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Genetic analysis of an ARS element from the fission yeast *Schizosaccharomyces pombe*

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ARS (autonomously replicating sequence) elements are DNA fragments that can function as origins of DNA replication in yeast. We report the first fine-structure analysis of *ars1*, an ARS element of the fission yeast *Schizosaccharomyces pombe*. Characterization of a series of nested deletion mutations indicated that the minimal fragment of DNA encompassing *ars1* is surprisingly large. No fragment <650 bp retained significant ARS activity. Analysis of deletion and substitution mutations scanning the entire minimal *ars1* identified a single essential 50 bp fragment (segment 1). Only one other 50 bp mutation reduced activity as much as 5-fold and most deletions were without effect. Thus, the minimal *ars1* is composed of two general types of genetic elements, a small segment that is absolutely required for efficient ARS activity and a much larger region that is tolerant of internal structural alterations. Higher resolution analysis of segment 1 defined a critical 30 bp A/T-rich segment which appears to contain redundant genetic elements. *Schizosaccharomyces pombe ars1* promoted high frequency transformation in the budding yeast *S.cerevisiae* but this heterologous activity was not dependent on segment 1. Our analysis indicates that the functional elements required for ARS function in *S.pombe* and *S.cerevisiae* are clearly different.

Keywords: ARS elements/origins of DNA replication/ *Saccharomyces cerevisiae*/*Schizosaccharomyces pombe*

Introduction

The budding yeast *Saccharomyces cerevisiae* is the only eukaryotic organism in which the nucleotide sequences required to specify chromosomal origin function have been defined. ARS (autonomously replicating sequence) elements were first identified in this yeast as short chromosomal DNA sequences which promote high frequency of transformation and extrachromosomal maintenance of plasmid DNA (reviewed in Campbell and Newlon, 1991). These defining properties of ARS elements are features expected of origins of DNA replication. Using 2-D mapping techniques to identify replication intermediates, it has been directly demonstrated that *S.cerevisiae* ARS elements co-localize with sites of initiation of DNA replication in plasmids (reviewed in Fangman and Brewer, 1991). It has also been shown that all chromosomal origins of DNA replication which have been analyzed are associated with ARS elements and that initiation of DNA replication in the chromosomal context is dependent on the same sequence elements required for ARS function in plasmids (Deshpande and Newlon, 1992; Rivier and Rine, 1992; Huang and Kowalski, 1993; Marahrens and Stillman, 1994; Theis and Newlon, 1994).

The plasmid transformation assay has been used extensively to characterize the *cis*-acting sequence requirements for the function of yeast ARS elements *in vivo* (see Campbell and Newlon, 1991). The sequences that mediate ARS function in *S.cerevisiae* are contained within 100–200 bp of DNA and are A/T-rich. Sequence comparisons have led to the identification of an 11 bp conserved sequence element referred to as the ARS core consensus sequence (ACS, defined as [5′-(A/T)TTTAT(A/G)TTT(A/T)-3′]. All DNA fragments with ARS activity contain an exact or very close match to the ACS, and single point mutations within it can reduce or eliminate origin function (reviewed in Newlon and Theis, 1993). The sensitivity of the ACS to point mutations make it a strong candidate for the recognition site of a sequence-specific initiator protein. Consistent with this proposed role, a DNA-binding activity has been identified that specifically recognizes the double-stranded form of the ACS in the presence of ATP (Bell and Stillman, 1992). Mutations in the ACS that disrupt ARS function *in vivo* reduce DNA binding *in vitro*. The activity is associated with a complex of proteins called the origin recognition complex (ORC), which binds to all ARS elements tested and likely plays a role in the initiation of DNA synthesis (reviewed by Rowley *et al.*, 1994; Fox *et al.*, 1995).

While it has been established that an intact ACS is necessary for ARS activity, the ACS is not sufficient to promote autonomous plasmid maintenance. Sequences located 3′ to the T-rich strand of the core consensus, referred to as domain B, are also essential for plasmid stability and chromosomal origin function (Marahrens and Stillman, 1992, 1994; Rao *et al.*, 1994; Theis and Newlon, 1994). Although the 3′-flanking regions of all ARS elements are A/T-rich, they display little sequence homology and do not contain common consensus sequence elements. Progressive external deletions that enter domain B gradually reduce and eventually abolish ARS activity. Recent mutational analysis has defined several subelements within the B domain, one of which (B1) is required for efficient ORC binding (Rao and Stillman, 1995; Rowley *et al.*, 1995). The B3 element, present in a subset of ARS elements, contains a consensus binding site for the yeast DNA-binding protein Abf1 which stimulates ARS activity. The precise role of the remainder of the B domain is not yet clear.

Genomic DNA fragments with properties analogous to ARS elements of budding yeast have also been identified in the fission yeast *Schizosaccharomyces pombe* (Beach

and Nurse, 1981; Sakaguchi and Yamamoto, 1982; Losson and Lacroute, 1983; Toda *et al.*, 1984; Maundrell *et al.*, 1985; Wright *et al.*, 1986; Johnston and Barker, 1987; Maundrell *et al.*, 1988; Olsson *et al.*, 1993; Caddle and Calos, 1994; Dubey *et al.*, 1994; Wohlgemuth *et al.*, 1994). ARS elements are present in the *S.pombe* genome at an estimated frequency of one per 20–50 kb (Maundrell *et al.*, 1985; Caddle and Calos, 1994; Wohlgemuth *et al.*, 1994) and like their budding yeast counterparts, promote high frequency of transformation and extrachromosomal plasmid maintenance. As in *S.cerevisiae*, fission yeast ARS elements co-localize with chromosomal origins of DNA replication and when tested were shown to be required for initiation of DNA synthesis at these sites (Caddle and Calos, 1994; Dubey *et al.*, 1994; Wohlgemuth *et al.*, 1994). Sequence analysis of a number of *S.pombe* ARS elements revealed the presence of an 11 bp consensus known as the *S.pombe* ARS consensus sequence (PACS) which is different from the ARS consensus sequence (ACS) identified in *S.cerevisiae* (Maundrell *et al.*, 1988). Genetic analysis indicates, however, that the PACS is not essential for plasmid ARS activity in *S.pombe* (Maundrell *et al.*, 1988; Zhu *et al.*, 1994).

A thorough understanding of the structure and function of eukaryotic origins of DNA replication may require characterization of origins from a number of organisms. Specific DNA sequences that are required for the initiation of replication in mammalian cells have not been identified (reviewed in Burhans and Huberman, 1994). However, considerable insight may come from detailed comparison of the highly divergent fission and budding yeasts. *S.pombe* has served as a particularly powerful model organism for analyzing cell cycle control mechanisms and shares a number of basic features in common with multicellular eukaryotes. This report describes the first fine-structure genetic analysis of an ARS element from fission yeast. The element chosen for study is *ars1*, an A/T-rich 1.2 kb sequence present in most *S.pombe* replicating vectors. Plasmids containing this sequence transform *S.pombe* at high frequency and are propagated as unstable extrachromosomal elements. We carried out a systematic mutagenesis of *S.pombe ars1* in order to define the nucleotide sequence requirements for ARS activity. Our data indicate that the minimal segment of DNA required for full ARS activity, which encompasses nearly 800 bp, is quite large compared with *S.cerevisiae* ARS elements. Two short internal sequence domains which contribute to ARS function were identified by scanning mutagenesis. One of these domains contains sequences that are required for *S.pombe* ARS activity and are similar to sequences found in other *S.pombe* ARS elements. The other contains sequence elements that stimulate ARS activity several-fold. To assess directly the functional conservation of ARS elements in fission and budding yeasts, we compared the activity of several ARS elements in the two hosts. Our data indicate that the functional requirements for ARS activity in *S.pombe* differ from that of *S.cerevisiae*.

