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Motifs in *Schizosaccharomyces pombe ars3002* important for replication origin activity in *Saccharomyces cerevisiae*

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Abstract

Ars3002 is an efficient single-copy replication origin in the fission yeast, *Schizosaccharomyces pombe*. In a previous study, we tested the effects of consecutive ~50-bp deletions throughout *ars3002* on the replication efficiency of those origins in *S. pombe*. Here we report the results of our use of the same ~50-bp deletions to test the hypothesis that some of the *cis*-acting sequences important for replication origin activity in fission yeast might be conserved in the evolutionarily distant budding yeast, *Saccharomyces cerevisiae*. We found that in most cases there was no correlation between the effects of particular mutations in *S. pombe* and in *S. cerevisiae*. We conclude that it is unlikely that any of the *cis*-acting sequences recognised by homologous replication proteins is conserved between these two yeast species.

Keywords: Autonomously replicating sequence; DNA replication; Plasmid stability; Replication origin; Transformation; Yeast

1. Introduction

Autonomously Replicating Sequence (ARS) elements act as replication origins, allowing plasmids to be maintained as extrachromosomal DNA molecules in yeast cells (Newlon et al., 1991). Most but not all of them serve as replication origins in yeast chromosomes (Dubey et al., 1996; Sharma et al., 2001; Vujcic et al., 1999). Genomic fragments with ARS activity have been cloned from many yeast species, including *Saccharomyces*, *Schizosaccharomyces*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Candida*, and *Yarrowia* (reviewed by Huberman, 1999). They have been used to construct cloning and expression vectors and their interspecies functionalities have in some cases been tested (reviewed by Romanos et al., 1992).

ARS elements of the budding yeast, *Saccharomyces cerevisiae*, contain two principal domains. The A domain (~20 bp) contains a near match to the 11-bp sequence 5′-(A/T)TTTA(T/C)(A/G)TTT(A/T)-3′, which is called ARS Consensus Sequence (ACS). The B domain is an A + T-rich sequence of

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around 100 bp downstream of the T-rich strand of the A domain. This arrangement of sequences is important for correct binding of the proteins responsible for replication initiation. These include the Origin Recognition Complex (ORC), Cdc6, Cdt1, the MCM proteins and Cdc7/Dbf4 (reviewed by Nishitani and Lygerou, 2002). The distantly related fission yeast, *Schizosaccharomyces pombe*, has ARS elements with less well-defined sequence requirements. Fission yeast ARS elements are longer than those of budding yeast, and they contain multiple asymmetric A + T-rich motifs which are essential for ARS activity in vivo (Clyne and Kelly, 1995; Kim and Huberman, 1999; Okuno et al., 1997) and for binding ORC in vitro (Kong and DePamphilis, 2001; Lee et al., 2001; Takahashi and Masukata, 2001). They may also contain additional motifs that are important for origin function but do not bind ORC (Kim and Huberman, 1999; Okuno et al., 1997).

Fission yeast *ars3002* is located in chromosome III and is part of the *ura4* origin cluster, consisting of *ars3003*, *ars3002*, and *ars3004* in that order upstream of the *ura4* gene (Dubey et al., 1996). *Ars3002* is the best studied of these three ARS elements. Rounds of systematic deletions and linker substitution mutations have demonstrated that multiple motifs, many of which are A + T-rich and asymmetric (and thus bind ORC; Kong and DePamphilis, 2001) are important for *ars3002* function (Dubey et al., 1996; Kim et al., 2001). Many of these important sequences are redundant with each other. Consequently, no single deletion of ~50 bp is capable of completely eliminating *ars3002* activity (Kim et al., 2001, Fig. 2).

