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The spatial arrangement of ORC binding modules determines the functionality of replication origins in budding yeast

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ABSTRACT

In the quest to define autonomously replicating sequences (ARSs) in eukaryotic cells, an ARS consensus sequence (ACS) has emerged for budding yeast. This ACS is recognized by the replication initiator, the origin recognition complex (ORC). However, not every match to the ACS constitutes a replication origin. Here, we investigated the requirements for ORC binding to origins that carry multiple, redundant ACSs, such as *ARS603*. Previous studies raised the possibility that these ACSs function as individual ORC binding sites. Detailed mutational analysis of the two ACSs in *ARS603* revealed that they function in concert and give rise to an initiation pattern compatible with a single bipartite ORC binding site. Consistent with this notion, deletion of one base pair between the ACS matches abolished ORC binding at *ARS603*. Importantly, loss of ORC binding *in vitro* correlated with the loss of ARS activity *in vivo*. Our results argue that replication origins in yeast are in general comprised of bipartite ORC binding sites that cannot function in random alignment but must conform to a configuration that permits ORC binding. These requirements help to explain why only a limited number of ACS matches in the yeast genome qualify as ORC binding sites.

INTRODUCTION

Although the mechanism underlying the initiation of DNA synthesis differs across species, a general theme has emerged to describe the activation of the replication process in prokaryotes, animal viruses and simple eukaryotes. The recognition of a binding site with multiple sequence elements is a key factor for the initiation of DNA replication in a number of organisms (1). For many of these sites, the spacing between sequence elements is crucial for the binding of the replication initiator and the subsequent initiation of DNA synthesis (2). It has been shown, for instance, that exact spacing of the bipartite initiator binding site is necessary for replication from *oriP* in Epstein-Barr virus (3) and the simian virus (SV) 40 origin (4). The situation in eukaryotic cells is less well defined, as replication initiates from multiple start sites distributed along chromosomes and the search for origin sequences, especially in the genomes of mammalian cells, has proven difficult, with only a few well-studied examples (5).

Saccharomyces cerevisiae remains the eukaryotic system in which replication origins are best understood. Origin sequences were first identified as autonomously replicating sequences (ARSs) by their ability to promote replication of plasmid DNA (6–8). ARSs carry multiple matches to an AT-rich, 11 bp ARS consensus sequence (ACS), 5′-(A/T)TTTA(C/T)(A/G)TTT(A/T)-3′ (8–10). Substitution mutations scanning origin regions with matches to the ACS have identified multiple functional elements (11–15). Elements that are essential for replication are called A elements, while those that are non-essential are referred to as B elements (14). In *ARS1*, A and B elements overlap with matches to the ACS (Figure 1A) and at least one B element in conjunction with the A element is required to form a functional origin (16,17). Other ARS sequences, including *ARS305* and *ARS307* (Figure 1B), contain similar clusters of a single, essential A element and multiple B elements, all of which are associated with an ACS (13,15,18). What has remained a conundrum is the fact that there are more than 10 000 matches to the ACS in the budding yeast genome, but only a small subset of these appear to be functional binding sites for the initiator, the origin recognition complex (ORC) (19). In an attempt to distinguish functional ACSs from non-functional ones, an extended ACS that comprises 17 bp, instead of 11, has been described (20). However, the predictive value of this 17 bp sequence has not been firmly established, and it seems that algorithms based on the 17 bp consensus sequence (21) have a limited capacity for correctly predicting all 430 potential origins in the yeast genome (19).

Footprinting analyses have shown that ORC interacts directly with the ACS (18,22–24). In contrast to origins such as *ARS1* and *ARS307*, which carry a single essential A

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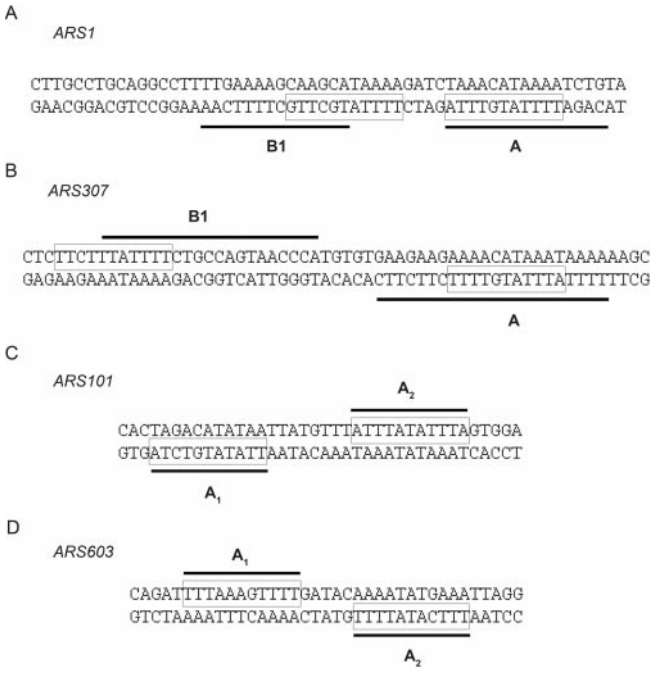

Figure 1. Core ACS sequences for four origins *ARS1*, *ARS307*, *ARS101* and *ARS603*. (**A** and **B**) The ACSs at *ARS1* and *ARS307* are shown in boxes. Previously defined A and B1 elements (14,15,17) are indicated by bold lines. (**C** and **D**) The ACSs at the compound origins *ARS101* and *ARS603* are shown in boxes (26,27), and the A_1 and A_2 elements are indicated by bold lines.

element, a second class of origins, branded compound origins, has been described (25,26). These consist of multiple ACS matches that appear to serve as redundant ORC binding sites because the origin was still active when one ACS was mutated (26). The best-studied examples for this class are *ARS101* (Figure 1C) and *ARS310* (26). Similarly, *ARS603* is also comprised of two ACSs (Figure 1D) (27). We have designated the ACS matches in *ARS101* and *ARS603* as A_1 and A_2, respectively (Figure 1C and D); however, it is important to point out that they are not directly equivalent to the A elements in *ARS1* and *ARS307*, which are necessary for ORC binding but not sufficient (15–18). Substitution mutations at either ACS in *ARS603* do not abolish origin activity, but combined mutations in both ACSs result in complete loss of replication function (27). Thus, *ARS603* has been proposed to function as a compound origin with multiple individual ORC binding sites (26).

