Roles for Internal and Flanking Sequences in Regulating the Activity of Mating-Type-Silencer-Associated Replication Origins in *Saccharomyces cerevisiae*

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Manuscript received July 6, 2000
Accepted for publication June 1, 2001

ABSTRACT

ARS301 and *ARS302* are inactive replication origins located at the left end of budding yeast (*Saccharomyces cerevisiae*) chromosome III, where they are associated with the *HML-E* and *-I* silencers of the *HML* mating type cassette. Although they function as replication origins in plasmids, they do not serve as origins in their normal chromosomal locations, because they are programmed to fire so late in S phase that they are passively replicated by the replication fork from neighboring early-firing *ARS305* before they have a chance to fire on their own. We asked whether the nucleotide sequences required for plasmid origin function of these silencer-associated chromosomally inactive origins differ from the sequences needed for plasmid origin function by nonsilencer-associated chromosomally active origins. We could not detect consistent differences in sequence requirements for the two types of origins. Next, we asked whether sequences within or flanking these origins are responsible for their chromosomal inactivity. Our results demonstrate that both flanking and internal sequences contribute to chromosomal inactivity, presumably by programming these origins to fire late in S phase. In *ARS301*, the function of the internal sequences determining chromosomal inactivity is dependent on the checkpoint proteins Mec1p and Rad53p.

In *Saccharomyces cerevisiae* a group of autonomously replicating sequence (ARS) elements—DNA sequences that function as replication origins in plasmids—is located near the left end of chromosome III (Figure 1). The ARS elements in this group, *ARS301–ARS304* plus *ARS320*, are inactive as replication origins in their normal chromosomal locations. Instead of firing on their own, they are passively replicated by the replication fork from the nearby active origin, *ARS305* (Dubey *et al.* 1991; Vujcic *et al.* 1999).

Two of these inactive origins, *ARS301* and *ARS302*, are parts of the *cis*-acting silencer elements (*HML-E* and *HML-I*, respectively) that are essential for repressing transcription of the mating type genes in the *HML* mating type cassette (Mahoney and Broach 1989; Mahoney *et al.* 1991). However, when silencing of the mating type cassettes is abrogated by mutation of the gene encoding Sir4p (one of the *trans*-acting proteins essential for silencing; reviewed in Laurenson and Rine 1992), *ARS301* and *ARS302* remain inactive as origins (Dubey *et al.* 1991). Thus, although *ARS301* and *ARS302* contribute to the formation of an altered chromatin structure that is essential for transcriptional silencing (reviewed in Lustig 1998; Stone and Pillus 1998), their inactivity as chromosomal replication origins does not appear to be a consequence of that altered structure. Furthermore, chromosomal inactivity is not a required feature of silencer-associated ARS elements. The ARS elements associated with the *HMR-E* and *-I* silencers near the right end of chromosome III are active (though inefficient) as origins (Rivier and Rine 1992; DeBeer and Fox 1999; Hurst and Rivier 1999; Rivier *et al.* 1999).

Results from three studies (Santocanale *et al.* 1999; Vujcic *et al.* 1999; Weinberger *et al.* 1999) suggest that *ARS301* is normally inactive because it is programmed to fire so late in S phase that it is passively replicated by the fork from early-firing *ARS305* before it has a chance to fire on its own (Figure 1). *ARS301* became partially active in its native chromosomal location when *ARS305* and *ARS306* were deleted (Vujcic *et al.* 1999). Under these conditions, the replication fork from the closest active origin, *ARS307*, did not reach *ARS301* until very late in S phase. The results indicate that in some cells *ARS301* fired on its own before the arrival of the fork from *ARS307* (Vujcic *et al.* 1999). In an independent study, Santocanale *et al.* (1999) attempted to activate *ARS301* by using *rad53* and *mec1* mutant cells. Mutations in these checkpoint genes had previously been shown to accelerate the firing of late origins (Santocanale and Diffley 1998; Shirahige *et al.* 1998). When such mutant cells were treated with hydroxyurea, which permits origin firing but blocks extensive fork progression, *ARS301* was partially activated (Santocanale *et al.* 1999). These results suggest that the combination of accelerated firing of late origins and inhibition of replication forks from early origins permitted *ARS301* to

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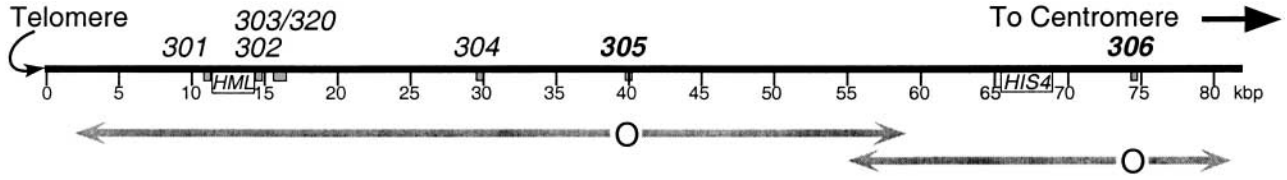


FIGURE 1.—Locations of origins and ARS elements, and directions of replication fork movement, in the leftmost quarter of *S. cerevisiae* chromosome III. The thick horizontal black line with scale in kilobase pairs represents distances along chromosome III, starting at the left telomere and extending toward the centromere. The positions of two genes (*HML* and *HIS4*) are indicated for reference; most genes are not shown. The positions of ARS elements (NEWLON *et al.* 1991; VUJCIC *et al.* 1999) are indicated by small boxes below the line and by corresponding numbers above the line. The numbers corresponding to the two ARS elements that are active as replication origins (HUBERMAN *et al.* 1988; ZHU *et al.* 1992) are indicated in boldface. The directions of the replication forks emanating from the two origins (O) are indicated by the gray arrows at the bottom (HUBERMAN *et al.* 1988; DUBEY *et al.* 1991; ZHU *et al.* 1992; additional unpublished results determined by neutral-alkaline 2-D gel electrophoresis).

fire before being inactivated by the fork from *ARS305*. Similarly, activation of *ARS301* was also observed (WEINBERGER *et al.* 1999) after hydroxyurea treatment in *orc2* mutant cells, in which the firing of late origins is also accelerated (SHIRAHIGE *et al.* 1998). Although these studies did not directly address *ARS302*, their results are consistent with the hypothesis that *ARS302*, like *ARS301*, is normally inactive because it is programmed to fire in very late S phase and is passively replicated by the fork from *ARS305* before it can fire on its own.