About half of tested ARS elements from *S. pombe* are active in *S. cerevisiae* (Maundrell et al., 1988), and some *S. pombe* ARS elements are active in *K. marxianus* (Antunes et al., 2000; De Souza Jr. and de Morais Jr., 2000). This cross-species activity can be exploited for biotechnological and evolutionary studies, and it may provide helpful clues regarding aspects of the initiation process. In this work we have tested the hypothesis that *cis*-acting sequence motifs within *S. pombe ars3002* may be conserved in *S. cerevisiae* by measuring the effects of systematic ~50-bp deletions throughout the length of *S. pombe ars3002* on the transformation frequency of *ars3002* in *S. cerevisiae*. We compared these results with previous measurements of the effects of the same deletions on transformation frequency in *S. pombe*. The results confirm that different sequences are important for transformation frequency in the two yeasts, suggesting that few if any *cis*-acting sequences are recognised by homologous replication proteins in these two distantly related organisms.

2. Materials and methods

2.1. Strains, plasmids, media, and cultivation

The *S. cerevisiae* strain MM10-2a (our laboratory) was used as host in genetic transformation experiments. *E. coli* XL1-Blue (Stratagene, San Diego, CA) was used for plasmid manipulation. Plasmids containing the extended *ars3002* of *S. pombe* and its 55-bp deletion derivatives are described in Kim et al. (2001). Briefly, plasmid pUra4Script:*ars3002* (ext) is pBluescript KS^+ (Stratagene) containing the *ura4* gene and the 1.2 kb-*ars3002*. From that plasmid, consecutive deletions of ~50 bp within *ars3002* were generated by PCR (Kim et al., 2001). Mutant plasmids Δ2 to Δ22 were used and compared to the intact 1.2-kb *ars3002* (Fig. 1).

Recombinant bacterial cells were cultivated in LB medium supplemented with ampicillin (100 μg/ml) and tetracycline (12.5 μg/ml). Yeast media were YPD (10 g/l yeast extract, 20 g/l peptone, and 20 g/l glucose) and SC-ura (6.7 g/l YNB, 20 g/l glucose, 20 μg/ml histidine, 60 μg/ml leucine, 30 μg/ml lysine, and 40 μg/ml tryptofan) as selective medium. Solid media contained 15 g/l agar (Ausubel et al., 1989). The cells were cultivated at 30 °C in an oscillating bath.

2.2. DNA manipulation and yeast transformation

Bacterial cells were transformed by the calcium chloride method, and plasmids were extracted by alkaline lysis (Ausubel et al., 1989). Plasmids were checked for correct construction by digesting with *Xba*I and *Hin*dIII followed by electrophoretic analysis in 1.5% agarose. Plasmid concentration