One prediction for compound origins is that they possess multiple, redundant replication start sites (26). Previous mapping studies of *ARS1* revealed a single, defined leading strand start site that maps adjacent to the ORC binding site (28,29). For origins with redundant, non-essential ACSs, it is therefore possible that more than one start site for replication exists. However, this has never been directly addressed.

To examine the origin structure of an ARS element containing more than one potential ORC binding site, we investigated replication initiation at the origin *ARS603*. Our studies revealed that replication initiates in close proximity to the ACS matches where ORC binding occurs. The initiation pattern of *ARS603* is similar to that of *ARS1* (29), suggesting that the two ACSs at *ARS603* do not function as individual origins. Because it was previously unknown if a single or multiple ORC complexes occupy the ACSs in compound origins, we employed electrophoretic mobility shift assays (EMSAs) to distinguish between these possibilities. Our results demonstrate that the two ACSs act in concert as a single bipartite ORC binding site and that their spacing and orientation affect ORC binding *in vitro* and *in vivo*. While substitution mutations similar to those introduced in earlier studies (27) retained some ORC binding activity, deletion of one ACS abrogated ORC binding. These studies argue that a single ACS is insufficient to define a functional ORC binding site, and we propose the inclusion of bipartite consensus sequences in future algorithms for the identification of replication origins in yeast. The dependence of ORC binding on the spacing of specific recognition sequences identifies another link between origin structure and activation to further our understanding of the DNA replication initiation process.

MATERIALS AND METHODS

Replication initiation point mapping

Replication initiation point (RIP) mapping was performed on the strain SP1 *MAT**a***, *ura3-52 his3 trp1-289 leu2-2,113 ade8 can1* (14). DNA was isolated by CsCl gradient centrifugation from asynchronously growing cultures, as described (30). As a source for the non-replicating DNA control, DNA was isolated from nocodazole-arrested cells. Approximately 2 μg of isolated DNA was digested with PstI, leaving a 5′ phosphorylated end, and then treated overnight with λ-exonuclease to eliminate nicked DNA (30). The sample was then used as a template for primer extension reactions using radiolabeled primers as described (30). Reactions were fractionated on a polyacrylamide gel next to corresponding sequencing reactions. The sequences for the primers utilized in this study were as follows: 161t (5′-GCTGGGAGAACTATTCTTCCAGAG-3′) for the top strand and 665b (5′-GAGCGATTCTGATGAAACGCACTG-3′) for the bottom strand.

In vitro ORC EMSA

Binding reactions were performed with DNA fragments generated by PCR using biotin-labeled primers. To reduce variability, one set of biotin-labeled primers (IDT) was used to recognize the flanking regions of the cloning site in the plasmid pSTBlue-1 (Novagen) (31). Annealed oligonucleotides spanning wild-type (WT) or mutant origin sequences of *ARS603*, *ARS101*, *ARS1* and *ARS307* were inserted into this vector. The length of the probes ranged from 81 to 100 bp to include core sequences for each origin (Figure 1) and 44 bp of vector flanking regions. Binding reactions with 10 fM of labeled probe and 10 nM baculovirus-purified yeast ORC, a generous gift from Dr B. Stillman, were incubated in the presence of 1.5 mM ATP, 1 mM DTT, 0.5 mM phenylmethlysulfonyl fluoride (PMSF), 20 mM KCl, 5 mM $MgCl_2$, 1 mM Tris (pH 7.5), 22.5 mM HEPES (pH 7.0), 0.2 mM EDTA, 2 mg/ml BSA, 2.5% glycerol and 25 mg/ml poly(dG–dC) as nonspecific competitor. These conditions were used to shift ~50% of the probe for WT *ARS603*. Biotin-labeled DNA was visualized using a chemiluminescent detection kit (Pierce).

Chromatin immunoprecipitation (ChIP)

Strains used for integration of *ARS603* into the *URA3* locus and mutation of endogenous *ARS603* by two-step gene replacement were derivatives of BF264-15DU *MAT**a** ura3 ade1 his2 leu2-3,112 trp1-12 Δns* (32). For integration at the *URA3* locus, 206 bp of *ARS603* was ligated into the SacI–SalI cloning region of pRS306. The vector was then linearized with StuI within the *URA3* marker and integrated into the chromosomal *URA3* locus. Chromatin samples were obtained from asynchronously growing cultures and processed as described (33). ORC-bound fragments were precipitated from pre-cleared chromatin with a mouse, anti-yeast ORC2 monoclonal antibody (RDI) and protein A sepharose beads (Sigma), which were pre-blocked in BSA. Immunoprecipitated DNA fragments were detected by PCR. Primer pairs for the ChIP analysis were specific to the following regions: *ARS1*: 630 (5′-GTTAGCTGGTGGACTGACGCCAGAAA -3′), 1010 (5′-GCCTGTGAACATTCTCTTCAACAAG-3′); *R11:* t(5′-CACCGATACGTACTTAAACTCT-3′), b(5′-GAGAAAGCTTAGTCCATTCGGC-3′); *ARS603/URA3:* pRS2100t (5′-CAGCTTTTGTTCCCTTTAGTGAG-3′), pRS-2613b (5′-CACCTCTGACTTGAGCGTCGAT-3′); *ARS603*: 161t (5′-GCTGGGAGAACTATTCTTCCAGAG-3′), 482b (5′-CTCCTTTGTTAGAGTTAATTTATGATC-3′).