Previous investigations of the nucleotide sequences required for the function of chromosomally active origins (MARAHRENS and STILLMAN 1992; RAO *et al.* 1994; RASHID *et al.* 1994; THEIS and NEWLON 1994; HUANG and KOWALSKI 1996) revealed a modular structure. An essential A domain containing the 11-bp ARS consensus sequence (ACS) is flanked by a multipart B domain on the 3′ side of the T-rich strand of the ACS (MARAHRENS and STILLMAN 1992; RAO *et al.* 1994; RASHID *et al.* 1994; THEIS and NEWLON 1994; HUANG and KOWALSKI 1996) and in some cases by a C domain on the 5′ side (WALKER *et al.* 1990). The protein complex, origin recognition complex (ORC), binds to the A and the nearby B1 domains (BELL and STILLMAN 1992; DIFFLEY and COCKER 1992; RAO and STILLMAN 1995; ROWLEY *et al.* 1995). Whether chromosomally inactive ARS elements or silencer-associated ARS elements have a similar modular structure has not previously been tested.

In this study, we defined the minimal sequences that are required for *ARS301* and *ARS302* function as plasmid replication origins, and we explored the roles of internal and flanking sequences in rendering these origins inactive in their normal chromosomal locations.

MATERIALS AND METHODS

Yeast strains and methods: Yeast strain CT7-11 (Table 1) was used for the deletion and linker substitution analyses of *ARS301* and *ARS302*. Two-step gene replacement (SCHERER and DAVIS 1979; BOEKE *et al.* 1987) was employed to insert ARS elements into new chromosomal locations in appropriate recipient strains (Table 1). Details of strain construction (SHARMA 2000) are available upon request.

Mutational analyses of *ARS301* and *ARS302*: The nucleotide sequences studied here are part of the complete nucleotide sequence of *S. cerevisiae* chromosome III, GenBank accession no. NC_001135. All of the constructs described here were confirmed by sequencing. Standard *in vitro* mutagenesis techniques were employed. Details of methodology and all primer sequences are available upon request.

The clustered point mutations introduced into *ARS301* are as follows (wild-type sequence is upper case; mutations are lower case): 11/11 ACS match, **TTaTATcTaTT**; 10/11 ACS match, **AtAtgATTAAA**; Rap1p binding site, **TATGAATGcGaaT**. The clustered point mutations introduced into *ARS302* are as follows: 11/11 ACS match, **TTTgAatTcTT**; 10/11 ACS match **a**, **AAAATgaattc**; 10/11 ACS match **b**, **gAAttcAAAAT**; 10/11 ACS match **c**, **ATTgAatTcTA**; Abf1p binding site, **ACGTTTG aAtTcATA**.

For the purpose of evaluating their replication efficiency, all mutant constructs were cloned into a plasmid, pMW311, which is a derivative of pRS306 (SIKORSKI and HIETER 1989) containing a centromere (*CEN5*) and a selectable marker (*URA3*).

Evaluation of replication efficiency: Standard methods were employed for yeast transformation and growth under selective (no uracil) and nonselective (complete medium) conditions (SHERMAN 1991). Single transformant colonies were restreaked onto selective plates, and single colonies from the restreaking were inoculated into 3 ml selective medium and incubated for 20–24 hr at 30°. About 250 cells were then plated on nonselective and selective plates to determine the percentage of plasmid-containing cells under selection (replication efficiency). For transformants with high plasmid loss rates, more cells were plated on the selective plates to achieve ~250 final colonies.

DNA isolation and two-dimensional gel analysis: DNA was isolated from logarithmically growing cells (1–1.5 × 10^7/ml) using the glass bead method (HUBERMAN *et al.* 1987), and the replicating DNA was enriched as described (HUBERMAN *et al.* 1987). The two-dimensional (2-D) gel analyses were as described (BREWER and FANGMAN 1987), as were Southern blotting, hybridization, and image processing (DUBEY *et al.* 1991).

RESULTS

We wished to determine the extent to which ARS elements that are silencer associated and inactive in chromosomes (such as *ARS301* and *ARS302*) differ from chromosomally active nonsilencer-associated ARS elements (such as *ARS1*, *ARS305*, and *ARS307*). This requires identifying and comparing the sequences within each ARS element that are important for its function as a replica-

TABLE 1

***S. cerevisiae* strains used in this study**

Strain	Genotype	Reference
CT7-11	**a** *leu2-3,-112 his 3Δ1 trp1 ura3-52 ade2-101 can1*	Saavedra and Huberman (1986)
YKS100	CT7-11 Δ*ars305::ARS301* (115 bp)	This article
YKS101	CT7-11 Δ*ars305::ARS301* (95 bp)	This article
YKS102	CT7-11 Δ*ars305::ARS302*	This article
YKS103	CT7-11 Δ*ars1::ARS302*	This article
Y300	**a** *can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1*	Allen *et al.* (1994)
YKS104	Y300 Δ*ars305::ARS301* (115 bp)	This article
Y301	**a** *can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 sad1-1 (rad53)*	Allen *et al.* (1994)
YKS105	Y301 Δ*ars305::ARS301* (115 bp)	This article
YMP10860	**a** *ura3 leu2 trp1 his3 slm1*	Paulovich *et al.* (1997)
YKS106	YMP10860 Δ*ars305::ARS301* (115 bp)	This article
YMP10848	**a** *ura3 leu2 trp1 his3 slm1 mec1-1*	Paulovich *et al.* (1997)
YKS107	YMP10848 Δ*ars305::ARS301* (115 bp)	This article
YCS37	**a** *can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1* Δ*sir4::kanr* Δ*ARS1::HIS3*	Santocanale *et al.* (1999)
YKS108	YCS37 Δ*ars301::ARS305*	This article
YCS38	**a** *can1-100 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1* Δ*sir4::kanr* Δ*ARS1::HIS3 sad1-1 (rad53)*	Santocanale *et al.* (1999)
YKS109	YCS38 Δ*ars301::ARS305*	This article

tion origin in a plasmid. The ability of an ARS element to serve as a replication origin in a plasmid is frequently referred to as its "ARS activity." Since the sequences important for the ARS activities of several chromosomally active nonsilencer-associated ARS elements were previously characterized in detail (Marahrens and Stillman 1992; Rao *et al.* 1994; Rashid *et al.* 1994; Theis and Newlon 1994; Huang and Kowalski 1996), it was sufficient for us to similarly characterize the sequences needed for ARS activity by *ARS301* and *ARS302*.

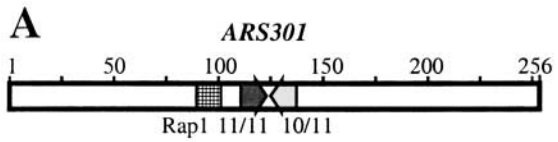
These two ARS elements were originally localized by deletion analyses (Broach *et al.* 1982). However, the regions defined by the deletions were not tested for independent ARS activity, leaving considerable uncertainty as to the boundaries of *ARS301* and *ARS302*. Consequently our first task was to accurately localize these ARS elements.