Results

ars1 plasmids transform *S.pombe* at high frequency and are maintained in the transformants as unstable extrachromosomal elements. It has previously been shown that *ars1* activity resides within a 1.2 kb DNA fragment (Heyer *et al.*, 1986). To refine further the structure of *ars1* we have analyzed the activity of mutant versions of the element generated by PCR techniques (see Materials and methods). All *ars1* derivatives were cloned into pRS305 (Sikorski and Hieter, 1989), a vector that contains the *S.cerevisiae LEU2* marker which complements *leu1* mutants of *S.pombe*.

In our studies we used both quantitative and qualitative measures to assess ARS function. The principal method was the plasmid transformation assay, which has been widely used to compare the activities of ARS mutations in *S.cerevisiae*. This assay is convenient and provides quantitative information. We also documented the relative sizes of the transformed colonies where appropriate. The *S.cerevisiae LEU2* gene in single copy complements *S.pombe leu1* mutants only weakly, resulting in reduced growth rates under selective conditions (Russell, 1989). Transformants containing low copy numbers of *LEU2* plasmids are therefore likely to give rise to colonies of reduced size. Thus, differences in colony size, while qualitative in nature, are a very sensitive indicator of differences in average plasmid copy number. Variable colony size of ARS deletion derivatives in *S.pombe* has been observed previously (Johnston and Barker, 1987; Caddle and Calos, 1994; Dubey *et al.*, 1994; Wohlgemuth *et al.*, 1994). In addition, 2-D mapping has provided some evidence that colony size is correlated with efficiency of initiation of plasmid DNA replication (Caddle and Calos, 1994). Although neither of the methods we employed gives an absolute measure of the efficiency of an ARS element in initiating DNA replication, both are useful for identifying genetic elements that contribute to ARS function.

In all of our experiments plasmid DNA was introduced into a *S.pombe leu1-32* strain by electroporation. Colonies were usually visible after 5 days at 30°C and scoring the number of transformants began 6 to 8 days after the transformation. Plasmids containing the complete 1.2 kb *ars1* fragment yielded ~10^5 transformants/μg DNA. The vector alone, which contained no *S.pombe*-derived sequences, gave very few background colonies, as did plasmids containing two different random fragments of *S.pombe* genomic DNA (data not shown).

Boundary analysis of S.pombe ars1

In initial studies we delineated the outer boundaries of *ars1* by determining the transformation frequencies of plasmids containing a series of nested *ars1* deletions (Figure 1). The data indicate that the minimal segment of DNA encompassing *ars1* is quite large. The smallest *ars1* fragment that retained wild-type levels of transformation efficiency was 782 bp (*ars1* nucleotides 350–1131; pRC46). The left boundary of *ars1* was quite sharply defined with transformation activity falling almost two orders of magnitude by deletion of only 23 bp (pRC50). Deletions from the right boundary, however, had a more gradual effect. Removing 59 bp from this end of the minimal ARS (pRC47) reduced transformation activity 5-fold and deletion of 129 bp (pRC48) reduced activity by ~50-fold. No segment <650 bp showed significant ARS activity.

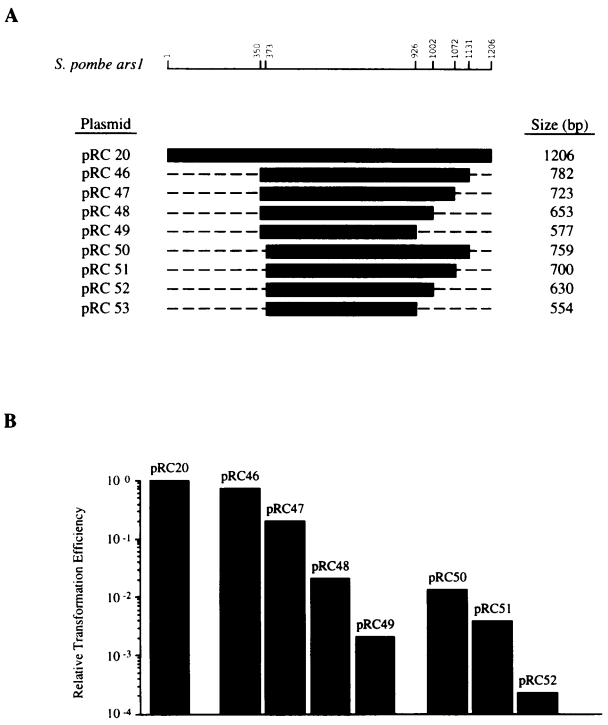


Fig. 1. Nested boundary analysis of *S.pombe ars1*. (**A**) Map depicting the nested boundary constructs. The full-length *ars1* is drawn at the top of the figure; numerals indicate nucleotide position. Filled bars represent the *ars1* sequence contained in each construct. Dashed lines indicate sequences that have been removed by deletion. pRC20 is the full-length wild-type *ars1* control. (**B**) Transformation frequencies of the plasmids depicted in (A) represented as the number of transformants/μg of plasmid DNA relative to the wild-type plasmid pRC20 to compensate for variation in transformation frequencies between experiments. Data shown were obtained by scoring the plates 6 days post-transformation. Plasmid reference numbers defined in (A) are indicated above each graph bar. pRC53 did not produce any transformants even after 9 days at 30°C. Based on the transformation frequency and growth properties of the transformants, pRC46 (*ars1* 350–1131) was determined to be the minimal *ars1* construct.