Quantitation by particle analysis was performed using the NIH *ImageJ* program, and fold enrichment was calculated by taking the ratio of immunoprecipitated to input signals for the origin region (*ARS1* or *ARS603*) divided by the same ratio for the non-origin region (R11) as described (34).

Plasmid stability assay

Origin sequences ~200 bp in length were cloned into pSTBlue-1 (Novagen) unless otherwise indicated. Site-directed mutagenesis reactions were performed according to the Stratagene QuikChange procedure. The sequences were then cloned into the pARS plasmid vector containing the *URA3* gene and a centromere (14). Yeast transformations into the SP1 strain (14) were performed with lithium acetate, and cells were plated onto SC–Ura plates. Primary transformants were further streaked out on an additional SC–Ura plate. Yeast cells containing the pARS plasmid with the given origin sequence were grown in SC–Ura medium to early log phase, counted by hemocytometer and plated to assess the initial number of plasmid-containing cells. Approximately 200 cells were released into 2 ml of complete medium for 30 h at 30°C. Cells were counted again, and equal amounts were plated on complete and SC–Ura medium to determine the percent of colonies that retained the pARS plasmid. The assay was duplicated for at least two independently isolated transformants of each strain.

RESULTS

Replication in *ARS603* initiates close to the two ACS elements

Previously, we investigated DNA replication events at *ARS1* and found that DNA synthesis initiates adjacent to the site of ORC binding (28,29). To determine the DNA replication initiation pattern for *ARS603*, we performed RIP mapping. This method allowed us to map the start sites for both leading and lagging strand synthesis with nucleotide resolution (30). *ARS603* has two core elements, a 10/11 and a 9/11 match to the ACS (27). Here we refer to the first ACS match as A_1 and the second ACS match as A_2 (Figure 1D). A_1 and A_2 are oriented in opposite directions, separated by 5 bp. For RIP mapping, DNA was isolated by CsCl gradient centrifugation from asynchronously growing cultures. Nocodazole-arrested cells served as the source for the non-replicating DNA control sample (30). Nascent DNA was enriched by treatment with λ-exonuclease (28,30). Nascent or non-replicating DNA was used as templates for primer extension reactions using radiolabeled primers that annealed ~250 bp up or downstream of the ACSs. Primer extension products were fractionated on polyacrylamide gels next to corresponding sequencing reactions (30). The ladder of bands represents replication intermediates (Figure 2A and B) and the position of the smallest band marks the initiation site for the leading strand [also referred to as the transition point (TP) between leading and lagging strands, TP; Figure 2A and B]. All other bands represent initiation sites for Okazaki fragments (29). Any faint bands that were not reproducible were not considered. We mapped the TP between leading and lagging strand synthesis within the A_2 element (Figure 2A and B). Replication initiated at nucleotide position 403 on the top strand and at nucleotide position 394 on the bottom strand, 9 bp apart. Subsequent Okazaki fragment start sites for lagging strand synthesis were spaced at intervals of ~50–60 bp or multiples thereof. DNA from nocadozole-arrested cells did not show any reproducible bands, as expected (Figure 2A and B, lane N). Positive controls for correct primer annealing were performed on restriction enzyme-digested DNA, where the restriction site was confirmed by RIP mapping (data not shown). A synopsis of the initiation map of *ARS603* is shown (Figure 2C). It is worthwhile to note that we did not observe a composite pattern (an overlay of two initiation maps with one replication start site in A_1 and another one in A_2), as might have been predicted for a compound origin (Figure 2D, bottom; Figure 2E, lane D). These results suggest that DNA replication at *ARS603* likely initiates from a single start site, similarly to *ARS1* (28,29).

Each ACS match at *ARS603* contributes equally to ORC binding

Based on our RIP mapping results and earlier studies on *ARS603* by others (27), we predicted that ORC binds to A_1 and A_2. Because this had never been tested directly, and it was unclear whether A_1 and A_2 could bind a single ORC or multiple complexes, we performed electrophoretic mobility shift analyses on *ARS603* using purified ORC. Initially, we titrated ORC to approximate binding of 50% of a ~90 bp probe (data not shown) and used these conditions for subsequent experiments (see Materials and Methods for details). ORC binding to *ARS603* was dependent on the presence of ATP (data not shown), as expected (24,35,36).

Previous analyses of *ARS603* used XhoI substitution mutations that converted the A_1 and A_2 sequences into 7/11 matches to the ACS. Either mutation at A_1 or A_2 alone did not affect origin activity; only the combined mutation of both elements resulted in complete loss of activity at *ARS603* (27).