Preliminary experiments revealed that a PCR-derived 256-bp stretch contained full *ARS301* activity (Figure 2A). This stretch contains one 11/11 and one 10/11 match to the ACS as well as a putative Rap1p binding site (Buchman *et al.* 1988). Similar experiments revealed that a 275-bp *Hin*cII-*Hin*dIII restriction fragment (Figure 2B) contains the bulk of *ARS302* activity. This fragment has one 11/11 match and three 10/11 matches to the ACS as well as putative Abf1p and Rap1p binding sites (Buchman *et al.* 1988).

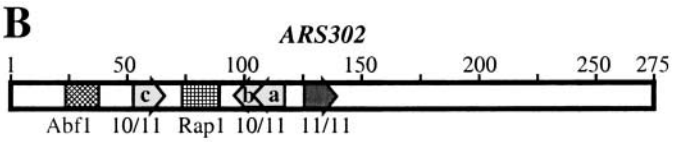
***ARS301* is similar to most other *S. cerevisiae* ARS elements:** Intact *ARS301* (Figure 2A, line 1) displays high replication efficiency, which is slightly higher than that of *ARS305*, a well-characterized strong ARS element (Huang and Kowalski 1993, 1996; Lin and Kowalski 1997; Figure 2A, line 18). To identify the essential ACS match and to assess the contribution of the putative Rap1p binding site to *ARS301* function, we generated clustered point mutations targeting the ACS matches and the Rap1p binding site (Figure 2A, lines 2–4). Mutation of the 11/11 ACS match abolished ARS activity, but mutation of the 10/11 match had no effect. Thus *ARS301*, like most other tested ARS elements, has only one essential ACS match. Mutation of the Rap1p binding site reduced ARS activity only slightly.

Progressive deletions from either side of the essential 11/11 ACS within the 256-bp stretch were generated by PCR, and each deletion was tested for ARS activity as above. Deletion of up to 60 bp from the 3′ side (Figure 2A, lines 10–12) did not greatly reduce ARS activity, but further deletions (lines 13–15) had significant effects. In contrast, none of the deletions 5′ of the ACS caused an appreciable change in ARS activity (Figure 2A, lines 5–9). Consistent with the directed mutation analysis (Figure 2A, line 4), complete deletion of the Rap1p binding site had no major effect (compare lines 8 and 9 and lines 16 and 17). Combination of the 3′ and 5′ deletions revealed that a short stretch of DNA—95 bp—comprising an essential ACS match and 3′ flanking sequences (Figure 2A, line 17) is about two-thirds as active as intact *ARS301*. Thus *ARS301*, like well-characterized chromosomally active nonsilencer-associated *S. cerevisiae* ARS elements (reviewed in Theis and Newlon 1996) consists primarily of an essential ACS match and 3′ flanking B-domain sequences.

***ARS302* requires A, B, and C domains:** Preliminary experiments (not shown) revealed that a 275-bp *Hin*cII-*Hin*dIII fragment contains the bulk of *ARS302* activity. However, intact *ARS302* (Figure 2B, line 1) has a much



Construct	Replication Efficiency
1) wildtype	79.5 ± 18.0
2) 11/11⁻	0
3) 10/11⁻	79.8 ± 15.3
4) RAP1⁻	69.6 ± 16.1
5) Δ1-21	77.1 ± 13.6
6) Δ1-41	73.8 ± 14.8
7) Δ1-61	67.5 ± 4.3
8) Δ1-81	57.8 ± 7.7
9) Δ1-101	55.5 ± 15.7
10) Δ234-256	72.5 ± 12.8
11) Δ217-256	69.0 ± 7.8
12) Δ197-256	59.1 ±13.0
13) Δ177-256	35.3 ±11.2
14) Δ157-256	28.3 ±9.3
15) Δ137-256	0
16) Δ1-81/Δ197-256	74.1 ± 9.0
17) Δ1-101/Δ197-256	53.3 ± 7.4
18) *ARS305*	75.1 ± 11.8



Construct	Replication Efficiency
1) wildtype	19.7 ± 5.8
2) 11/11⁻	0
3) 10/11a⁻	16.1 ± 4.5
4) 10/11b⁻	13.7 ± 3.4
5) 10/11c⁻	15.1 ± 5.0
6) ABF1⁻	10.1 ± 2.7
7) Δ1-39	9.4 ± 0.8
8) Δ1-74	5.3 ± 3.5
9) Δ1-109	0
10) Δ237-275	9.2 ± 1.8
11) Δ217-275	9.3 ± 2.4
12) Δ197-275	8.1 ± 3.0
13) Δ177-275	5.1 ± 2.6
14) Δ157-275	0

FIGURE 2.—Characterization of *ARS301* and *ARS302*. (A) *ARS301*. The starting nucleotide is 11136 in GenBank NC_001135. (B) *ARS302*. The starting nucleotide is 14565 in GenBank NC_001135. Replication efficiency was measured as the percentage of plasmid-bearing cells under selection (MATERIALS AND METHODS). "0" indicates that the cells could not grow under selection due to total lack of plasmid replication. The results shown are an average of three independent experiments with standard deviations. The *ARS305* plasmid employed as control in A, line 18, was constructed by cloning the 550-bp *Nru*I-*Cla*I fragment containing *ARS305* into the same centromere-bearing plasmid used to evaluate *ARS301* and *ARS302* activity.

lower replication efficiency than *ARS301* (Figure 2A, line 1) or *ARS305* (Figure 2A, line 18).

A linker substitution mutation that disrupted the 11/11 ACS match inactivated *ARS302* (Figure 2B, line 2). Similar mutations within each of the 10/11 matches did not greatly affect replication efficiency (lines 3–5). A mutation within the putative Abf1p binding site reduced replication efficiency by 50% (line 6). Mutations within the putative Rap1p binding site reduced replication efficiency even farther (Figure 3).

PCR-generated deletions from the 5′ side of the essential ACS (Figure 2B, lines 7–9) all reduced ARS activity. Construct Δ1-39, which deletes the Abf1p binding site, reduced ARS activity to a level similar to that of the linker-substituted Abf1p site (Figure 2B, lines 6 and 7). Further deletions resulted in greater loss or total absence of ARS activity (Figure 2B, lines 8 and 9). Thus, C-domain sequences are required for *ARS302* function even in the presence of an intact B domain. Progressive deletions within the B domain also compromised ARS activity (Figure 2B, lines 10–14).

Thus *ARS302* contains essential A, B, and C domains. Although not individually essential, sequences at the extreme left and right ends of the 275-bp *Hinc*II-*Hin*dIII fragment contribute significantly to ARS function. For this reason we used the complete restriction fragment in subsequent experiments.

***ARS302* is modular:** Because *ARS302* is unusually large and has an unusually stringent requirement for C-domain sequences, we conducted higher resolution analysis of its sequence requirements by progressive linker substitution (ls). To accommodate the large size of *ARS302*, we initially used a large 14-bp *Kpn*I linker (G + C rich) to generate 20 linker substitutions spanning the 275-bp fragment. Six substitutions (ls7 and ls9–13) completely abolished ARS activity (data not shown). To identify the important sequences within these regions at higher resolution, 7-bp *Kpn*I linkers were employed. The 7-bp linker substitutions are designated ls7a, ls7b, etc. in Figure 3. Despite repeated efforts, we were unable to clone ls13a in bacterial cells. We did not subdivide ls10, which includes the essential ACS.