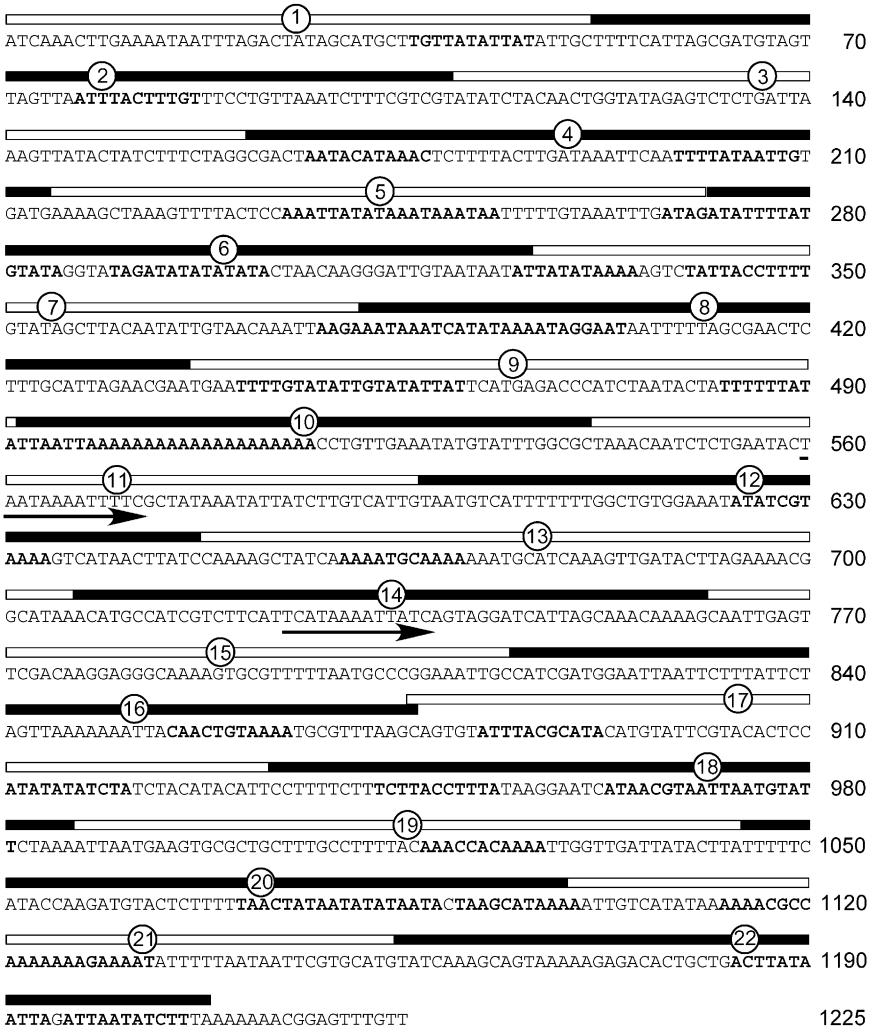

Fig. 1. Nucleotide sequence of *ars3002* of *S. pombe*. The numbered black and white horizontal bars indicate the regions removed by each of the ~50-bp deletions. The nucleotides without an overlying horizontal bar at the 3′ end of the sequence are part of *ars3002* but were not deleted. Near ($\geqslant$9/11) matches to the *S. cerevisiae* ACS are indicated by bold type. Note that some of these regions contain more than one overlapping match to the ACS and are, therefore, longer than 11 bp. A 13-bp directly repeated motif found in regions 11 and 14 is indicated by italic type and also by underlining with an arrow.

was calculated by comparing the signal from the linear molecule with signals from standard λ-*Hin*dIII fragments.

Yeast cells were transformed using the lithium acetate high-efficiency protocol (Gietz and Woods, 1994), and the frequency of transformation was calculated as the total number of transformants per microgram of DNA. The relative frequency was the ratio between the transformation frequencies of the mutant and control plasmids.

Mitotic plasmid stability was measured by duplicated-plate method described by Futcher and Cox (1984). The instability rate was calculated from the slope of the exponential decay of plasmid stability curves, and it is expressed as the percentage of plasmid-free cells generated from a

population of plasmid-containing cells per cell generation (Futcher and Cox, 1984).

3. Results

The control plasmid containing intact *ars3002* transformed *S. cerevisiae* cells at 2×10^3 transformants/μg DNA. All of the mutant plasmids also transformed yeast cells, although with different frequencies (black bars in Fig. 2). In most cases, the effects of particular deletions on *S. cerevisiae* transformation frequencies (black bars in Fig. 2) were different from those of the same deletions on *S. pombe* transformation frequencies (grey bars in Fig. 2; Kim et al., 2001). Most deletions (Δ2, Δ8, Δ9, Δ10, Δ11, Δ13, Δ14, Δ15, Δ16, Δ17, Δ18, Δ19, Δ21, and Δ22) suppressed transformation frequency, but some (Δ4, Δ5, Δ7, Δ12, and Δ20) had no significant effect, and others (Δ3 and Δ6) were stimulatory.