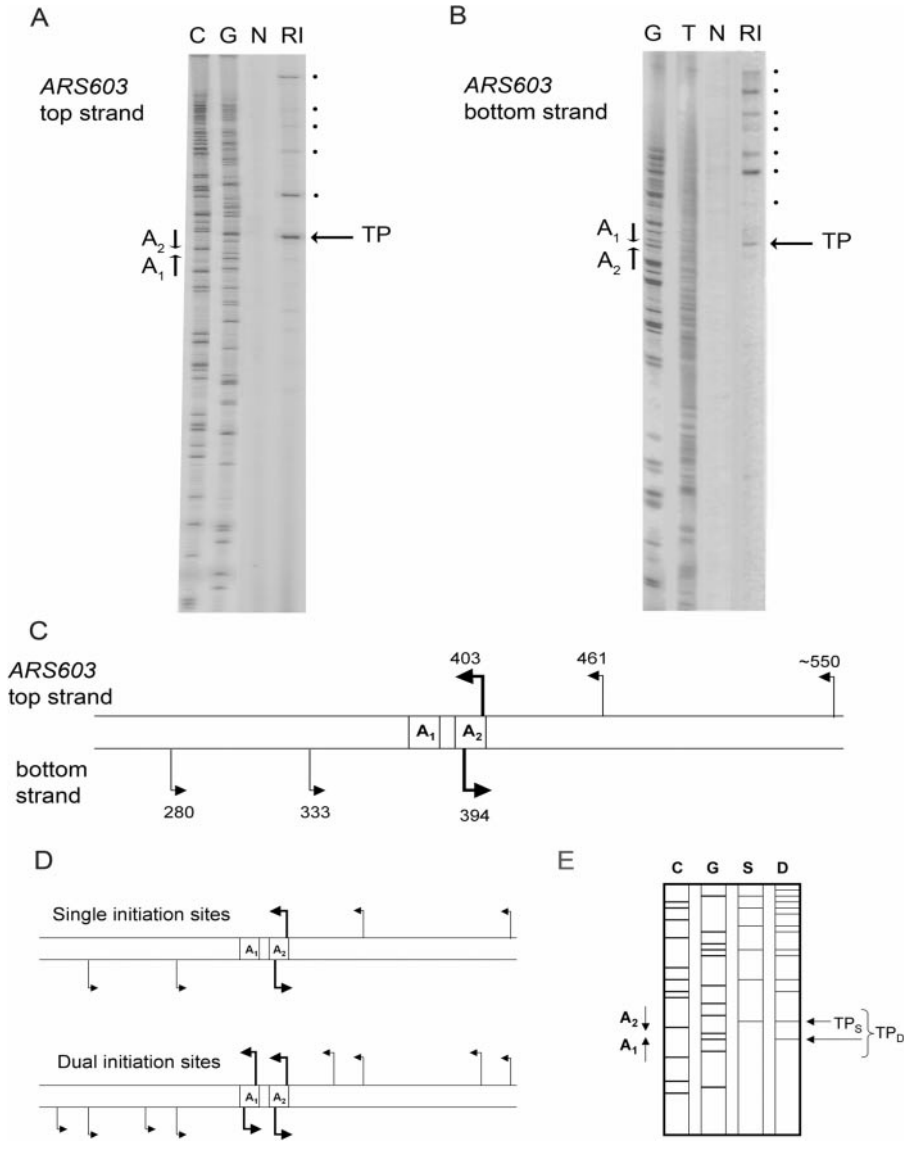

Figure 2. Initiation pattern of *ARS603*. (**A**) RIP mapping on the top strand of *ARS603* shows the transition point (TP) between leading and lagging strand synthesis at the A_2 element. Replication intermediates (RIs) are fractionated on a polyacrylamide gel next to corresponding Sanger sequencing reactions (C,G). Okazaki fragment initiation sites are indicated above the TP by black dots. Arrows denote the position and orientation of the A_1 and A_2 elements. As a negative control, DNA from nocodazole-arrested cells (N) was used as a template. (**B**) RIP mapping on the bottom strand of *ARS603* reveals the transition point (TP) at the A_2 element, and the Okazaki fragment initiation sites are also marked by black dots. Replication intermediates (RIs) are shown next to corresponding Sanger sequencing reactions (G,T). DNA from nocodazole-arrested cells (N) was used as a negative control. (**C**) A synopsis of the initiation sites at *ARS603* is shown. Bold arrows indicate the transition points (TPs) on the top and bottom strands, and the other arrows indicate lagging strand initiation sites. Nucleotide positions of individual start sites are shown. (**D**) Initiation maps for *ARS603* with a single TP (bold arrows, top) or hypothetical dual TPs (bold arrows, bottom) if the origin initiates replication at either position with similar frequencies. For dual initiation sites, two closely spaced (~10 nt) TPs in A_1 and A_2 are shown, as predicted for a compound origin with two independent ORC binding sites. Lagging strand initiation sites also lie ~10 nt apart, a distance too short to accommodate consecutive Okazaki fragments. (**E**) A cartoon of hypothetical RIP mapping results shows a polyacrylamide gel with either single (S) or dual (D) initiation sites on the top strand of *ARS603*. Sequencing reactions (C,G) identify the position of the ACSs indicated by arrows (A_1, A_2), as in (A). The location of the TP for the single initiation site scenario (TP_S) is indicated by a horizontal arrow, and the location of the TPs for the dual initiation site scenario (TP_D) is bracketed to encompass both arrows.

Using these exact substitutions, here labeled S4, we found that mutations at A_1 or A_2 caused a decrease in ORC binding in comparison to ORC binding at the WT *ARS603* sequence (Figure 3A). Moreover, the combined mutation of both A_1 and A_2 resulted in the dramatic reduction of ORC binding, consistent with the reported loss of origin activity (27). These results suggested that ORC binding is indeed affected by mutations in A_1 or A_2.

Since the XhoI substitution mutations resulted in a gain of GC content, we tested a variety of base substitutions that deviated from the ACS, but kept the GC content of A_1 and A_2 the same. Not surprisingly, the increase in mismatches to the ACS correlated with the decrease in the amount of shifted probe (Figure 3B), as expected from prior findings (24).