As in the case of linker substitutions targeting the ACS in other tested ARS elements (MARAHRENS and STILLMAN 1992; RAO *et al.* 1994; RASHID *et al.* 1994; THEIS and NEWLON 1994; HUANG and KOWALSKI 1996), ls10 abolished ARS function (Figure 3). But in contrast to other tested ARS elements, three additional linker substitutions also led to complete loss of ARS activity (Figure 3, ls9b, ls11b, and ls12a).

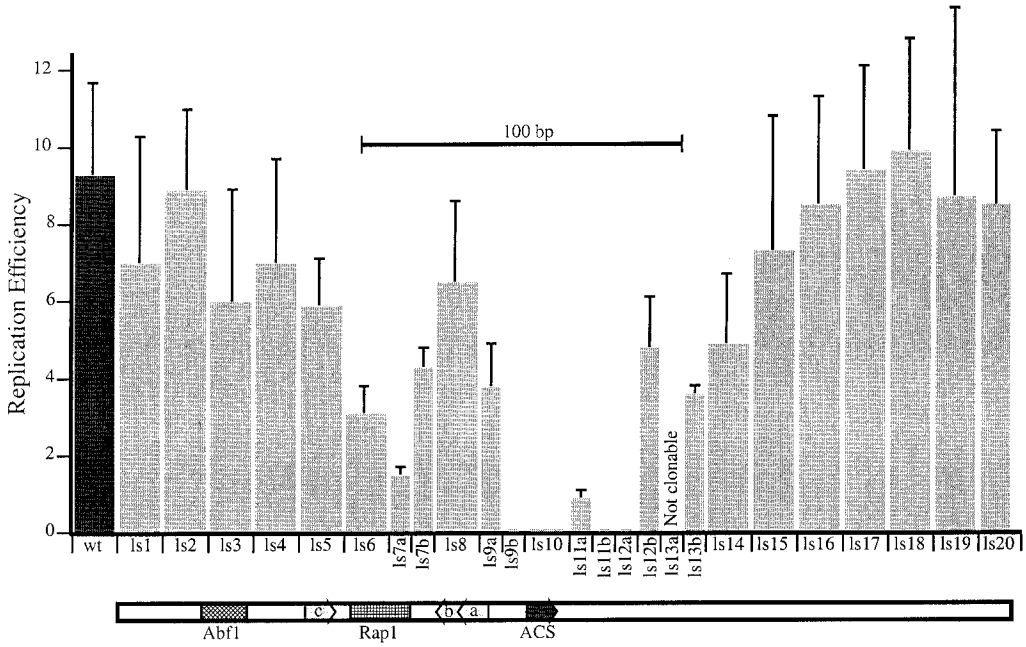


FIGURE 3.—Linker substitution analysis of *ARS302*. A 14-bp *Kpn*I linker was used to scan the 275-bp *ARS302* fragment in head-to-tail fashion, generating a total of 20 clones (ls20 has a 10-bp substitution). Additional substitutions were performed for the five regions covered by ls7, ls9, and ls11–ls13, utilizing a 7-bp *Kpn*I linker. The results plotted are the averages of three independent experiments with standard deviations. In spite of repeated efforts, we were unable to clone ls13a in bacterial cells and therefore could not assess the effect of that substitution. We do not know why wild-type *ARS302* replication efficiency was lower in this series of experiments (~9%) than in the series reported in Figure 2B (~20%), which was performed several months earlier. We note that within each series repetitions of the wild-type control were consistently high (Figure 2B) or low (Figure 3).

Consistent with Figure 2B, linker substitution revealed that sequences within the C domain contribute significantly to *ARS302*. ls3, which overlaps the putative Abf1p binding site, resulted in a small reduction in ARS activity, and ls6 and ls7a, which overlap the putative Rap1p binding site, had a greater effect (Figure 3). ls7b and ls9a also reduced ARS activity significantly, suggesting that the substituted sequences are important for *ARS302* function.

Thus our mutational analysis of *ARS302* revealed that it resembles chromosomally active nonsilencer-associated ARS elements in being modular, with an essential ACS and B domain. It differs from previously characterized ARS elements in having an *essential* C domain. Since *ARS301* does not have an essential C domain (Figure 2A), it is unlikely that this feature of *ARS302* is relevant to its chromosomal inactivity or its role in silencing. Rather, this feature is more likely to be another of the many possible variations in sequence and organization of *S. cerevisiae* ARS elements (RASHID *et al.* 1994; THEIS and NEWLON 1997).

The sequences flanking *ARS301* can inactivate *ARS305*: SANTOCANALE *et al.* (1999), VUJCIC *et al.* (1999), and WEINBERGER *et al.* (1999) previously demonstrated that *ARS301* is inactive as an origin at its native chromosomal location because it is extremely late firing at this position. To test the role of flanking sequences in rendering *ARS301* inactive, we replaced *ARS301* with *ARS305*, which is an active, early-firing origin at its native chromosomal position (HUBERMAN *et al.* 1988; REYNOLDS *et al.* 1989; DUBEY *et al.* 1991; HUANG and KOWALSKI 1993; BOUSSET and DIFFLEY 1998). Specifically, we deleted the sequences corresponding to the 115-bp version of *ARS301* (Figure 2A, line 16) and replaced them with a 550-bp *Nru*I-*Cla*I restriction fragment containing *ARS305* (HUANG and KOWALSKI 1993). The cartoon (not to scale) in Figure 4A shows the structure of the modified chromosome with an extra copy of *ARS305* at the position of *ARS301*. Two-dimensional gel analysis of DNA from exponentially growing cells of this strain revealed that the extra copy of *ARS305* generated a Y arc but not a bubble arc (Figure 5, A and B), indicating that in this new context *ARS305* is passively replicated. A control experiment using the same DNA preparation (Figure 5C) revealed a strong bubble arc and weaker Y arc from *ARS305* at its native position. Thus the absence of a bubble arc in Figure 5B could not be attributed to loss of bubble intermediates during DNA preparation. Since *ARS301* at its normal location is inactive because it fires late in S phase (SANTOCANALE *et al.* 1999; VUJCIC *et al.* 1999; WEINBERGER *et al.* 1999), we assume that inactivation of *ARS305* at this position is accomplished in a similar fashion—by forcing it to fire so late that it is passively replicated by the fork from the active copy of *ARS305* before it can fire on its own.