Scanning mutagenesis of ars1 defines an essential segment

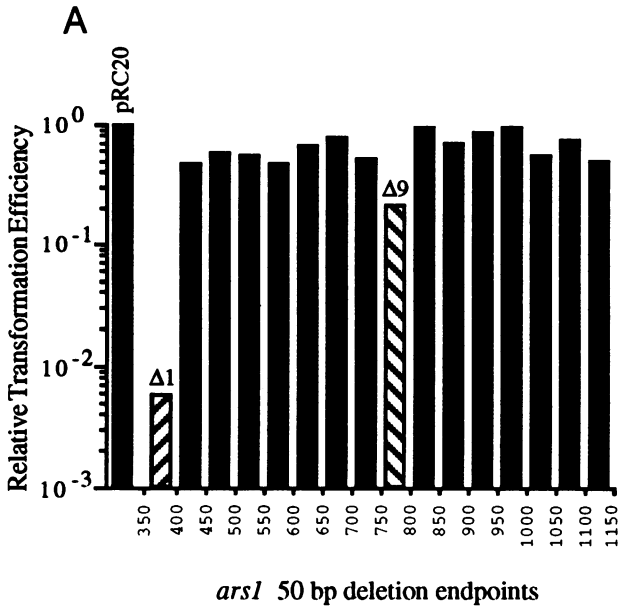
To probe the internal structure of *ars1* we constructed a systematic series of deletion and substitution mutations that scanned the entire minimal ARS which had been defined by the nested deletion analysis. The results obtained with the internal deletion series are shown in Figure 2. Deletion of the segment between positions 350 and 399 at the extreme left end of the minimal *ars1* (segment 1) decreased transformation frequency by an average of nearly 200-fold (Δ1, Figure 2A and B). This result is consistent with the data obtained by nested deletion analysis where removal of the first 23 bp of this segment also resulted in a dramatic loss of ARS activity (pRC50, Figure 1B). The effect of deleting segment 1 was not simply due to altering the spacing of other critical elements, since ARS activity was similarly reduced by substitution with 50 bp of random DNA sequence (pRCR1; Figure 2B). The colonies that resulted from both the 50 bp deletion and substitution mutations in segment 1 included some small and pinpoint-size transformants (Figure 2B).

Although the number of transformants observed with the deletion of segment 1 (Δ1) was only a small fraction of the full-length *ars1* control, it was greater than the number of transformants observed with the vector pRS305 which does not contain any *ars1* sequences (Figure 2B). Plasmid structure in a sample of five of these Δ1 transformants was examined. Total DNA was prepared from the yeast transformants (grown under selective conditions) and plasmids were analyzed by restriction enzyme digestion followed by Southern blotting. We found that two of the five transformants contained plasmids that had undergone obvious sequence rearrangements (data not shown). Our analysis probably underestimated the fraction of plasmids with such rearrangements since a relatively small number of restriction enzymes was used. Control plasmids recovered from cells transformed by the wild-type *ars1* did not show such rearrangements. The tendency of non-ARS plasmids to rearrange in *S.pombe* has been previously observed (Gaillardin *et al.*, 1983; Maundrell *et al.*, 1985; Heyer *et al.*, 1986; Wright *et al.*, 1986). A reasonable interpretation is that the plasmids recovered from Δ1 transformants had acquired sequences with ARS activity by internal rearrangement or recombination with chromosomal DNA. Thus all of our data are consistent with the hypothesis that segment 1 contains a genetic element(s) that is essential for ARS function.

Deletion of the 50 bp segment between nucleotides 750 and 799 (segment 9) also caused a significant reduction (5-fold) in transformation frequency (Δ9, Figure 2A). The transformants were heterogeneous in size with many pinpoint-size colonies (data not shown). The effect of this deletion, like that of segment 1, was not due to an alteration of the spacing of surrounding elements since similar results were obtained with a 50 bp substitution mutation (data not shown).

Interestingly, most of the 50 bp deletions scanning the minimal *ars1* had little effect on ARS function (Figure 2A). Since nested deletions that remove all of the *ars1* sequences to the right of nucleotide 926 reduce ARS activity nearly 500-fold (pRC49, Figure 1B), the simplest interpretation of these data is that *ars1* contains essential genetic elements in addition to segment 1, but that such elements are dispersed over a large region. In support of this, we observed that several deletions of larger segments of DNA within the minimal *ars1* (≥100 bp) resulted in reduced transformation frequencies and heterogeneous colony size (data not shown). The most severe of these deletions removed sequences between segments 1 and 9 (data not shown). Except for the obvious difference in scale, this is reminiscent of the organization of budding yeast ARS elements described in the Introduction. The possible implications of this unusual genetic organization will be addressed in the Discussion.

Relative to the overall 55% A+T composition of the *S.pombe* genome (Shapiro, 1970), the nucleotide sequence of segment 1, like that of the rest of the minimal *ars1*, is A/T-rich (Figure 3). Eighty-eight per cent of the residues of this 50 bp segment are A or T, and it has the highest A/T content of the 50 bp segments that were analyzed. Examination of the sequence revealed several interesting features. A palindromic sequence near the left end of segment 1 (TATAATTATA) is also present with a single mismatch in segment 9, the other 50 bp segment shown to contribute to *ars1* function. The biological significance of this sequence motif is unclear, however, since it can



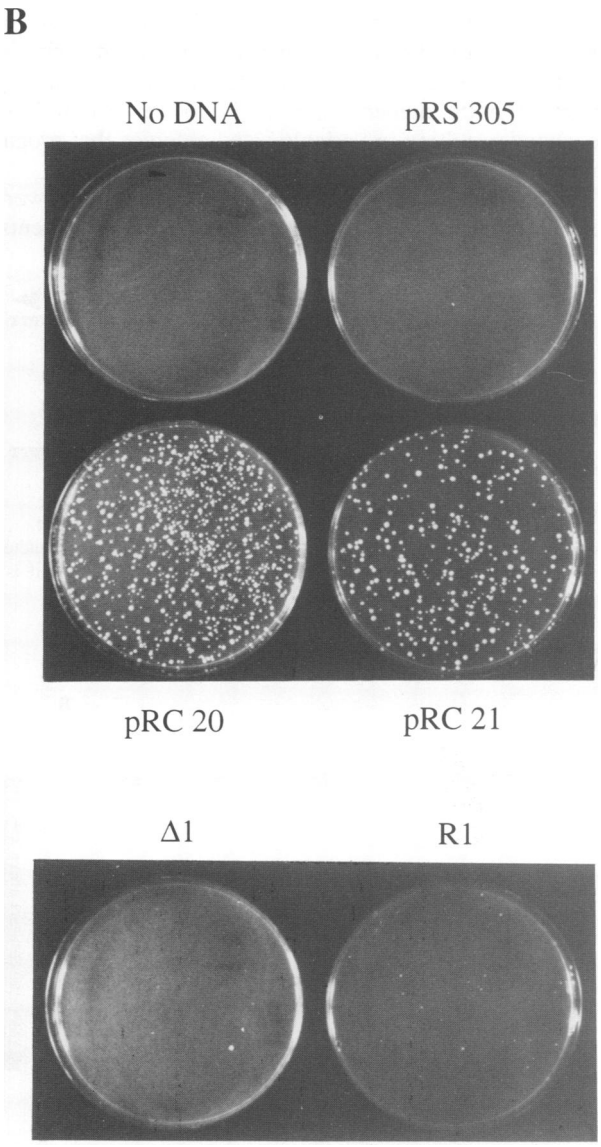


Fig. 2. Fifty bp linker scanning mutagenesis of *ars1*. (**A**) ARS activity of the 50 bp deletions determined by the transformation assay. Data shown are the average of two independent experiments scored 6–8 days post-transformation. Transformation efficiency is represented as the number of transformants/μg deletion plasmid relative to the wild-type plasmid pRC20 to compensate for variation in transformation frequencies between experiments. *ars1* endpoints of each deletion are indicated on the horizontal axis. Segment 1 is *ars1* nucleotides 350–399 and segment 9 is *ars1* 750–799. Deletions of these two segments are indicated by striped graph bars and labelled Δ1 and Δ9, respectively. All of the 50 bp deletions were constructed in the context of the 1.2 kb full-length *ars1*. (**B**) Phenotype of the 50 bp deletion (Δ1) and substitution (R1) mutants of *ars1* segment 1. Photographs of this representative experiment were taken 7 days after transformation. pRS305 is the vector control used for these studies. pRC20 and pRC21 are full-length wild-type *ars1* plasmids.

be deleted in either segment 1 or segment 9 without great effect on *ars1* function (see below; Figure 4 and data not shown). Figure 3 also indicates that the segment from nucleotides 370–399 contains three repeats of a 9 bp sequence [TTGTA(T/A)TTT]. It is possible that the presence of these repeats is significant; however, exact copies of the repeats are not generally found in other *S.pombe* ARS elements.