Interestingly, however, these 7/11 matches retained much less ORC binding capacity than the 7/11 matches in the S4

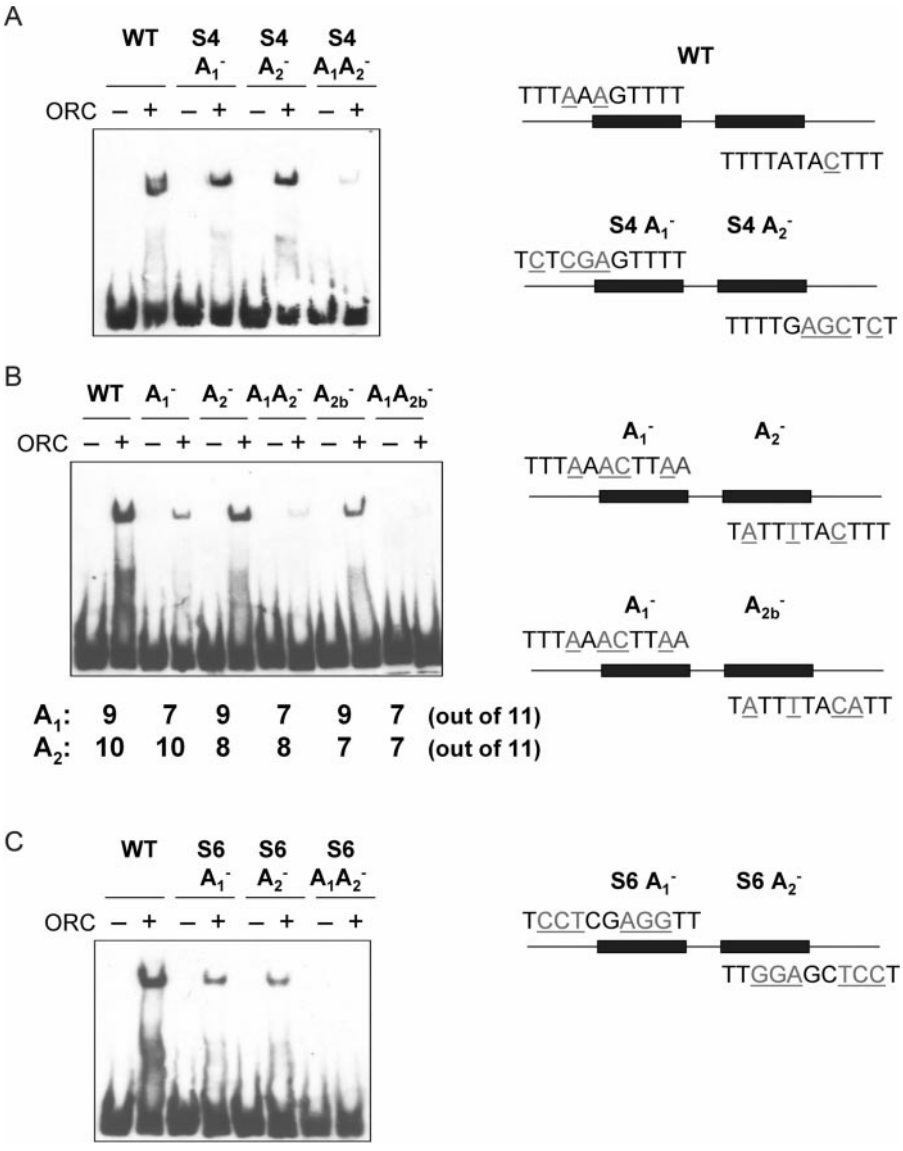

Figure 3. A_1 and A_2 contribute equally to ORC binding at *ARS603*. EMSAs were performed in the presence and absence of purified ORC on probes containing the core *ARS603* sequence (Figure 1D) flanked by common vector sequences (see Materials and Methods). (**A**) Mutations that substituted 4 bp in the ACS reduced ORC binding equally at A_1 and A_2. XhoI substitution mutations (S4) were introduced in single elements (A_1^-, A_2^-) or in combination ($A_1A_2^-$). Mismatches to the ACS are in gray and underlined. (**B**) ORC binding ability correlated with the number of matches to the ACS. Mutations with conservation of GC content still affected ORC binding. Notations below the figure indicate the number of matches to the 11 bp ACS. (**C**) Mutations that replaced 6 bp of the ACS reduced ORC binding equally at A_1 and A_2. Substitution mutations with the sequence CCTCGAGG (S6) were introduced at single elements (A_1^-, A_2^-) or in combination ($A_1A_2^-$).

A_2^- and S4 A_1^- probes (compare Figure 3A and B). The decrease in binding activity may have been a result of mutating the highly conserved position 10 (8) within the ACS of A_1^- and A_{2b}^- probes. In contrast, the S4 mutations retain a stretch of Ts in positions 8–10, leaving the most highly conserved nucleotides intact (8). To further disrupt each A element in *ARS603*, we utilized substitution mutations with the linker sequence (5′-CCTCGAGG-3′). These linker sequences replaced 8 bp of sequence and were used in earlier studies to dissect *ARS1*, *ARS307* and *ARS305* (13–15,18). We called these mutations S6 because they replaced 6 bp of the ACS, reducing the match at these elements to 5/11 (Figure 3C). The ability of ORC to retard the electrophoretic mobility of the *ARS603* fragment was severely decreased for both S6 fragments (Figure 3C, lanes A_1^- and A_2^-). In contrast to the S4 probes, ORC binding was reduced by >50% for individual S6 probes [similar to the A_1^- and A_{2b}^- fragments (Figure 3B)]. Equivalent mutations in A_1 and A_2 resulted in similar reduction of ORC binding, suggesting that the individual ACSs play an equal role in ORC binding at *ARS603*. These results suggested to us that A_1 and A_2 contribute synergistically to ORC binding, either as two individual but cooperative ORC binding sites or as a single ORC binding site.