Replication origin activity in *S. cerevisiae* requires at least a 9/11 match to the ACS [(A/T)TTTA(T/C)(A/G)TTT(A/T)]. Twenty four separate stretches containing such matches are present within *ars3002*. They are indicated by bold type in Fig. 1. The presence of so many near matches to the ACS appears to explain why no single ~50-bp deletion completely prevents transformation of *S. cerevisiae* (Fig. 2). Probably *ars3002* acts like a cluster of independent origins in *S. cerevisiae*. Deleting any single origin has little effect on overall transformation frequency, because the other origins in the cluster remain functional.

The only deletion with similar effect (positive or negative) in the two yeasts is Δ10, which inhibits transformation in both organisms (Fig. 2). However, whereas Δ10 is the *only* deletion with significant inhibitory effect in *S. pombe*, it is one of many deletions with inhibitory effect in *S. cerevisiae* (Fig. 2). Like many other regions in *ars3002*, region 10 contains multiple near matches to the *S. cerevisae* ACS (bold type, Fig. 1). These overlap with a long stretch of A residues, which is a preferred ORC-binding motif in *S. pombe* (Kong and DePamphilis, 2001; Lee et al., 2001; Takahashi and Masukata, 2001). Thus in both cases the effect on replication is likely to be due to partial loss of ORC binding, but this does not mean the two organisms have identical ORC binding sites. The similar effects in this case are probably a consequence of the fact that the *S. cerevisiae* ACS resembles the more loosely defined sequences important for ORC binding in *S. pombe* in being A + T-rich and having an excess of A residues in one strand and T residues in the complementary strand.

Deletions of regions 11 and 14 suppressed transformation frequency most severely (Fig. 2).

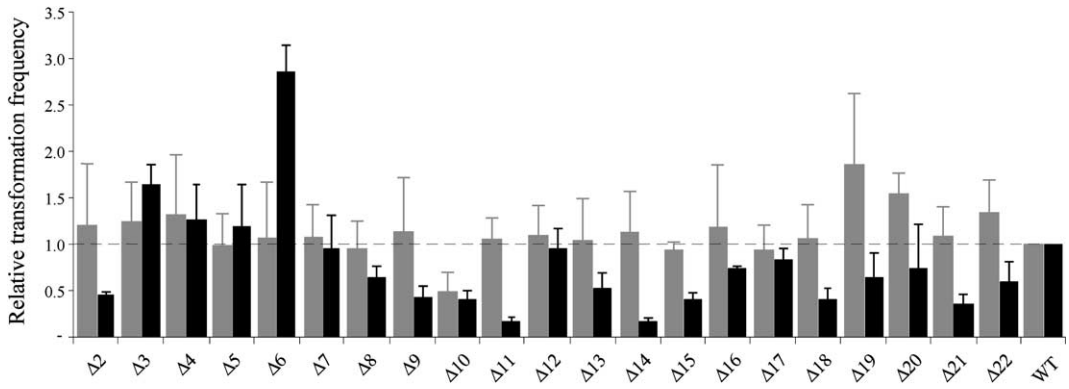

Fig. 2. Comparison of the relative transformation frequencies of the ~50-bp deletion plasmids for *S. cerevisiae* (black) with those for *S. pombe* (gray). All of the *S. cerevisiae* experiments were done in triplicate. The black columns represent mean values and the error bars show standard deviations. The *S. pombe* data are from Kim et al. (2001). The thin dashed line shows the position of a relative transformation frequency of 1.0.

Interestingly, neither of these regions contains a near match to the ACS (Fig. 1). Instead, both regions contain a 13-bp A + T-rich directly repeated motif T(A/C)ATAAAATT(T/A)TC (italicized, underlined nucleotides in Fig. 1). In future studies it will be important to test the effects of mutations in this 13-bp sequence on the replication origin activity of *ars3002* in *S. cerevisiae*.