Fine structure mapping of segment 1

Since segment 1 clearly contains sequences essential for *ars1* function, we studied the effects of smaller deletions and substitutions within this region in order to define important sequence element(s). In initial experiments, five 10 bp substitution mutations covering the entire 50 bp segment were constructed. All five constructs exhibited approximately the same transformation frequency as the wild-type *ars1*, although the cells transformed with the two right-most 10 bp deletions showed somewhat smaller colony size (data not shown). More significant effects on ARS activity were observed with 20 and 30 bp deletions (illustrated in Figure 4A). Four 20 bp deletions covering segment 1 at 10 bp intervals were generated. Although none of these deletion mutations dramatically reduced the transformation frequency, the mutations located in the right side of segment 1 (Δ1B, Δ1C and Δ1D) gave rise to colonies of significantly reduced size (Figure 4B). If capable of further growth when restreaked onto selective medium, most of the transformants gave rise to colonies of variable size (data not shown).

Thirty bp deletion mutations in segment 1 (Δ1E, Δ1F and Δ1G) all showed slightly reduced transformation frequencies. The most dramatic effects were observed with 30 bp mutations which removed sequences over the right half of segment 1 (Δ1F and Δ1G). Both mutants showed reduced transformation frequency (4- to 10-fold), delayed colony formation and dramatically reduced average colony size (Figure 4C and data not shown). The pinpoint-size colonies, which predominated among the transformants (see Figure 4C), usually failed to grow further when restreaked onto selective medium, suggesting that the plasmids were extremely unstable. Those transformants that were capable of further growth generally yielded colonies that were heterogeneous in size. As

described above, colony size is a qualitative measure of ARS function and has been correlated with efficiency of initiation (Caddle and Calos, 1994). Plasmids containing defective ARS elements are likely to be lost at high frequency, resulting in variably sized colonies that propagate poorly under selective conditions.

To establish that the effects of the 30 bp deletions were not due to alterations in the spacing of genetic elements,

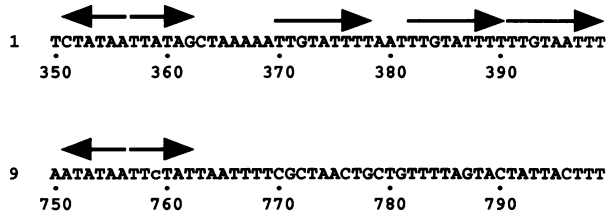


Fig. 3. Nucleotide sequence of *ars1* segments 1 and 9. Nucleotide position is marked below each sequence and repeat motifs are depicted by arrows. The mismatch in segment 9 to the inverted repeat motif is indicated by lower case.

30 bp substitutions in segment 1 (R1E, R1F and R1G) were also examined with similar results (Figure 4C). Although none of these substitution mutations completely reproduced the effect of deleting or substituting the entire 50 bp segment 1, it is clear that the deletions and substitutions that enter the right-most 30 bp of segment 1 (Δ1C, Δ1D, Δ1F, Δ1G, R1F and R1G) have dramatic effects on ARS activity. These results indicate the presence of an important genetic element within this sequence. As mentioned above, this sequence contains three direct repeat motifs (see Figure 3) which may contribute to *ars1* function.

Interestingly, none of the internal mutations completely abolished the activity of segment 1. All of the mutations tested retained some ARS function, since significant numbers of small or pinpoint colonies were observed. This observation suggests that there may be some functional redundancy of the genetic elements required for *ars1* function even within segment 1. It should also be noted that the effects of deleting certain regions of segment 1