***ARS301* and *ARS302* are not activated when moved to positions occupied by active origins:** The above results suggest that the sequences flanking *ARS301* are capable, on their own, of forcing an otherwise early-firing ARS element to fire late in S phase. We suspected, however, that sequences *within ARS301* and *ARS302* might also contribute to determination of late firing. To test this possibility, we moved *ARS301* and *ARS302* to other locations normally occupied by active replication origins. We chose the location of *ARS305* on chromosome III (as dia-

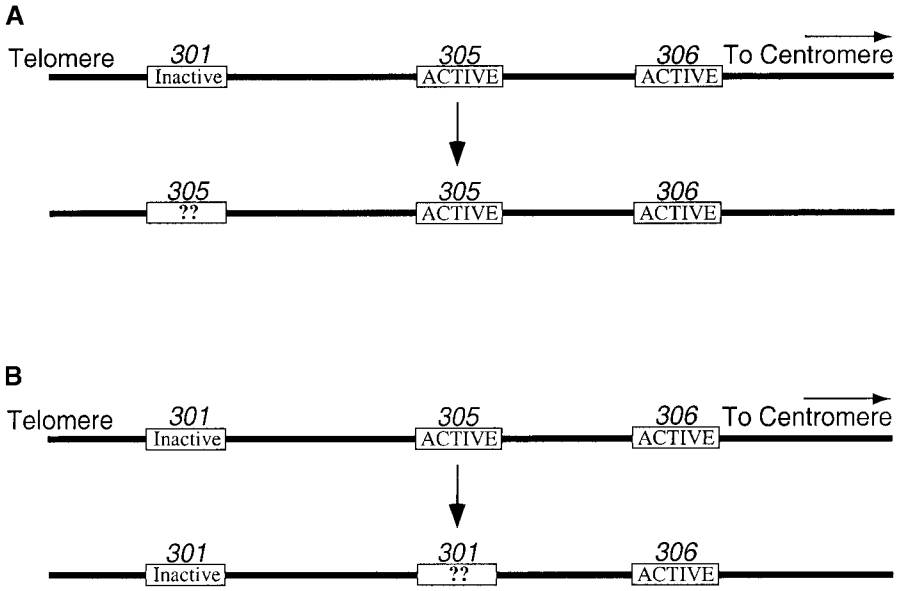


FIGURE 4.—Cartoons summarizing ARS element transplacement experiments (not to scale). (A) Transplacement of a copy of *ARS305* to the position normally occupied by *ARS301*. The experiment was designed to test whether *ARS305* at the position of *ARS301* would be active or inactive at its new location (question marks). (B) Transplacement of a copy of *ARS301* to the position normally occupied by *ARS305*. The experiment was designed to test whether *ARS301* at the position of *ARS305* would be active or inactive at its new location (question marks).

grammed in Figure 4B) and the location of *ARS1* on chromosome IV as recipient sites for transposition, since both *ARS305* and *ARS1* are well-characterized, early-firing, chromosomally active origins (MARAHRENS and STILLMAN 1992; HUANG and KOWALSKI 1993, 1996; LIN and KOWALSKI 1997). In addition, the *ARS1* location offered the opportunity to test whether origin inactivation is unique to chromosome III or also applies to other chromosomes.

Two-dimensional gel analyses (Figure 5, D–I) revealed that neither *ARS301* nor *ARS302* is significantly activated by transposition to the new locations. Constructs of 115 bp and 95 bp containing *ARS301* (with or without its Rap1p binding site) at the *ARS305* location display primarily Y arc signals, indicating that *ARS301* at this position is passively replicated (presumably by the fork from *ARS306*; Figures 1 and 4B) most of the time (Figure 5, D and F). Restriction fragments containing *ARS302* at the *ARS305* position (Figure 5G) or at the *ARS1* position (Figure 5H) were also primarily passively replicated. In all cases, control experiments demonstrated the presence of bubble arcs at known active origins in the DNA preparations (Figure 5, E and I, and data not shown).

Thus, these results suggest that sequences within *ARS301* and *ARS302* are sufficient to prevent them from firing even after they have been moved to chromosomal locations usually occupied by active early-firing origins. Presumably these internal sequences dictate late origin firing. The fact that *ARS302* is inactive at the position of *ARS1* on chromosome IV demonstrates that the phenomenon of ARS element inactivation is not restricted to chromosome III.

***ARS305* is not activated at the position of *ARS301* in *rad53* mutant cells:** SHIRAHIGE *et al.* (1998) demonstrated that a minimally active ARS element on chromosome VI, *ARS608*, could be further activated by mutation of the checkpoint gene, *RAD53*. This activation was due to accelerated firing of *ARS608* in the mutant strain. In contrast, the results of SANTOCANALE *et al.* (1999) showed that *ARS301* at its native location could not be activated solely by a *rad53* mutation. The inability of *ARS301* to function in the *rad53* mutant strain is presumably a consequence of the combined effects of late-firing determinants within *ARS301* itself and in its flanking sequences. Since *ARS305* fires early at its native location, it is likely that *ARS305* does not contain internal sequences specifying late firing. We therefore asked whether a copy of *ARS305* moved to the position of *ARS301*—which is presumably rendered late firing only by flanking sequences—could be activated by a *rad53* mutation.

We moved the 550-bp fragment containing *ARS305* (the same fragment used in Figure 5B) to the position of *ARS301* in *rad53* mutant cells. Like the *RAD53* strain (Figure 5B), the *rad53* mutant strain generated only a Y arc (Figure 6A). When a 2-D gel of replication intermediates from the same DNA preparation was probed for *ARS305* at its normal position, the usual bubble arc characteristic of *ARS305* origin function was detected (Figure 6B). Thus the absence of a bubble arc in Figure 6A was not due to a defective DNA preparation. Instead, the presence of a Y arc without a bubble arc suggests that, even in a *rad53* mutant strain, the sequences flanking *ARS301* render *ARS305* at the position of *ARS301* sufficiently late firing that it cannot fire on its own before being passively replicated by the fork from *ARS305* at its native position, where it is early firing.