A single ACS match is insufficient to support ORC binding

Although both ACSs in *ARS603* appeared to contribute equally to ORC binding, we never detected a second band,

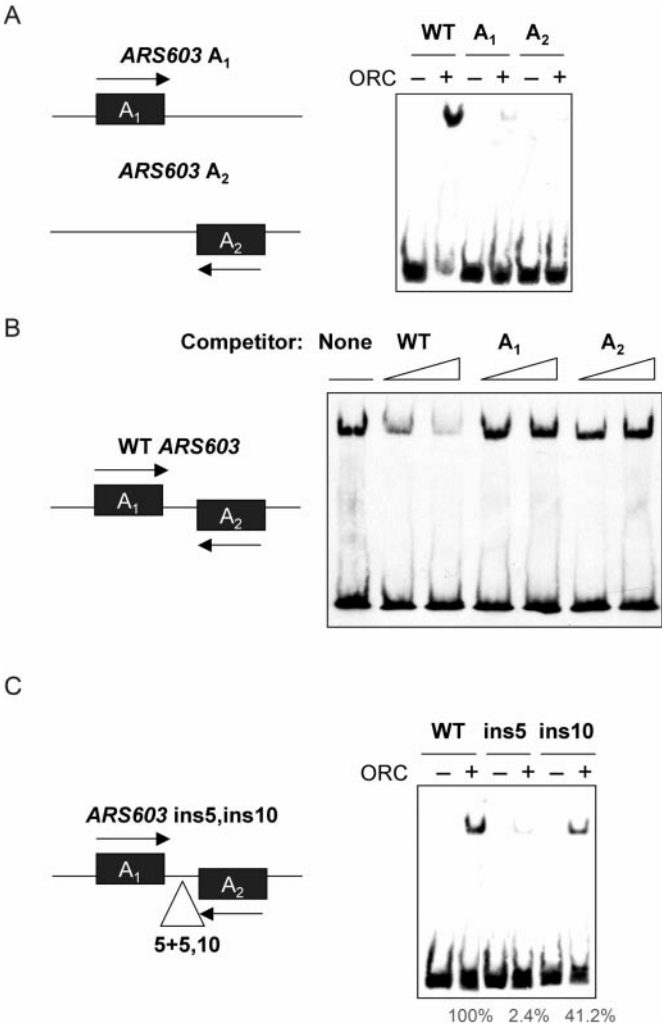

Figure 4. Both A_1 and A_2 are required for ORC binding to *ARS603*. EMSAs were performed in the presence and absence of purified ORC. (**A**) A single match to the ACS (A_1 or A_2) did not support ORC binding. (**B**) 50- and 500-fold unlabeled wild-type *ARS603* competed for ORC binding, but single A element fragments were unable to compete against wild-type *ARS603*. (**C**) Insertions between the A elements were constructed and tested for ORC binding. While half of a helical turn (ins5) disrupted ORC binding, a full helical turn (ins10) restored some binding. Percentages below the figure indicate the amount of probe shifted by ORC (with wild-type levels set to 100%), as quantified by particle analysis (*ImageJ*).

even at very high concentrations of ORC (50 nM/reaction, data not shown), as would have been expected if two ORCs could bind to the WT probe. To further define the roles of A_1 and A_2, we constructed fragments of the same length as the WT *ARS603* fragment, containing either element A_1 or element A_2 flanked by random sequences that were unable to bind ORC by themselves (data not shown). Subsequent *in vitro* binding assays revealed no significant binding of ORC to either A_1 or A_2 alone (Figure 4A). The consequences of deleting A_1 or A_2 contrasted with the effect of substitution mutations that still allowed for ORC binding as long as one ACS remained intact (Figure 3), explaining earlier reports on the origin activity of *ARS603* mutants (27). In addition, we used unlabeled A_1 or A_2 containing fragments as well as a WT control in a competition assay measuring ORC binding to a labeled WT *ARS603* fragment. Consistent with the results shown in Figure 4A, 50- and 500-fold excess of A_1 or A_2 sequences did not impede ORC binding to WT *ARS603* (Figure 4B). These data support the notion that both A_1 and A_2 are required for ORC binding to *ARS603* and cannot function as individual ORC binding sites.

ORC binding at *ARS603* requires a defined helical phase

Examination of the *ARS603* sequence revealed that A_1 and A_2 are separated by 5 bp. Because A_1 and A_2 lie on opposite strands, this particular spacing would place the two elements on the same side of the DNA double helix. To explore whether this specific configuration is a requirement for ORC binding, we inserted sequences of different lengths between the A_1 and A_2 elements. The insertion of 5 bp, approximately half a helical turn, caused a severe reduction in ORC binding (Figure 4C). However, insertion of an additional 5 bp for a total of 10 bp, or close to a full helical turn, partially restored ORC binding (Figure 4C). Based on these results, A_1 and A_2 require alignment to a defined helical phase to function as an efficient ORC recognition sequence.