Four transformed cells were chosen for further analysis according to their effect on the transformation efficiency comparing to intact extended *ars*3002: slight(Δ3) and severe (Δ6) stimulatory effect, and slight (Δ17) and severe (Δ11) inhibitory effect. The severe inhibitory effect of the Δ11 region deletion was also observed for the smaller size of the transformed colonies (Table 1). However, this response was not conclusive for measuring of plasmid replication efficiency, since similar results were found for other deletions (data not shown). Besides to their differences in transformation efficiency, those deleted plasmid showed practically the same high plasmid loss rate (Table 1) and low plasmid stability under selective (Table 1) and non-selective (Fig. 3) growth condition. Both parameters, which were only slightly higher for the intact *ars3002*-containing plasmid, were very indicative that all plasmid tested behaved like replicative plasmids in *S. cerevisiae* cells (Futcher and Cox, 1984). Therefore, neither of those regions contain nucleotide sequence useful for efficient plasmid segregation, i.e., functioning as centromere in *S. cerevisiae*. Finally, although *ars3002* plasmid seemed not to be efficiently segregated for *S. cerevisiae* daughter cells, specific growth rate are not severely affected by the presence of that episomal DNAs (Table 1). It means that no metabolic burn was imposed by the presence of extrachromosomal cluster of replication origin.

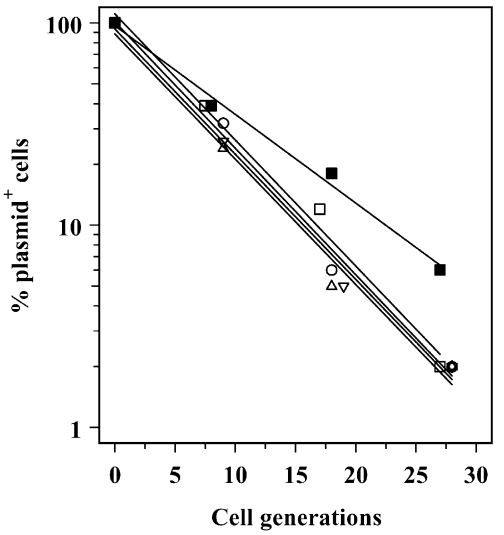

Fig. 3. Plasmid stability of transformed cells grown in non-selective medium. Yeast cells transformed with plasmids containing extended *ars3002* (■) or deleted plasmids Δ3 (□), Δ6 (◯), Δ11 (△), and Δ17 (▽) were cultivated in selective medium for 16 h and transferred to non-selective medium as described by Futcher and Cox (1984). The population of plasmid-containing cells after selective growth (see Table 1) represented 100% of plasmid$^+$ cells at the beginning of the stability experiments. The values represent the average of three experiments.

4. Discussion

It is not surprising that the ~50-bp deletions in *ars3002* (Fig. 1) should affect *S. cerevisiae* trans-

Table 1
Morphological and kinetics characteristics of *S. cerevisiae* cells harbouring plasmids containing extended *ars3002* or some of its deleted derivatives

Plasmid	Colony size (%)		P_S[a](±SD)	μ[b](±SD)	L[c](±SD)
	∅ < 1.0 mm	∅ > 1.0 mm			
ars3002	0	100	61 ± 8	0.36 ± 0.05	0.10 ± 0.01
Δ3	50	50	80 ± 19	0.31 ± 0.08	0.14 ± 0.03
Δ6	50	50	90 ± 10	0.37 ± 0.03	0.15 ± 0.01
Δ11	95	5	59 ± 11	0.37 ± 0.04	0.14 ± 0.03
Δ17	95	5	74 ± 6	0.38 ± 0.03	0.13 ± 0.01

[a] Proportion of plasmid-containing cells during exponential growth in selective medium.
[b] Specific growth rate measured under selective growth conditions (h^{-1}).
[c] Plasmid loss rate.

formation frequencies differently from *S. pombe* transformation frequencies (Fig. 2; Kim et al., 2001). These two yeasts are evolutionarily distant from each other (Kaufer and Potashkin, 2000). Although there are some similarities in sequence requirements for origin function in the two yeasts, there are also distinct differences. In both organisms, origins are located in A + T-rich intergenic regions containing stretches with an excess of A residues in one strand and T residues in the complementary strand. However, compared to fission yeast, budding yeast replication origins are relatively small (100–200 bp in contrast to 500–1500 bp) and require a single near match to a well-defined consensus sequence (in contrast to the requirement for multiple poorly-defined A + T-rich motifs in fission yeast).