***ARS301* at the location of *ARS305* is activated in *rad53* and *mec1* mutant cells:** We next wanted to check the effects of *rad53* and *mec1* mutations on the late firing determined by sequences *internal* to *ARS301*. For that purpose, we moved *ARS301* (115-bp construct; Figure 2A,

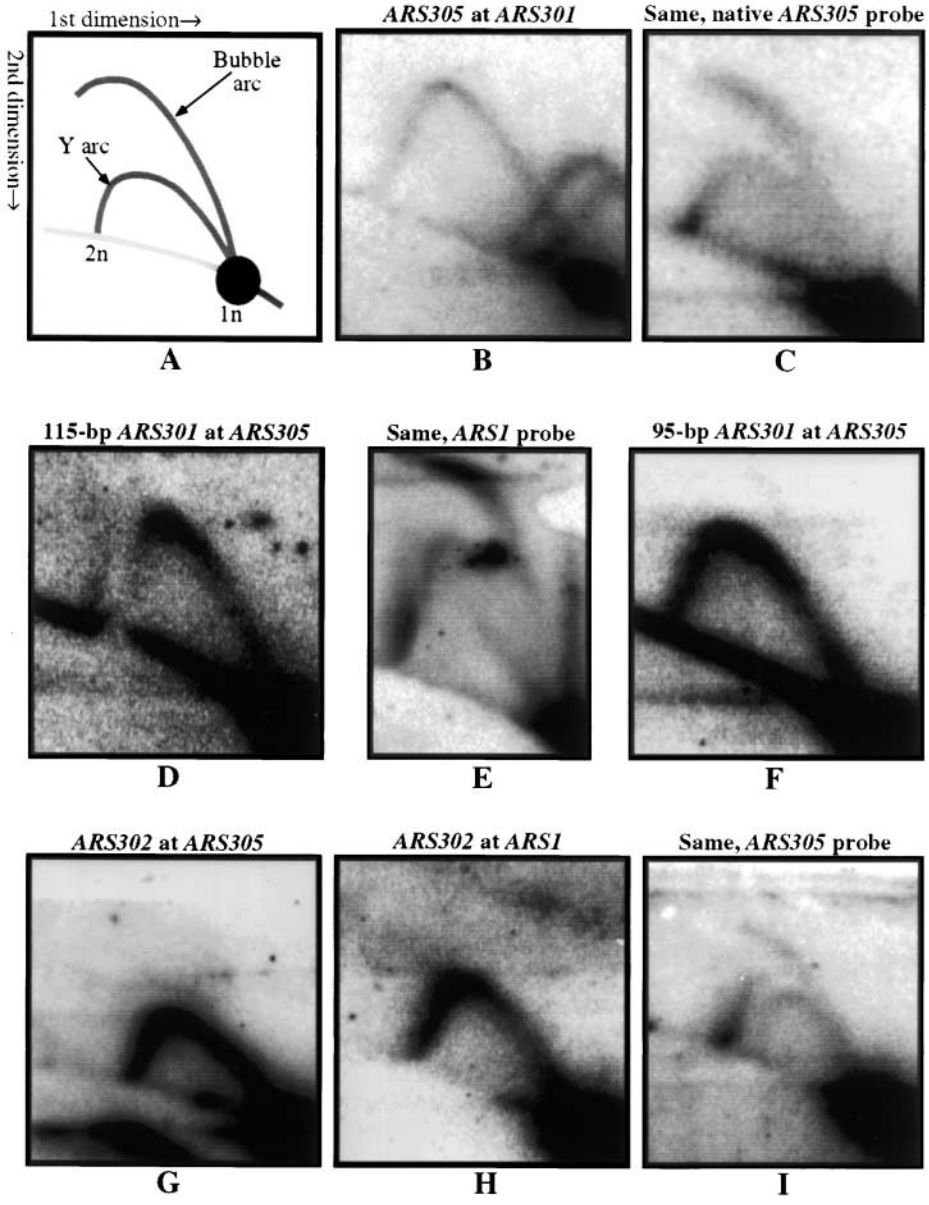


FIGURE 5.—Two-dimensional gel evaluation of the activity of potential replication origins moved to new locations. (A) Diagram illustrating the positions of Y arcs (indicative of passive replication) and bubble arcs (indicative of active origin firing) after neutral-neutral two-dimensional gel electrophoresis (BREWER and FANGMAN 1987). (B and C) *ARS305* is not active in the position of *ARS301*. A 550-bp *Nru*I-*Cla*I fragment containing *ARS305* was moved to the position of *ARS301* (strain YKS108; Table 1). DNA was isolated from this strain and separated into two portions. The first portion was digested with *Eco*RV and *Bam*HI and then subjected to 2-D gel electrophoresis. The blot was probed with a 1.2-kbp *Xba*I fragment covering *ARS301* (B). This probe detects a 4.2-kbp *Eco*RV-*Bam*HI fragment centered on the ectopic copy of *ARS305*. It also detects two smaller restriction fragments containing sequences similar to those flanking *ARS301*. These also generate Y arcs, which are partially visible in the right half of B. The second DNA portion was digested with *Eco*RV and *Hin*dIII and subjected to 2-D gel electrophoresis (C). The blot was probed with the 550-bp *Nru*I-*Cla*I fragment containing *ARS305*. This probe detects a 4.7-kbp restriction fragment centered on *ARS305* at its native location. Because the ectopic copy of *ARS305* at the *ARS301* position was inserted into that position as a *Hin*dIII cassette, it is excised by *Hin*dIII digestion as a small fragment that migrates out of the detectable range. (D–F) *ARS301* is inactive at the position of *ARS305*. The 115-bp (D) and 95-bp (F) fragments containing *ARS301* (Figure 2A, lines 16 and 17) were moved to the position of *ARS305* (strains YKS100 and YKS101; Table 1). DNA was digested with *Nco*I and then subjected to 2-D gel electrophoresis. A 2.1-kbp *Bam*HI-*Pst*I fragment covering *ARS305* was used to detect a 4.6-kbp *Nco*I restriction fragment centered on the transplaced copy of *ARS301*. In E, DNA from strain YKS100 was probed with a 1.4-kbp *Eco*RI fragment containing *ARS1*. This probe detects a 4.7-kbp *Nco*I fragment centered on *ARS1*. (G–I) *ARS302* is not activated in the position of *ARS305* or *ARS1*. The 275-bp *Hinc*II-*Hin*dIII fragment containing *ARS302* was moved to the locations of *ARS305* and *ARS1* (strains YKS102 and YKS103; Table 1). Isolated DNA was cut with the indicated restriction enzymes and subjected to 2-D gel electrophoresis. A 4.6-kbp *Eco*RI fragment centered on the copy of *ARS302* at the *ARS305* position was detected by probing with a 2.1-kbp *Bam*HI-*Pst*I fragment covering *ARS305* (G). A 4.7-kbp *Nco*I fragment centered on *ARS302* at the *ARS1* position was detected by probing with a 1.4-kbp *Eco*RI fragment covering *ARS1* (H). The same blot was reprobed with the 2.1-kbp *Bam*HI-*Pst*I fragment covering *ARS305* to reveal the 5.1-kbp *Nco*I fragment centered on *ARS305* (I).

line 16) to the position of *ARS305* in cells carrying mutant *rad53* and *mec1* genes. *ARS301* became partially active in both the *rad53* and *mec1* mutant strains (Figure 6, C and D). However, when *ARS301* was moved to the position of *ARS305* in the corresponding wild-type strains, it remained inactive (Figure 6, E and F), as in strain YKS100 (Figure 5D). Thus, in the *rad53* and *mec1* mutant strains, *ARS301* at the *ARS305* location is rendered sufficiently early firing that in some cells it can fire before being inactivated by the fork arriving from early-firing *ARS306*.