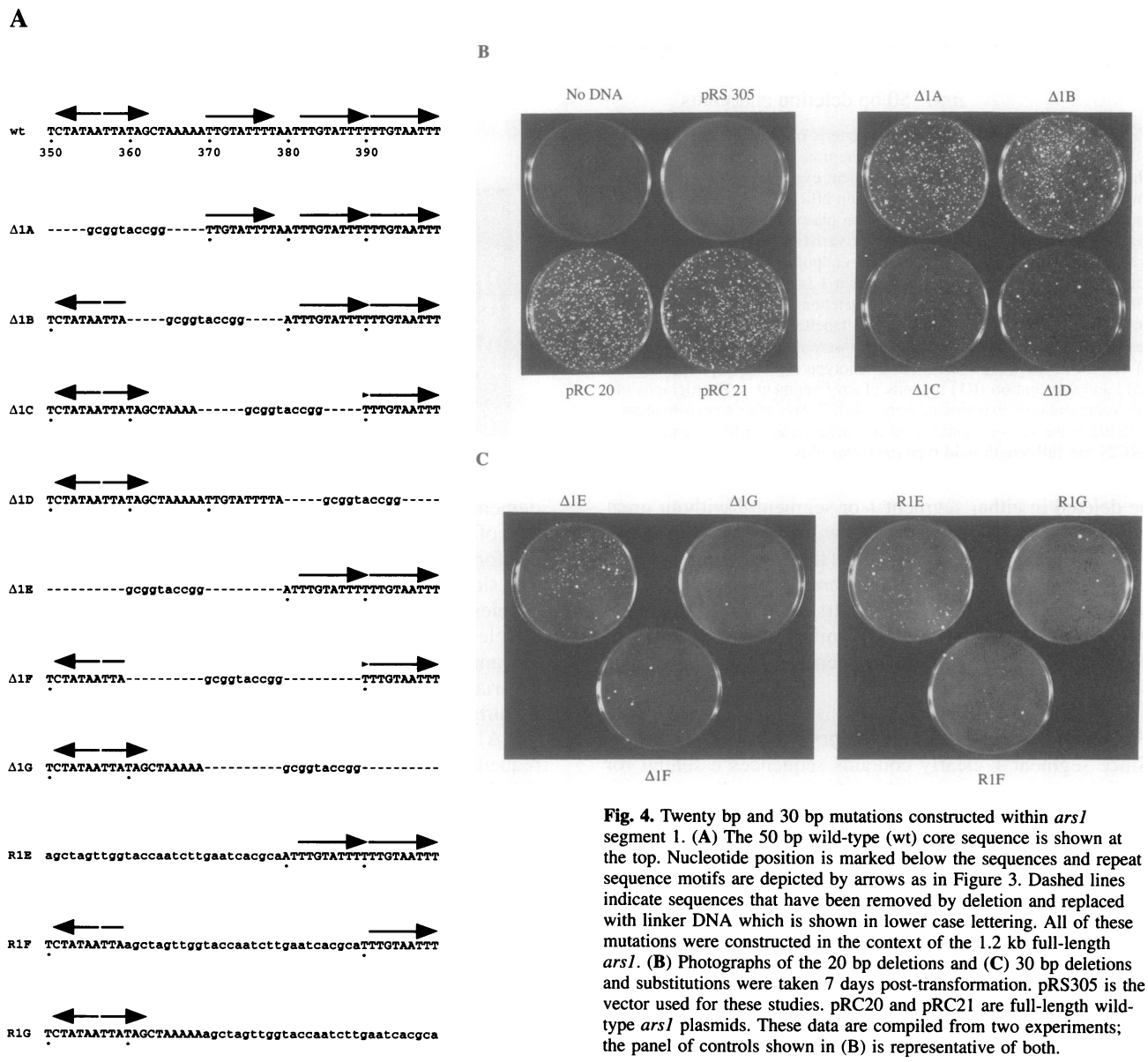


Fig. 4. Twenty bp and 30 bp mutations constructed within *ars1* segment 1. (**A**) The 50 bp wild-type (wt) core sequence is shown at the top. Nucleotide position is marked below the sequences and repeat sequence motifs are depicted by arrows as in Figure 3. Dashed lines indicate sequences that have been removed by deletion and replaced with linker DNA which is shown in lower case lettering. All of these mutations were constructed in the context of the 1.2 kb full-length *ars1*. (**B**) Photographs of the 20 bp deletions and (**C**) 30 bp deletions and substitutions were taken 7 days post-transformation. pRS305 is the vector used for these studies. pRC20 and pRC21 are full-length wild-type *ars1* plasmids. These data are compiled from two experiments; the panel of controls shown in (B) is representative of both.

may depend on the nature of the flanking sequences. As an example, an internal deletion of the left-most 20 bp of segment 1 (Δ1A, which removes *ars1* nucleotides 350–369) had relatively little effect on ARS function (Figure 4B), while a larger deletion that removed the left-most 23 bp of segment 1 plus all of the leftward flanking DNA (pRC50) was profoundly defective (Figure 1B). As previously observed (Sakaguchi and Yamamoto, 1982), it appears that the sequence requirements for ARS function in *S.pombe*, as in budding yeast (Bouton and Smith, 1986), are dependent on vector context.

The right-most 30 bp of segment 1 are extremely A/T-rich (90%) and also exhibit a highly asymmetric distribution of A and T residues with most of the T residues (70%) on the top strand as it is written in Figure 3. This strand bias is similar to A/T-rich regions in known origins of DNA replication such as budding yeast ARS elements and the SV40 origin [reviewed in Campbell and Newlon (1991) and DePamphilis (1993), respectively]. Sequences similar to the right-most 30 bp of segment 1 (the *ars1* core sequence element) are readily found in previously described *S.pombe* ARS elements and are listed in Figure 5. Most ARS fragments have several regions with significant sequence homology to this part of segment 1. The segments shown in Figure 5 were selected because the extent of nucleotide sequence identity was ⩾67% (at least 20 bases of identity with the 30 bp *ars1* core sequence). Interestingly, a number of the segments selected in this way are found within regions shown to be required for ARS function on the basis of deletion analysis (Maundrell *et al.*, 1988; K.Maundrell, personal communication). Preliminary evidence indicates that a 30 bp replacement of the sequence in *ars727* also disrupts ARS activity in *S.pombe* (data not shown). However, a rigorous assessment of the significance for ARS function of these sequences, which may be conserved among *S.pombe* ARS elements, will require further genetic analysis. Moreover, the high A/T content of fission yeast ARS elements clearly increases the probability that such sequences will be present on a random basis.

ars1	TTGTATTTTAATTTGTATTTTTTGTAATTT
ars17	ATTTATTTTAATTTTTGTTGATTTAAATTT
ars727	ATTTATTTTATTTTATTTTTTTTTTTATTTA
ars745	ATTTATTATTTTTATTATTTATTTTCATTT
ars747	ATTTTATTTATTTAGTATATTTATTTATTT
ars756	TTATATTTTATAGAATATTTTTTTTTATTT
ars766	ACCAATTTCAATTTATTTTTTTATTTATTT
ars767	TTTATTTATTTATTTTTTTTTTTTTTAATT
ars772	ATGTTTCTAATTCTCTATTTTTTCTAAATT

Fig. 5. Homologous sequences found in *S.pombe* ARS elements. ARS sequences were identified by homology search using the right-most 30 bp of *ars1* segment 1 (shown on top line). Only those sequences which contained at least 20 bases of identity (67%) were accepted. The listed sequences correspond to the following nucleotides: *ars1*, 370–399; *ars17*, 766–795; *ars727*, 266–295; *ars745*, 749–778; *ars747*, 190–219; *ars756*, 618–647; *ars766*, 973–1002; *ars767*, 538–567; *ars772*, 111–140. In all cases, deletion of a region containing the listed sequence resulted in at least a 10-fold reduction in ARS activity which was determined by dividing the reported number of transformants/μg deletion derivative by the number of transformants/μg wild-type ARS plasmid (Maundrell *et al.*, 1988; K.Maundrell, personal communication). *ars744* did not contain any sequence elements that satisfied our criterion for homology, although 17 and 18 base matches were found. *ars745* and *ars767* each contained one other sequence element and *ars747* contained two other elements, however, their significance could not be determined from the available genetic analyses. *ars766* contained one other sequence element which was also located within the deleted region and one element whose significance could not be determined. *ars3002* and *ars3003* were not analyzed.

The sequences required for ARS function in S.pombe and S.cerevisiae are different

Previous studies have indicated that a subset of *S.pombe* ARS elements are able to function in *S.cerevisiae* (Maundrell *et al.*, 1985). To examine this observation further we transformed *S.cerevisiae* with the plasmid pRC20 containing the wild-type 1.2 kb *S.pombe ars1*. As shown in Figure 6, pRC20 transformed *S.cerevisiae* at efficiencies similar to those of control *S.cerevisiae* ARS elements (*ARS1* and *ARSH4*), although the pRC20 transformants were smaller than the control transformants. To examine the sequence requirements for *ars1* function in *S.cerevisiae* we analyzed the activity of several deletion mutants. Interestingly, deletion of either segment 1 or segment 9 did not have significant effects on the observed transformation frequency although the same mutations reduced transformation frequency in *S.pombe* by an average of 200- and 5-fold, respectively. This observation suggests that the activity of *S.pombe ars1* in *S.cerevisiae* is not dependent on the same genetic elements that are required for function in the native host. Sequence analysis revealed that the *S.pombe ars1* fragment contains four 10/11 bp matches and twenty-two 9/11 bp matches to the *S.cerevisiae* ARS consensus, and it seems likely that the observed activity of *ars1* in *S.cerevisiae* is fortuitous. Deletion of the previously described *S.pombe* ARS consensus sequence (PACS; Maundrell *et al.*, 1988) had no effect on transformation in *S.pombe* or in *S.cerevisiae*. Finally, we tested whether *S.cerevisiae* ARS elements can function in *S.pombe*. In keeping with similar observations by others (Beach and Nurse, 1981) we found that neither of the *S.cerevisiae* ARS elements tested was able to transform *S.pombe*. We therefore conclude from these studies that the specific sequence elements required for ARS function in *S.pombe* and *S.cerevisiae* are different.