Since only one (Δ10) of the deletions with strong inhibitory or stimulatory effects in budding yeast had a similar effect in fission yeast (Fig. 2), we conclude that it is unlikely that proteins important for replication in budding yeast recognise *cis*-acting sequences identical to those recognised by homologous proteins in fission yeast. Similar conclusions have previously been reached by other investigators studying different ARS elements in the two yeasts (Luehrsen et al., 1988; Maundrell et al., 1988).

Given the smaller size of budding yeast replication origins, it is surprising that many of the ~50-bp deletions had a significant effect on *ars3002* transformation frequencies in budding yeast (Fig. 2). One might have anticipated that *ars3002*, which is over 1200 bp and which contains 24 separate regions with one or more near matches to the ACS, would have behaved in budding yeast like a collection of clustered, independent, efficient replication origins, each of typical budding yeast size (100–200 bp). If that had been the case, then any single ~50-bp deletion within *ars3002* should not have significantly affected transformation frequency. This cumulative effect of single independent origins can explain the high transformation efficiency found for pDblet plasmid, which contain a duplicated 0.8-Kb core region of *ars3002*, in both *S. cerevisiae* and *K. marxianus* budding yeast (De Souza Jr. and de Morais Jr., 2000).

There are at least two possible explanations for this unexpected behaviour. First, it is possible that—although multiple regions within *ars3002* may serve as origins in budding yeast—none of these origins is efficient. If so, then mutating any one of the origins would be expected to significantly reduce overall origin activity. Second, it is possible that although *ars3002* is efficiently replicated in budding yeast, it is not efficiently segregated to daughter cells. Indeed, the plasmids employed here contain no sequence known to be capable of functioning as a centromere in budding yeast. Centromeres are not required for reasonably efficient plasmid segregation in fission yeast, but they are essential for efficient segregation of replicative plasmids in budding yeast (reviewed by Huberman, 1999). If (as seems likely), inefficient segregation is one of the factors limiting *ars3002* transformation efficiency in budding yeast, then a possible explanation for the effects of some, if not all, of the ~50-bp deletions is that they affect segregation efficiency, either negatively (in most cases) or positively (Δ3 and Δ6).In previous report, we shown that pDBlet, a replicative plasmid containing a doublet of the 800-bp core region of *ars3002*, transformed MM10-2a cells with an efficiency of 2.2×10^5 transformants/μg plasmid DNA (De Souza Jr. and de Morais Jr., 2000). In the present report, control plasmid containing single intact *ars3002* transformed the same *S. cerevisiae* strain at 2×10^3 transformants/μg DNA. Both plasmids presented the same high plasmid lost rate, which indicates that the problem can be related to replicator strength and not to plasmid segregation. Therefore, amplification of small replicator elements should increase the possibility of recognition by the *S. cerevisiae* replication proteins.

The possibility that some (or perhaps all) of the ~50-bp deletions affect segregation rather than replication is strengthened by the observation that the deletions that most strongly inhibited *ars3002* transformation of *S. cerevisiae* (Δ11 and Δ14) do not contain any close matches to the *S. cerevisiae* ACS. Nevertheless, plasmid deleted in region 11 displayed the same high plasmid lost rate as other mutants, and even the intact *ars3002* (Table 1). They do, however, have in common a 13-bp motif, T(A/C)ATAAAATT(T/A)TC, whose significance is currently unknown. Additional studies will be

required both to determine whether this motif is important for *ars3002* transformation of budding yeast and also to determine whether the deletions that affect transformation frequency do so by modulating replication efficiency, segregation efficiency, or both.

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