DISCUSSION

Until this study, the chromosomal locations of *ARS301* and *ARS302* had been determined only approximately (BROACH *et al.* 1982). Nevertheless, on the basis of this

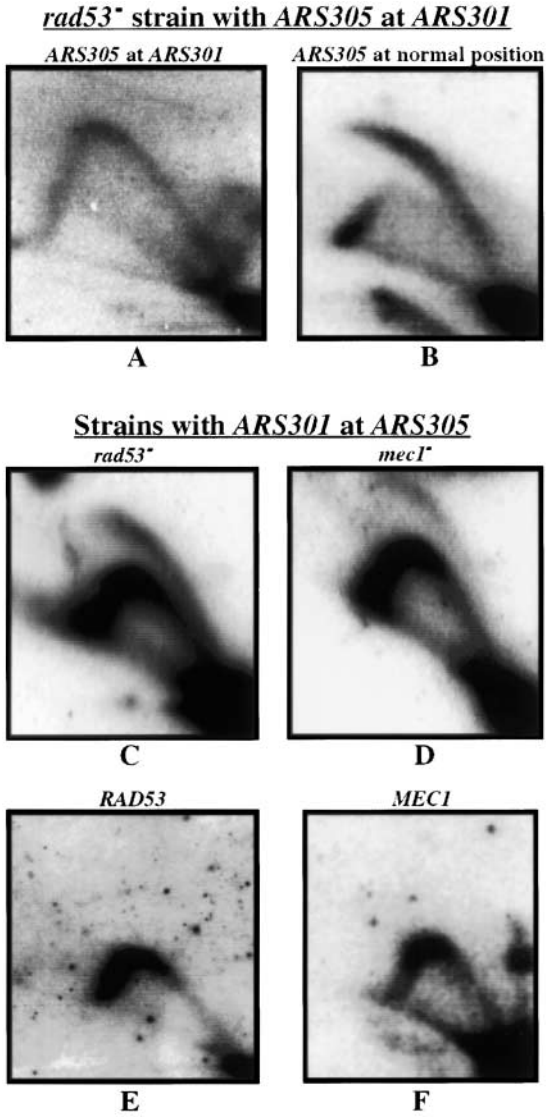


FIGURE 6.—Effects of checkpoint mutations on activities of transplaced origins. (A) *ARS305* at the position of *ARS301* is not activated by *rad53* mutation. The 550-bp fragment containing *ARS305* activity was moved to the *ARS301* location in *rad53* mutant cells (strain YKS109; Table 1). The restriction enzymes and probe used were the same as in Figure 5B. (B) *ARS305* is active at its native location in *rad53* mutant cells. The same DNA preparation employed in A was digested with *Eco*RV-*Hin*dIII and was probed as in Figure 5C. (C and D) *rad53* and *mec1* mutations result in partial activation of *ARS301* at the position of *ARS305*. The 115-bp fragment containing *ARS301* (Figure 2A, line 16) was moved to the position of *ARS305* in *rad53* or *mec1* mutant cells (strains YKS105 and YKS107; Table 1). The restriction fragments and probe utilized for the 2-D gel analysis are as in Figure 5, D and F. (E and F) *ARS301* is inactive at the *ARS305* position in the wild-type strains from which the *rad53* and *mec1* mutants used in C and D were derived. These analyses employed DNA from strains YKS104 (E) and YKS106 (F). Restriction fragments and probes were as in C and D.

approximate localization, it was possible to conclude that *ARS301* and *ARS302* are associated with the *HML-E* and *-I* silencers and are normally inactive as chromosomal replication origins (Figure 1; DUBEY *et al.* 1991). Recently, it was demonstrated (SANTOCANALE *et al.* 1999; VUJCIC *et al.* 1999; WEINBERGER *et al.* 1999) that the inactivity of *ARS301* and probably *ARS302* is due to these ARS elements being programmed to fire extremely late in S phase. The experiments described here were designed to more precisely determine the locations of *ARS301* and *ARS302*, to test whether their sequence organizations differ significantly from those of active nonsilencer-associated origins, and to evaluate the contributions of both internal and flanking sequences to their inactivity as chromosomal origins.

***ARS301* and *ARS302* resemble other ARS elements:** Our analysis of *ARS301* (Figure 2A) revealed that a stretch of 95 bp (the essential ACS match, 10 bp 5′ of the ACS and 74 bp 3′ of the ACS) is sufficient for ARS activity. In contrast to our results for *ARS301* (Figure 2A) and to results from other labs for other ARS elements (reviewed in THEIS and NEWLON 1996), our analysis of *ARS302* revealed that sequences 5′ of the essential ACS match (C domain) as well as sequences 3′ of the ACS (B domain) are absolutely required for activity. For this reason, *ARS302* is larger than the typical *S. cerevisiae* ARS element.

Systematic linker substitutions of *ARS302* (Figure 3) revealed that it has a modular structure similar to those of other ARS elements (MARAHRENS and STILLMAN 1992; RAO *et al.* 1994; RASHID *et al.* 1994; THEIS and NEWLON 1994; HUANG and KOWALSKI 1996). The A domain of *ARS302* (ls9b and ls10) is essential, and so are the sequences that correspond in position to the B1 component of other ARS elements (ls11b and ls12a). Additional B-domain sequences (ls13 and ls14) that correspond in position to the B2 element of *ARS1* and *ARS307* are also important. Important C-domain sequences include ls9a, which is adjacent to the A domain. Additional experiments are needed to determine whether ls9a inhibits the ORC binding function of the adjacent A domain. It seems likely that ls6 and ls7a in the C domain inhibit *ARS302* by interfering with Rap1p binding. Similarly, the mild inhibition by ls3 may be due to interference with Abf1p binding. The causes of the inhibitions by ls1, ls5, and ls7b are not clear. Thus, although *ARS302* differs from other ARS elements in some respects, both *ARS301* and *ARS302* resemble them in others, especially with regard to sequences required for ORC binding. Consistent with this observation is the demonstration that ORC binds to *ARS301* and *ARS302* *in vitro* (BELL *et al.* 1993), and a prereplicative complex containing ORC and additional proteins is assembled at *ARS301 in vivo* (SANTOCANALE and DIFFLEY 1996).

Although our survey of the sequence requirements for plasmid origin function by *ARS301* and *ARS302* did not reveal unusual structural features of these ARS elements (Figures 2 and 3), the survey did emphasize that both ARS elements are located close to binding sites for transcription factors (Abf1p and Rap1p) that have

been implicated in transcriptional silencing (Brand *et al.* 1987; Mahoney and Broach 1989; Mahoney *et al.* 1991). It seems likely that it is the spacing and density of ORC, Rap1p, and Abf1p binding sites that distinguishes nonsilencer-associated from silencer-associated origins. The results presented here indicate that the Rap1p and Abf1p binding sites close to silencer-associated origins may (as in the case of the Rap1p site in *ARS302*; Figure 3) or may not (as in the case of the Rap1p site near *ARS301*; Figure 2) contribute significantly to plasmid replication origin activity.

