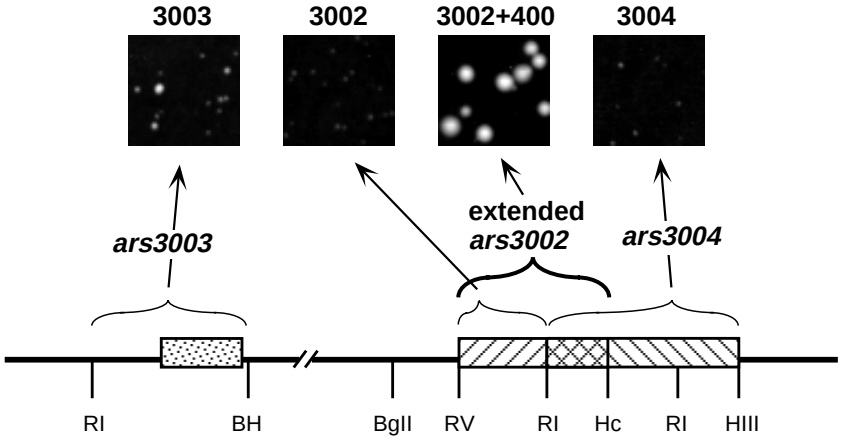

Figure 9. Comparison of the ARS activities of the three ARS elements of the *ura4* origin region. Top: Portions of Petri plates (same magnification). The indicated ARS elements were cloned into YIp5. Note that the plasmids containing *ars3002* (short form), *ars3003* and *ars3004* are the same as used in Figure 2. The extended *ars3002* (3002 + 400) was cloned between the *Eco*RI and *Nru*I sites of YIp5 and is the same construct used in Figure 7. See Materials and Methods for cloning details. Bottom: Diagram of the *ura4* origin region showing the previously defined ARS elements (thin braces) along with the re-evaluated *ars3002* (thick brace). RI, *Eco*RI; Bam, *Bam*HI; BgII, *Bgl*II; RV, *Eco*RV; Hc, *Hinc*II; HIII, *Hin*dIII.

location (Dubey *et al*., 1994). Our study shows that interference also occurs between two different *S. pombe* ARS elements in a plasmid context.

It was striking that *ars3002* and *ars3003* showed similar initiation frequencies in the bi-ARS plasmids, because *ars3002* is much more active than *ars3003* in the chromosome (Dubey *et al*., 1994). This apparent contradiction led us to identify an *ars3002*-stimulating element that had not been apparent from previous studies of ARS activities and the effects of chromosomal deletions (Dubey *et al*., 1994). We found that the 400 bp *Eco*RI-*Hinc*II fragment adjacent to *ars3002* greatly stimulates *ars3002* in a mono-ARS plasmid (Figures 7(c) and 9) and is important for reproducing the chromosomal hierarchy of origin function in a tri-ARS plasmid (Figures 5 and 9), yet is not an essential part of *ars3002* (Figure 3(b) and (c)). In addition, the 400 bp stretch stimulates the activity of other ARS elements such as *ars3003* (one of the ARS elements within the *ura4* origin region) and *ars3001,* which is located outside of the *ura4* origin region. The stimulatory effects are relatively orientation-independent and are detectable even when the 400 bp stretch is ~600 bp distant from the essential part of *ars3003* (Figure 8). These properties are those expected of a replication enhancer (Walker *et al*., 1990). Therefore the 400 bp DNA stretch appears to be the first replication enhancer found in *S. pombe*.

The properties of this *S. pombe* replication enhancer appear somewhat different from those of the known *S. cerevisiae* replication enhancers. Each of the studied *S. cerevisiae* replication enhancers stimulates only a small subset of tested replication origins (Fox *et al*., 1993; Raychaudhuri *et al*., 1997). In contrast, the 400 bp *S. pombe* replication enhancer stimulates all three of the tested origins.