A single base pair deletion between the ACS matches in *ARS603* and *ARS101* abolishes ORC binding

Replication initiation at a number of viral origins is severely influenced by the spacing between recognition sites (3,4). Because the configuration of functional elements and associated ACSs varies from one origin to another in budding yeast (37), it is widely assumed that the distance between ACSs is not relevant for origin function. To test this hypothesis directly, we examined the effect of a single bp deletion between A_1 and A_2. Surprisingly, ORC no longer bound to the *ARS603* fragment (Figure 5A). The effect of the single bp deletion, which removed an A-T bp, was attributed to change in spacing rather than the requirement for that particular bp or a change in G-C content, because the substitution of the same A-T bp with a C-G bp produced no discernable change in ORC binding (data not shown). Thus, the severity in the loss of ORC binding upon deletion of 1 bp contrasted strongly with the number of substitution mutations within the ACS matches required to achieve the same effect (Figure 3). To determine whether the effect of a single bp deletion was specific to *ARS603* or could be generalized to other origins, we chose *ARS101* to perform a similar experiment. This origin also possesses two ACS matches, one of which (A_2 in Figure 1C) has been reported to support replication individually (26). A single bp deletion between the two ACSs, originally 8 bp apart, severely compromised ORC binding at *ARS101*, even though we added twice as much ORC to these binding reactions as we used for our experiments with *ARS603* fragments (Figure 5B). We concluded that the spacing between the A elements in *ARS603* and *ARS101* is crucial for ORC binding.

Spacing of bipartite elements at *ARS1* and *ARS307* does not affect ORC binding

The ACS matches in both *ARS603* and *ARS101* were reported to be redundant with respect to their ability to bind ORC or confer ARS activity (26,27). In contrast to these compound

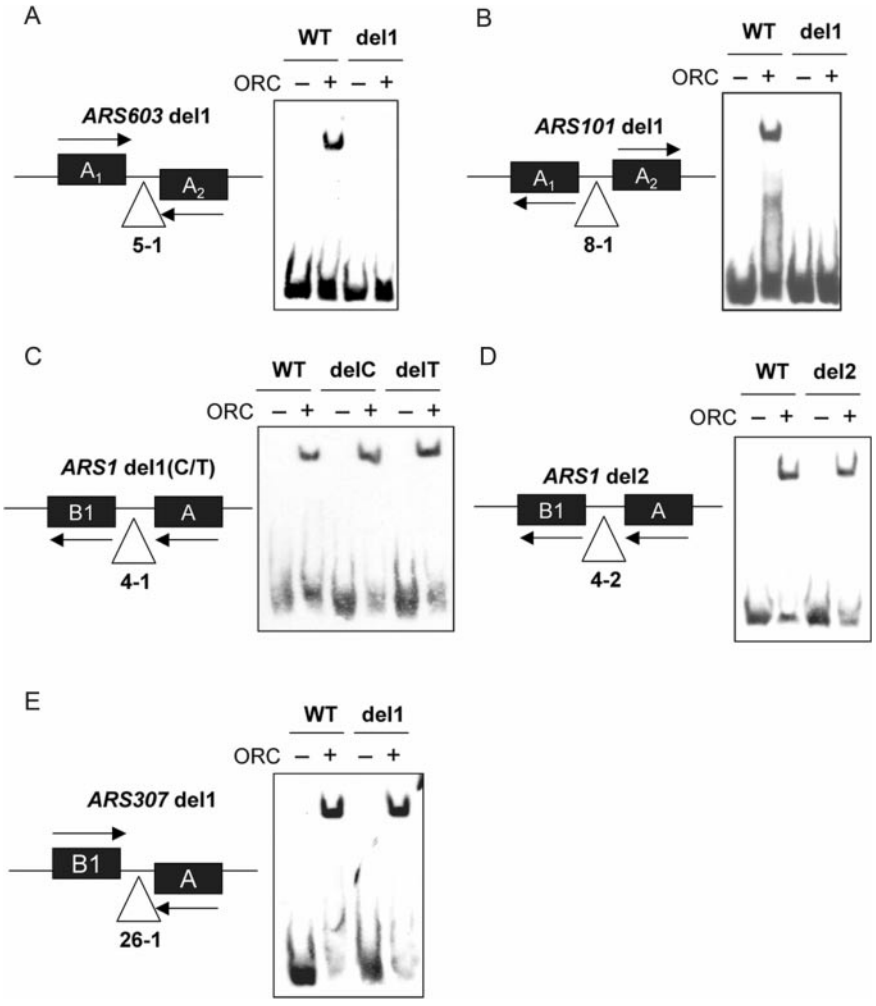

Figure 5. A single bp deletion eliminates ORC binding at *ARS603* and *ARS101*, but not at *ARS1* and *ARS307*. EMSAs were performed in the presence and absence of purified ORC. (**A**) ORC binding was abolished by a single bp deletion between the ACSs at *ARS603*. A single bp deletion (del1) was introduced between the A elements converting GATAC to GATC. (**B**) A single bp deletion between the ACSs at *ARS101* also severely decreased ORC binding. The 8 bp spacing was reduced to 7 bp (TTATGTTT to TTAGTTT) between the ACSs at *ARS101* (del1). (**C**) Single bp deletions between ACSs associated with A and B1 elements of *ARS1*, where the original spacing was 4 bp, did not show a significant decrease in ORC binding ability. Two different single bp deletions were made with either a C-G bp (delC) or a T-A bp (delT). (**D**) Deletion of 2 bp (del2) between the ACSs at *ARS1* also did not significantly affect ORC binding. (**E**) Deletion of a single bp (del1) between ACSs associated with the A and B1 elements at *ARS307* did not alter ORC binding.