Discussion

Fine-structure analysis of a S.pombe ARS element

Dissection of the sequence elements required for the function of eukaryotic origins of DNA replication has so far been limited largely to the budding yeast *S.cerevisiae*. This report describes the functional organization of *S.pombe ars1*. Our data indicate that the minimal *ars1*

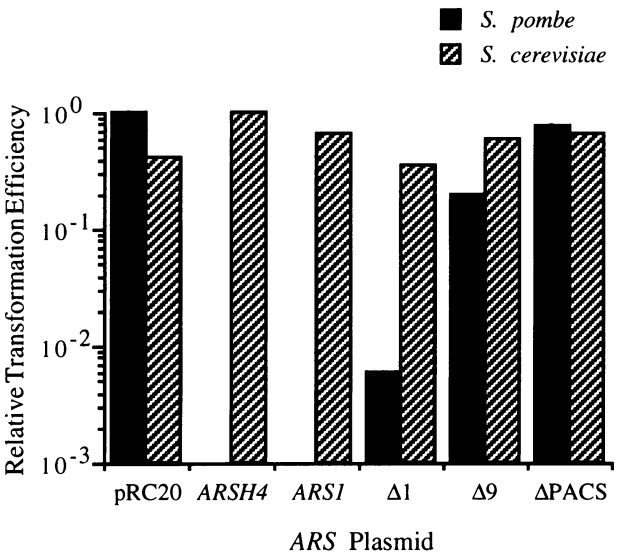


Fig. 6. Comparison of ARS activity in *S.pombe* and *S.cerevisiae*. Transformation efficiency is represented as the number of transformants/μg plasmid DNA relative to the control plasmids pRC20 and pRC*ARSH4* for *S.pombe* and *S.cerevisiae*, respectively. Δ1 is a 50 bp deletion of *S.pombe ars1* nucleotides 350–399 and Δ9 deletes *ars1* nucleotides 750–799. ΔPACS is an 11 bp substitution of the previously reported *S.pombe* ARS consensus sequence (PACS; Maundrell *et al.*, 1988). *S.cerevisiae* ARS-containing plasmids pRC*ARSH4* and pRC*ARS1* produced no *S.pombe* transformants even after 10 days at 30°C. All data shown are the average of two independent experiments scored 5 and 6 days after *S.pombe* transformations and 4 and 5 days after *S.cerevisiae* transformations.

segment sufficient for wild-type ARS function in *S.pombe* is 782 bp. Analysis of internal 50 bp deletion and substitution mutations scanning the entire minimal *ars1* sequence demonstrated the existence of a single segment at one end that is essential for ARS activity (segment 1). Most of the remaining 50 bp deletions had little effect on ARS activity. Only one other (segment 9) reduced ARS activity as much as 5-fold. Thus, the minimal *ars1* comprises two general types of genetic elements, a small segment (segment 1) that is required for efficient ARS activity and a much larger region that is tolerant of internal structural alterations. All mutations that removed substantial portions of segment 1 greatly reduced ARS function. However, 10 bp substitutions within the segment had little effect on ARS activity, indicating that there may be some functional redundancy of the genetic elements required for full *ars1* function. Further analysis indicated that the A/T-rich segment spanning the right-most 30 bp contains sequences that make significant contributions to ARS function. This core sequence has a marked asymmetry of A and T residues with most of the Ts on one strand and most of the As on the other. Sequences similar to this 30 bp core sequence element can be found in functionally important regions of other *S.pombe* ARS elements, but their significance remains to be assessed.

A number of *S.pombe* DNA fragments with ARS activity have been previously identified (Beach and Nurse, 1981; Sakaguchi and Yamamoto, 1982; Losson and Lacroute, 1983; Toda *et al.*, 1984; Maundrell *et al.*, 1985; Wright *et al.*, 1986; Johnston and Barker, 1987; Maundrell *et al.*, 1988; Olsson *et al.*, 1993; Caddle and Calos, 1994; Dubey *et al.*, 1994; Wohlgemuth *et al.*, 1994). Although few of these fragments have been studied in any detail, some general structural features of *S.pombe* ARS elements have begun to emerge which are confirmed by the present study. One striking generalization that can be made is that the sequences required for efficient replication in *S.pombe* appear to be distributed over a large region. Unlike the compact ARS elements of *S.cerevisiae*, ARS function in *S.pombe* appears to be dependent on contributions from elements that span at least several hundred base pairs (Olsson *et al.*, 1993; Caddle and Calos, 1994; Dubey *et al.*, 1994; Wohlgemuth *et al.*, 1994). For example, all of the DNA fragments with ARS activity recovered in one random screen of *S.pombe* genomic DNA were >800 bp (Maundrell *et al.*, 1988) and it has been reported that the activity of *ars17* is dependent on 1–2 kb of DNA (Johnston and Barker, 1987). *ars17* also contains an essential core element and an extensive flanking domain which seems to behave similarly to that of *ars1*. A second common feature of *S.pombe* ARS elements is that they are highly A/T-rich relative to the 55% A+T composition of the genome (Shapiro, 1970; Johnston and Barker, 1987; Maundrell *et al.*, 1988; Zhu *et al.*, 1994). For example, the 782 bp minimal *ars1* has an overall A+T content of 74%. The A/T-rich segments of *S.pombe* ARS elements also contain many short stretches in which most of the Ts are on one strand and most of the As are on the complementary strand (Zhu *et al.*, 1994). While the functional importance of this strand asymmetry remains to be determined, one possibility is that it establishes or constrains the structure or flexibility of the DNA at the origin.

Comparison of ARS elements in the fission and budding yeasts

Physical mapping studies by 2-D gel analysis have demonstrated that sites of initiation of DNA replication in the chromosomes of both *S.pombe* and *S.cerevisiae* co-localize with ARS elements (Fangman and Brewer, 1991; Caddle and Calos, 1994; Dubey *et al.*, 1994; Wohlgemuth *et al.*, 1994). ARS elements in both yeasts have a high A/T content, and both have a modular construction consisting of at least two domains that make different contributions to ARS function (Marahrens and Stillman, 1992; Rao *et al.*, 1994; Theis and Newlon, 1994; Rao and Stillman, 1995; Rowley *et al.*, 1995). Since such a modular structure is observed with certain viruses as well (DePamphilis, 1993), it may represent a general feature of the organization of origins of DNA replication in eukaryotic cells. Both fission and budding yeast ARS elements also contain easily unwound regions that may contribute to the efficiency of initiation of DNA replication (Natale *et al.*, 1993; Zhu *et al.*, 1994).