How does this *S. pombe* replication enhancer influence origin activity? It may serve as binding site(s) for proteins which somehow activate a nearby origin. It might be a binding site for a transcription factor, such as OBF1/ABF1 in *S. cerevisiae*. The mechanisms by which transcription factors stimulate replication in *S. cerevisiae* are not yet known, but appear to be different at different origins (Wiltshire *et al*., 1997). One possibility is that a transcription factor (or other enhancer-binding protein) may interact directly with a major component of the replication apparatus, such as the origin recognition complex (ORC; Bell & Stillman, 1992). Alternatively, an enhancer-binding protein may influence local chromosome structure in a way favorable for origin function. An enhancer could also stimulate replication by virtue of some protein-independent property of its nucleotide sequence, such as ease of unwinding. The enhancing effect might also be due simply to reiteration of sequences similar to those required by origins, as described earlier. It is obvious that some specific sequence stretch, not just A + T-rich sequences, is important for enhancing replication (Figure 7). What those specific sequences might be is not evident from the primary sequence of the 400 bp stretch (GenBank sequence AF040271). Although some of the A + T-rich asymmetric motifs previously detected within important regions of *S. pombe* ARS elements (Kim & Huberman, 1998) are located within the 400 bp stretch, there are no clusters of three or more such motifs in the same orientation (Figure 6, top, line A). Our previous study (Kim & Huberman, 1998) suggested that unoriented or smaller clusters are unlikely to contribute to ARS activity. High-resolution mapping of the sequences within the 400 bp stretch required for enhancer activity will help to distinguish among these possibilities.

The results presented here will help to resolve a long-standing problem in the interpretation of ARS activity measurements in *S. pombe*. Because *S. pombe* centromeres are very large (Takahashi *et al*., 1992) and possess ARS activity of their own (Smith *et al*., 1995), it is not possible to employ centromere-containing plasmids for measurement of ARS activity. The absence of centromeres in the plasmids used for ARS activity measurements in *S. pombe* leaves open the possibility that variations in ARS activity may be due to variations in segregation efficiency, instead of or in addition to variations in replication efficiency. The results presented here show that for three *S. pombe* ARS elements (*ars3003, ars3004,* and the extended *ars3002*; Figure 9), there is a correlation between relative ARS activity measurements (Figure 9) and relative replication efficiencies, as judged by 2D gel electrophoresis (Figure 5; see also Dubey *et al*., 1994). This correlation suggests that standard ARS activity measurements in *S. pombe* reflect primarily replication, not segregation, efficiency.

The studies presented here provide us with an improved picture of the structure of a compound origin region containing three origins, two of which overlap (Figure 9). The overlapping origin region has at least two resolvable initiation sites: the preferred one at *ars3002* and the minor one at *ars3004* (Dubey *et al*., 1994). The 400 bp enhancer-like element located between the two ARS elements stimulates *ars3002* and is indispensable for *ars3004*. Therefore, the 400 bp DNA stretch plays a critical role in determining the characteristics of the *ura4* origin region.

Wohlgemuth *et al*. (1994) isolated an origin region which appears to have at least two closely located ARS elements. After separation, one had strong origin activity and the other was very weak. But closely spaced, interacting origins are not universal in *S. pombe*. A single ARS element, *ori2004,* which can function by itself as a highly efficient chromosomal replicator, has also been reported (Okuno *et al*., 1997). Therefore, it seems possible that there are two different arrangements for replication origins in *S. pombe*; isolated, as is usually the case in *S. cerevisiae,* and clustered, possibly as occurs frequently in higher eukaryotes (Delidakis & Kafatos, 1989; Heck & Spradling, 1990; Liang *et al*., 1993; Vaughn *et al*., 1990; Little *et al*., 1993; Dijkwel *et al*., 1994; Aladjem *et al*., 1998). To determine the frequency of these two types of origin arrangement in *S. pombe*, more chromosomal origins need to be characterized.

Characterization of additional *S. pombe* origin clusters will also reveal the frequency with which replication enhancers, such as the one described here, coordinately influence the activities of neighboring replication origins. It is likely that further study of such enhancers will help to reveal the mechanism by which they coordinately regulate neighboring origins. Understanding this mechanism may provide clues to understanding the regulation of clustered origins in animal cells (Aladjem *et al*., 1998; Kalejta *et al*., 1998).