Our results and those from earlier studies (Newlon *et al.* 1991, 1993) indicate that only one origin, *ARS301*, is closely associated with the *HML-E* silencer. In contrast, the *HML-I* silencer is closely associated with three origins: *ARS302*, which is part of *HML-I*, and *ARS303* and *ARS320*, which are <2 kbp away (Figure 1). *ARS302, ARS303*, and *ARS320* are all individually weak ARS elements. Several individually weak origins are similarly clustered close to the *HMR-E* silencer near the right end of chromosome III (DeBeer and Fox 1999; Hurst and Rivier 1999). It is possible that interactions between closely spaced weak origins may contribute to silencer function in some cases (DeBeer and Fox 1999; Hurst and Rivier 1999).

In addition to the evidence provided here, further evidence that ability to function as a replication origin in a plasmid does not correlate with ability to serve as a replication origin in chromosomes comes from the following observations: (1) *ARS301* and *ARS305* are both efficient replication origins in plasmids (Figure 2A, lines 1 and 18), yet *ARS305* is a chromosomal origin and *ARS301* is not; and (2) *ARS302* and *ARS307* are both inefficient plasmid origins (Figures 2B and 3; Theis and Newlon 1994), yet *ARS307* is a chromosomal origin and *ARS302* is not. Taken together, the results summarized above suggest that other factors—not ARS structure and not plasmid origin efficiency—dictate whether or not an origin can fire in the chromosome.

Flanking sequences contribute to maintaining *ARS301* inactive: Santocanale *et al.* (1999), Vujcic *et al.* (1999), and Weinberger *et al.* (1999) have recently shown that these other factors involve control of replication timing. *ARS301* and *ARS302* are rendered inactive because they are programmed to fire extremely late during S phase. For this reason, they are always passively replicated by the fork from early-firing *ARS305* before they have a chance to fire on their own.

To address the contribution of sequences flanking *ARS301* to rendering it inactive, we moved *ARS305*, which normally fires very early (Reynolds *et al.* 1989; Bousset and Diffley 1998), to the position of *ARS301* (Figure 4A). We found that *ARS305* was inactivated at its new location (Figure 5B). Even in *rad53* mutant cells, *ARS305* at the position of *ARS301* was inactive (Figure 6A). The simplest explanation of these observations is that sequences flanking *ARS301* are capable of rendering any origin inserted at the position of *ARS301* so late firing that, before it can fire on its own, it is passively replicated by the fork from *ARS305*. This effect is not blocked even when the firing of late origins is accelerated due to *rad53* mutation. Since the sequences flanking *ARS301* influence the activity of *ARS305* when it is substituted for *ARS301*, it is reasonable to infer that these sequences contribute to maintaining *ARS301* inactive in the chromosome.

***ARS301* and *ARS302* may contain internal late-firing determinants:** Earlier experiments suggested that the default time for origin firing is early and that ARS elements within certain regions of the *S. cerevisiae* genome fire later than others due to the influence of flanking sequences (Ferguson and Fangman 1992; Friedman *et al.* 1997). That this might not be the case for *ARS301* was suggested by Bousset and Diffley's (1998) observation that a plasmid (pCS1) containing *ARS301* with short 5′ and 3′ flanks replicates in late S phase. Our measurements of *ARS301* location and size (Figure 2A) now permit the conclusion that the 208-bp insert in late-replicating pCS1 includes the 95-bp minimal *ARS301* (Figure 2A, line 17), 93 bp of 5′ flanking sequence, and 20 bp of 3′ flanking sequence. These measurements raise the possibility that the determinants of late origin firing in pCS1 may lie (at least in part) within the minimal ARS element rather than within the short flanking sequences.

To assess the role in replication timing and origin activity of sequences within minimal *ARS301* and *ARS302*, we moved minimal versions of both ARS elements to the position of *ARS305* (Figure 4B) and asked if either was now activated. In addition, we also transposed *ARS302* to the position of *ARS1* on chromosome IV to address the possible role of specific chromosomal context in its inactivity. The results in Figure 5 show that, even at positions normally occupied by active origins, minimal *ARS301* and *ARS302* are unable to significantly initiate replication. This suggests that, even though they have now been separated from their normal flanking sequences, their time of firing at the ectopic locations is not sufficiently advanced to allow them to initiate replication before being passively replicated by forks from nearby early-firing origins. Since both *ARS305* and *ARS1* are active early-firing replication origins at their normal locations, it is unlikely that the sequences flanking them dictate late origin firing. Thus, the internal sequences within minimal *ARS301* and *ARS302* appear to be capable of specifying late origin firing on their own. This capability is not confined to chromosome III, as *ARS302* is also inactive at the position of *ARS1* on chromosome IV.

To address the regulation of late firing specified by the internal sequences, we asked if *ARS301* could be activated when moved to the *ARS305* position in *mec1* and *rad53* checkpoint-deficient cells. We observed that *ARS301* at the *ARS305* location became partially active in *rad53* and *mec1* mutant strains (Figure 6, C and D). This obser-

vation suggests that *ARS301* is prevented from firing at the *ARS305* position by an active checkpoint mechanism dependent on the Mec1 and Rad53 proteins and this mechanism acts at least in part on the minimal *ARS301* sequence that was transposed to the *ARS305* position. Presumably this is the same Mec1p- and Rad53p-dependent mechanism that was previously demonstrated to retard the activation of late-firing origins (Santocanale and Diffley 1998; Shirahige *et al.* 1998).

These results imply that *ARS301* is inherently late firing, and some of the sequences responsible for that late firing are located within a stretch of 95 bp. This provides an excellent starting point for the precise localization of *cis*-acting sequences responsible for determining the time of firing of a eukaryotic origin.

Both internal and flanking sequences contribute to rendering *ARS301* and *ARS302* inactive in the chromosome: Thus our results suggest that in their normal chromosomal positions *ARS301* and *ARS302* are inactivated by two effects, each of which contributes to late firing. Late firing is imposed first by *internal* sequences within the ARS element itself, which prevent minimal *ARS301* and *ARS302* from being activated at the *ARS305* position, and second by *flanking* sequences, which are capable of rendering even a normally early-firing origin (*e.g.*, *ARS305*) late firing. It is not surprising, therefore, that activation of *ARS301* and *ARS302* in their normal chromosomal positions requires draconian measures (Santocanale *et al.* 1999; Vujcic *et al.* 1999; Weinberger *et al.* 1999).

We thank Rolf Sternglanz, John Diffley, and Deborah Mahoney for providing yeast strains; and we thank the anonymous referees for helpful suggestions. This research was supported by grants R01-GM49294 and R21-CA84302 from the National Institutes of Health and by a 1999 Developmental Funds award from the Roswell Park Cancer Center Support Grant (P30 CA16056-24).

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Communicating editor: J. Rine