We have shown that *S.pombe ars1* is organized into two domains with different characteristics. The core sequence element (segment 1) is a short sequence that is absolutely required for ARS function. The large domain flanking the essential core element encompasses most of the 782 bp minimal ARS. Fifty bp deletion mutations in this domain have little effect on activity, although larger internal deletions can reduce the efficiency of ARS function. The properties of this region have some similarities to those of the B domain of *S.cerevisiae* ARS elements which flanks the ACS, although the *S.pombe* region is much larger. Linker scanning analysis of the B domain of

budding yeast *ARS1* showed that no single subsegment was absolutely required for efficient ARS activity, although deletions or substitutions reduced ARS function (Marahrens and Stillman, 1992).

While the role of the large domain flanking the *S.pombe ars1* core sequence element is of considerable interest, it is not possible at this point to distinguish among several possible functions. The domain may contain multiple or redundant binding sites for protein(s) that stimulate ARS function. In budding yeast, for example, sequence elements in the B domain contribute to the binding of ORC and Abf1 (Campbell and Newlon, 1991; Rao and Stillman, 1995; Rowley *et al.*, 1995). Abf1 is not required for ARS function, but can contribute to overall ARS activity. It has also been suggested that the B domain may bind additional factors that remain to be identified (Rao and Stillman, 1995; Rowley *et al.*, 1995). Further work will be required to determine whether the flanking domain of *S.pombe* ARS elements binds factors similar to those observed in budding yeast. A second possible role for the flanking domain is to facilitate DNA unwinding, since an origin of replication must serve as the site of initial unwinding of the duplex. Application of a thermodynamic model to the sequences of several *S.pombe* ARS elements demonstrated that they all contain segments of DNA which are likely to have low intrinsic helical stability (Zhu *et al.*, 1994). Progressive deletion of the most easily unwound sequences in two tested cases was correlated with severe reduction in ARS activity, suggesting that easily unwound DNA may be important for ARS function in *S.pombe* (Zhu *et al.*, 1994). Studies in budding yeast indicate that easily unwound regions are tolerant of small substitutions and can be functionally substituted by unrelated sequences that exhibit helical instability (Umek and Kowalski, 1988; Huang and Kowalski, 1993). These properties of easily unwound DNA might explain the fact that budding yeast ARS elements share little sequence homology over most of their lengths. Finally, the ARS flanking sequences may contribute to the formation of a local chromatin structure that facilitates origin function. This kind of model is particularly attractive given the large size of the flanking domain and its relative insensitivity to localized sequence perturbations. *In vitro* studies with the SV40 DNA replication system have provided evidence that the assembly of nucleosomes at an origin of replication can profoundly inhibit initiation of DNA synthesis (Cheng and Kelly, 1989). It has also been demonstrated that the inappropriate positioning of a nucleosome over the *S.cerevisiae ARS1* can inhibit ARS activity *in vivo* (Simpson, 1990). It is possible that intrinsic periodicities in the sequence of the flanking domain influence the organization of nucleosomes in the vicinity of the core sequence element of *S.pombe ars1* as well. A specific chromatin organization might be important to allow access of initiation factors to the origin or perhaps to facilitate the initial local unwinding. It is worth noting that the above models are not all mutually exclusive, and it may prove difficult to distinguish between them experimentally.

While it is apparent that there are some general similarities in the organization of ARS elements in *S.pombe* and *S.cerevisiae*, the present study as well as previous work indicates that the specific nucleotide sequences that mediate autonomous plasmid replication in the two yeasts are fundamentally different (Beach and Nurse, 1981; Gaillardin *et al.*, 1983; Maundrell *et al.*, 1985; Johnston and Barker, 1987; Maundrell *et al.*, 1988) and are apparently not conserved. *S.pombe ars1* was found to permit high frequency of transformation in the budding yeast *S.cerevisiae* but this heterologous activity was not dependent on the genetic elements required for function in the native host. In particular, the core sequence element in segment 1 was completely dispensable for function in *S.cerevisiae*. Other *S.pombe* DNA fragments with ARS activity can also function in budding yeast (Maundrell *et al.*, 1985) but our data strongly suggest that this may be due to the chance occurrence in such fragments of sequences that can mediate ARS activity in *S.cerevisiae*. It has previously been observed that a subset of *S.pombe* ARS fragments contain the ARS consensus sequence (ACS) which is essential for autonomous replication in *S.cerevisiae* (Maundrell *et al.*, 1988). *Saccharomyces pombe ars1* contains a number of near matches to this consensus sequence. Consistent with previous observations (Beach and Nurse, 1981), neither of the two *S.cerevisiae* ARS elements that we tested was able to promote autonomous plasmid maintenance in *S.pombe*. We conclude from these studies that the specific sequence requirements for ARS function in *S.pombe* and *S.cerevisiae* are significantly different.

Molecular interactions at ars1 in vivo

By analogy with budding yeast, the short domain which is absolutely required for *ars1* activity is a good candidate for the binding of a sequence-specific DNA binding factor(s) involved in the initiation of DNA replication. The essential segment 1 of *ars1* is A/T-rich (88%) and has notable strand bias (56% of the T residues on one strand). The essential ACS common to *S.cerevisiae* ARS elements is also an asymmetric A/T-rich sequence. Evidence suggests that ORC, the proposed *S.cerevisiae* initiator protein, binds to the ACS *in vitro* (Bell and Stillman, 1992; Rao and Stillman, 1995) and *in vivo* (Diffley and Cocker, 1992; Rowley *et al.*, 1995). The *ars1* core sequence similarly may be the recognition site for the cellular factor(s) involved in origin recognition and initiation of DNA synthesis in *S.pombe*.

As in other systems, it is likely that the first step in the initiation of DNA replication in *S.pombe* is the specific recognition of the essential sequence element(s) by an initiator protein. A complete understanding of origin function and its regulation will require knowledge of both the protein factors and the DNA sequences with which they interact. The characteristics of the *ars1* segment 1 which we have described make it an excellent candidate for the recognition site of an origin-binding protein in *S.pombe*; it contains one or more genetic elements that are essential for its ARS function in fission yeast and may play a role similar to the core consensus sequence of *S.cerevisiae* ARS elements. In budding yeast some single point mutations within the essential ARS consensus sequence abolish ARS and origin activity (reviewed in Newlon and Theis, 1993), a feature expected of a unique sequence element recognized by a site-specific DNA-binding protein. In the case of *S.pombe ars1*, however, even larger sequence alterations in the essential core element (segment 1) do not abolish ARS function. While

it is possible that origin activity is not controlled by specific protein–DNA interactions in *S.pombe*, we consider this extremely unlikely given the ample precedents in budding yeast and eukaryotic viruses. In addition, the demonstration that origins of replication in *S.pombe* are localized (Caddle and Calos, 1994; Dubey *et al.*, 1994; Wohlgemuth *et al.*, 1994) is suggestive of a mechanism of initiation directed by sequence-specific protein recognition. It seems likely that our observations are attributable to the presence of redundant elements within the essential sequence of *ars1*. Identification and characterization of the protein–DNA interactions that occur *in vivo* are likely to provide details about the functional contributions made by these elements. We plan to exploit the information derived from the genetic analysis of *ars1* to identify and characterize functionally the protein(s) that interact with *S.pombe* origins of DNA replication. We expect that this biochemical approach to elucidating the molecular events that occur at *ars1* will complement the genetic studies presented here.