Materials and Methods

Strains and media

Escherichia coli DH5α (Life Technologies) or XL1-blue (Stratagene) were used for all subcloning. The *S. pombe* strain *ura4*-D18 (*ura4*-D18 *leu1*-32 *end1* h^-; Grimm *et al*., 1988) was used as the recipient strain for transformations. *S. pombe* cells were grown in EMM (Moreno *et al*., 1991) supplemented with 150 mg/l each of uracil and leucine when not under selection, or 150 mg/l leucine when under selection for uracil prototrophy.

Plasmid construction

For constructing mono and bi-ARS plasmids, two different vectors, pRS306 (Sikorski & Hieter, 1989) and YIp5 (Struhl *et al*., 1979) were used. The fragments shown in Figure 1 were ligated into the appropriate restriction sites within the vectors. For the pRS:3002 mono-ARS plasmid, the 0.8 kb *Eco*RV-*Eco*RI fragment was blunt-ended and ligated into the *Sma*I site within the multiple cloning site of pRS306. The final orientation of *ars3002* was such that its *Eco*RI end was closer than its *Eco*RV end to the *Bam*HI site within the multiple cloning site. Then the *Eco*RI-*Bam*HI fragment containing *ars3002* was cut out from the vector and ligated between the *Eco*RI and *Bam*HI sites within the YIp5 vector to construct YIp5:3002. For the mono-ARS plasmids containing *ars3004*, the 1.58 kb *Nsi*I-*Hin*dIII fragment was ligated into the *Pst*I and *Hin*dIII sites of pRS306 to obtain pRS:3004, then the *Bam*HI-*Hin*dIII fragment containing *ars3004* was cut out and ligated between the same restriction sites in the YIp5 vector. To obtain YIp5:3002opposite or YIp5:3003opposite, the same ARS element-containing fragments were blunt-ended and ligated into the *Pvu*II site of the YIp5 vector. For the constructs containing the *ura4* gene instead of *URA3* in the YIp5 vector (YIp5:3003U4 or YIp5:3002U4), YIp5:3003 or YIp5:3002 were cut with *Sma*I and partially digested with *Pst*I, and then the 5.8 kb (for 3003) or 5.1 kb (for 3002) fragments were gel-purified and ligated with the 1.8 kb *Pst*I-*Hin*cII fragment containing the *ura4* gene from the pura4script vector (Dubey *et al*., 1996).

All the deletion constructs used for localization of *ars3004* within the 1 kb *Eco*RI fragment (Figure 6) were cloned into the pRS306 vector. The boundaries of some deletion inserts (Figure 6(d) and (h)) were produced by PCR using primers containing *Bam*HI sites at their 5′ ends, and those of the others were made with appropriate restriction enzymes (*Hin*cII for E and F, *Fok*I for G). These inserts were prepared using appropriate restriction enzymes from the pBluescript (Stratagene) construct containing the 1 kb *Eco*RI fragment, then inserted into matching sites within the multiple cloning site of pRS306 while maintaining orientation. The primer sequences are available upon request.

To test the *ars3002*-stimulating effect of the 1.15 kb, 400 bp and control 400 bp (72% A + T-rich) segments (Figure 7), we used multiple manipulations to insert all the fragments shown in Figure 7 into the same position (between the *Eco*RI and *Nru*I sites within the YIp5 vector) with the same orientation. The 400 bp control 72% A + T stretch was prepared from the region downstream of the *S. cerevisiae GAL7* gene by PCR. The primer

sequences and manipulation details are available upon request.

To construct YIp5:*ars3001* (Figure 8), the ~600 bp *Cla*I-*Bam*HI fragment of pura4script:rDNA-573 (containing a 573 bp *ars3001* fragment with full ARS activity; Kim & Huberman, 1998) was ligated into the *Cla*I-*Bam*HI sites in the YIp5 vector. The 400 bp *Eco*RI-*Hin*cII enhancer fragment was first cloned into the pBluescript vector, and then cut out with enzymes appropriate for ligation to the right or left side of the ARS element in YIp5:*ars3003* or YIp5:*ars3001* (Figure 8). The details are available upon request.

Transformation frequency assay

S. pombe D18 cells lacking the *ura4* gene were transformed (Gietz *et al*., 1992) with equal amounts of DNA (100 ng) from the plasmids under test and then grown under selection for uracil prototrophy. After five to six days, plates were imaged with a digital scanner, and the number and mean area of colonies were calculated using an image processing program that permitted objective discrimination between the larger colonies and the smaller background colonies produced by vector alone (Huberman, 1999). All results shown are an average of two independent experiments, with data range indicated by error bars.

Determination of plasmid structure and copy number in *S. pombe* cells

The transformation procedure described above was used with the mono or bi-ARS plasmids. A ligation mixture containing the tri-ARS plasmid was used as substrate for transformation by the same procedure (Gietz *et al*., 1992). Cells from transformant colonies were regrown in liquid medium for two to three days, and total DNA was prepared (Hoffman & Winston, 1987) so that plasmid concentration and structure could be checked by Southern blot analysis using appropriate monomer plasmids as controls. To determine the average copy number of the plasmids inside cells (see the text), total DNA (genomic + plasmid) was prepared and cut with *Hin*dIII, producing the 7.7 kb genomic copy and linearized plasmid copy of different size. After Southern blotting, the 3.9 kb *Eco*RI fragment, shared by both the genomic and plasmid fragments, was used for hybridization probe. Two independent experiments were done, and the average copy number values were calculated.

DNA isolation and neutral/neutral 2D gel electrophoresis

DNA was isolated from logarithmically growing cells (1.0×10^7 to 1.5×10^7 cells/ml) using the glass bead method (Huberman *et al*., 1987). Just before harvesting, we added sodium azide to a final concentration of 0.1 % as described (Dubey *et al*., 1994; Sanchez *et al*., 1998). For 2D gel analysis, all steps were basically as described (Brewer & Fangman, 1987). About 100-120 μg of DNA was used as starting material for one experiment when replicating DNA was enriched by BND-cellulose fractionation (Huberman *et al*., 1987). When the enrichment step was skipped, 15-20 μg of total DNA was used (Figure 5(a) and (b)). For larger fragments (⩾6 kb), the gel electrophoresis conditions for the second dimension were modified (0.6 % gel, 2V/cm, 14-16 hours) to obtain better separation of bubble signals from Y signals. Southern blotting, hybridization, and image processing were as described (Dubey *et al*., 1994).

Plasmid retention and plasmid loss rate assay

All experiments were done with transformants containing stable monomer plasmids. The assays were performed as described (Dani & Zakian, 1983) with the following modifications. The transformants were grown in selective medium (EMM-uracil) to 1.2×10^7 to 1.5×10^7 cells/ml. Then equal numbers of cells (200-300 cells) were plated onto selective (EMM-uracil) and non-selective (EMM + uracil) plates to determine the fraction of cells containing plasmid. Cultures were also diluted and inoculated into non-selective medium (1×10^4 cells/ml). Since *S. pombe* cells have a longer lag time than *S. cerevisiae*, we carefully monitored the growth rate of the cells after the dilution. When they started to grow logarithmically, appropriate numbers of cells (200-300 cells/plate) were plated onto the selective and non-selective plates to obtain B (% of cells with plasmid at start). Cells were allowed to grow further (N; about 10-12 generations), while being maintained in logarithmic phase. Then equal numbers of cells were plated onto selective and non-selective plates to determine A (% of cells with plasmid after N generations). The percentage of cells that lose plasmid per generation (X) was determined as follows: $X = 1 - e^r$, where $r = \ln(A/B)/N$. Two different colonies were tested for each construct.

DNA sequence determination and analysis

The 1.45 kb *Eco*RI-*Hin*dIII fragment was dissected by *Eco*RI to produce a 1 kb *Eco*RI and a 450 bp *Eco*RI-*Hin*dIII fragment, and these fragments were cloned into the pBluescript KS^+ vector separately. Then the inserts were sequenced from both ends by an automated DNA sequencer, which provided clear signals up to at least 600 bp. Primers based on the overlap region in the 1 kb fragment were then used to complete the double-stranded sequencing of this fragment. The nucleotide sequence is available in GenBank (accession number: AF040271). All consensus sequence searches (Figure 6) were done with MacVector software.

Acknowledgments

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