Materials and methods

Strains and media

S.pombe strain *h⁻ leu1-32* was grown in supplemented Edinburgh minimal medium (EMM, Bio 101) at 30°C. All supplements were obtained from Sigma. EMM+5S was EMM with 250 μg/ml each of adenine, uracil, leucine, lysine and histidine. EMM–leu was the above without addition of leucine. *S.cerevisiae* strain YPH 499 (*MATa ura3-52 lys2-801 ade2-101 his3Δ200 trp1Δ63 leu2Δ1*) was grown at 30°C in YEPD (1% yeast extract, 2% peptone, 2% dextrose) supplemented with 160 μg/ml tryptophan. SC and SC–leu media were prepared essentially as described (Rose *et al.*, 1990) without addition of inositol or *para*-aminobenzoic acid. All solid media contained 2% agar.

Construction of plasmids

pREP3X (Forsburg, 1993; Maundrell, 1993) was the source of the full-length wild-type *S.pombe ars1*. Mutant versions of *ars1* were generated by PCR techniques. Oligonucleotide primers were designed with *Bam*HI or *Hin*dIII restriction enzyme recognition sites at the 5′ termini. PCR products were simultaneously digested overnight with *Bam*HI and *Hin*dIII (New England Biolabs) for subsequent cloning. A *S.cerevisiae ARS1*-containing fragment was generated by *Hin*dIII digestion of YRp17 (Botstein and Davis, 1982; Stinchcomb *et al.*, 1982). The *S.cerevisiae ARSH4*-containing fragment was obtained by *Bam*HI and *Hin*dIII digestion of pRSS83 (Sikorski and Hieter, 1989). Following restriction enzyme digestion all DNA fragments were purified from agarose gels using the GENECLEAN kit (Bio 101) and cloned into appropriately digested pRS305 (Sikorski and Hieter, 1989). This vector is a derivative of pBLUESCRIPT (Stratagene) that contains the *S.cerevisiae LEU2* marker which complements *leu1* mutants of *S.pombe*.

Nested boundary mutations contained no *S.pombe ars1* sequences external to the endpoints given. All other mutations were constructed within the full-length *ars1* as follows. Internal oligonucleotide primers were designed with *Kpn*I restriction enzyme recognition sites at the 5′ termini. These were paired with primers complementary to the ends of the full-length *ars1* to generate left and right PCR products. These fragments were co-ligated and then subcloned into pRS305 as described above. Fifty bp deletions replaced 50 bp of *ars1* sequence with the 20 bp linker sequence ATGCATTGGTACCACCATGG. Each 50 bp substitution construct was generated by first digesting the cognate deletion plasmid to remove the linker sequence. Two synthetic oligonucleotides were then pre-annealed prior to ligation to the deletion plasmid fragment. This strategy replaced 50 bp of *ars1* with the sequence ATGCATAGTTAGCTAGCTAGTTGGTACCAATCTTGAATCACGCACCATGG. This substitution sequence was designed to minimize direct and indirect repeats while approximating the 55% A+T base composition of the *S.pombe* genome (Shapiro, 1970). Ten, 20 and 30 bp deletions were designed to replace 10, 20 or 30 bp of *ars1* sequence with the 10 bp linker sequence GCGGTACCGG. 30 bp substitution constructs replaced 30 bp of *ars1* with the linker sequence AGCTAGTTGGTACCAATCTTGAATCACGCA. The 20 and 30 bp mutations are summarized in Figure 4A. Deletion of the *S.pombe* ARS consensus sequence (PACS) replaced *ars1* nucleotides 916-926 (ATAAATAAATT) with the linker sequence GCGGTACCCGG.

Plasmid DNA used for yeast transformations was purified using QIAGEN plasmid DNA purification kits (QIAGEN). pRC20 is pRS305 containing a full-length *S.pombe ars1* insert subcloned from pREP3X (Forsburg, 1993; Maundrell, 1993). pRC21 is pRS305 containing the full-length *ars1* sequence generated by PCR. These two plasmids display similar activities in standard ARS assays (described below).

PCR

Reaction mixtures contained 10 ng template DNA, 0.4 μM each primer, 200 μM deoxynucleoside triphosphates (dNTPs), 2.5 U purified or recombinant *Pfu* polymerase (Stratagene) and 1× buffer (Stratagene) in 100 μl. PCR conditions were 1 cycle of 94°C for 5 min, 40°C for 3 min and 72°C for 3 min followed by 25 cycles of 94°C for 1 min, 40°C for 3 min and 72°C for 3 min. These cycles were followed by a final extension step of 72°C for 7 min.

Electroporation

Transformation of *S.pombe* by electroporation was carried out as described (Prentice, 1992) with some modifications. Cells were grown in EMM+5S with shaking at 30°C and harvested at an $OD_{600\ nm}$ of 0.5–0.6 by centrifugation. Cells were then washed once each with ice-cold water then ice-cold 1 M sorbitol and resuspended in 1 M sorbitol to a density of 1×10^9 cells/ml. Forty μl of the cell suspension were mixed with 100 ng of plasmid DNA in 1 μl and incubated on ice for 5 min. Forty μl of the mixture were transferred to an ice-cold 0.2 cm cuvette (Bio-Rad) immediately before electroporation. Cells were pulsed at 2.0 kV, 200 Ω and 25 μF using a Bio-Rad gene pulser. Observed time constants varied slightly about the theoretical value of 5.0 ms. Immediately after the pulse, 0.9 ml of ice-cold 1 M sorbitol was added to the cuvette. Aliquots of the transformation were plated onto EMM–leu for selection. Colonies became visible after 3–5 days at 30°C. Electrocompetent *S.cerevisiae* cells were prepared and transformed as previously described (Becker and Guarente, 1991) using 100 ng plasmid DNA in 1 μl. Aliquots of the transformation were plated onto SC–leu plates. Colonies became visible after 2 or 3 days at 30°C. Using these methods typical control transformation efficiencies for *S.pombe* were 10^5 transformants/μg DNA and 10^3 transformants/μg DNA for *S.cerevisiae*.

ARS activity assays

Cells were transformed by electroporation and the number of colonies on selective plates was generally scored for up to 1 week after control transformants became visible. Activity of each sample was expressed as the transformation efficiency (number of transformants/μg DNA) relative to control (pRC20 and pRC*ARSH4* for *S.pombe* and *S.cerevisiae*, respectively) to account for variation between experiments. All transformation experiments were performed at least twice.

Dideoxynucleotide sequencing

Nucleotide sequences were determined using the Sequenase DNA sequencing kit (United States Biochemical) based on the method of Sanger *et al.* (1977). The endpoints of the nested boundary constructs were confirmed. The sequences of the entire *ars1* inserts of pRC21, pRCΔ1 and pRCΔ9 were determined. For all other constructs the sequence of at least the 50 bp segment encompassing the mutation was confirmed. Analysis of our constructs revealed a discrepancy from the published *ars1* sequence (Maundrell *et al.*, 1988). All plasmids analyzed, including pREP3X (the source of the wild-type *ars1*), pRC20 and pRC21, contain a 2 bp insertion (AT) after nucleotide position 969. The full-length *ars1* sequence used in our studies is therefore 1206 bp in length.

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