origins, *ARS1* and *ARS307* contain only a single essential ACS match (the A element). Previous studies showed that the A element is required but not sufficient for ORC binding and that ORC interacts with both the A and B1 elements in *ARS1* (18,24,35). To address whether the spacing between A and B1 is crucial for ORC binding at *ARS1* and *ARS307*, we introduced deletion mutations between these elements. These deletions also reduced the spacing between the associated ACSs. ORC binding at both *ARS1* and *ARS307* was not significantly affected by the removal of a single bp (Figure 5C and E). Furthermore, no effect was seen at *ARS1* regardless of the removal of either a C-G or a T-A bp (Figure 5C). Indeed, the deletion of even two out of the 4 bp between the ACSs at *ARS1* was not sufficient to significantly impair ORC binding (Figure 5D). In *ARS307*, the distance between the two ACSs is rather large, spanning 26 bp (Figure 1B). Therefore, we also tested whether the removal of 5 bp had any effect on ORC binding. However, we did not detect any changes (data not shown). Therefore, at *ARS1* and *ARS307*, small changes in spacing between the ACSs (and thus between the A and B1 elements) do not appear to be critical for ORC binding, supporting prior ARS assay results on *ARS1* mutants (38). This is also consistent with earlier reports suggesting that the A element at *ARS1* can cooperate with any of the non-essential B elements (16).

Orientation of the A elements affects ORC binding at *ARS603*, *ARS101* and *ARS1*

Early comparisons of ARS element configurations revealed that the orientation of ACSs differs from origin to origin (6). This prompted us to investigate whether ORC binding was sensitive to changes in the orientation of individual ACSs at *ARS603*, *ARS101* and *ARS1* (Figure 1). Previous analyses have noted a favored ACS orientation in some instances for the support of plasmid replication (6,38,39).

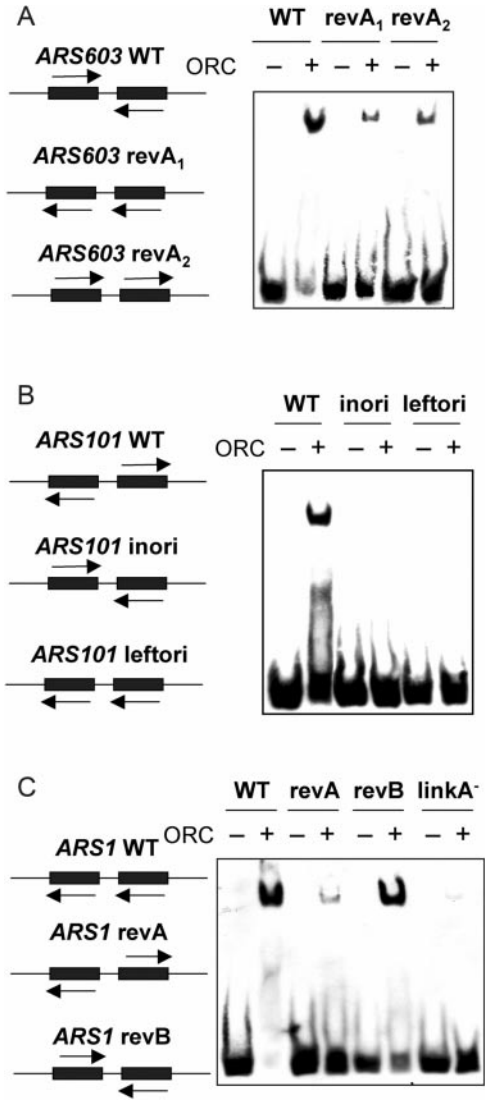

Figure 6. Reversal of the A element affects ORC binding at *ARS603*, *ARS101* and *ARS1*. EMSAs were performed in the presence and absence of purified ORC. (**A**) Changes in the orientation of A_1 or A_2 signficantly decreased ORC binding to *ARS603*. Mutations were introduced to reverse the A_1 element ($revA_1$) or the A_2 element ($revA_2$) in *ARS603*. (**B**) Changes in the orientation of A_1 or A_2 in *ARS101* abrogated ORC binding. ACS orientations were converted to face inward (inori), or leftward (leftori). (**C**) ORC binding was sensitive to the reversal of the ACS within the A element of *ARS1*. Mutations created a reversal of the ACS associated with the A element (revA), or the ACS associated with the B1 element (revB). The negative control ($linkA^-$) for ORC binding was the linker substitution mutation at positions (858–865) of element A in *ARS1* (18).

At *ARS603*, the reversal of either A_1 or A_2 resulted in a significant reduction of ORC binding (>50%, Figure 6A). The reversal of either ACS match at *ARS101* resulted in a complete loss of ORC binding (Figure 6B). Meanwhile, at *ARS1*, the reversal of the ACS within the essential A element caused a severe reduction in ORC binding (Figure 6C, lane revA), similar to a linker substitution mutation in the A element (Figure 6C, lane link A^-). However, a change in the orientation of either the ACS that is associated with the B1 element (Figure 6C, lane revB) or the reversal of the entire B1 element (data not shown) reduced ORC binding only slightly. According to these results, ORC binding is sensitive to the orientation of the ACS within the respective A elements of all tested origins. This further supports the notion that a single ACS cannot function as an ORC binding site but acts in concert with its flanking region.

ORC binding *in vivo* reflects ORC binding *in vitro*

Because the *in vitro* DNA mobility shift assays using purified ORC revealed that the spacing between A_1 and A_2 was critical for ORC binding at *ARS603*, we next examined the effect of the single bp deletion *in vivo* by ChIP using ORC2-specific antibodies. Regardless of whether *ARS603* was inserted at the *URA3* locus or present at its endogenous locus, a single bp deletion between the A elements abrogated ORC binding *in vivo* (Figure 7A and C). In contrast, ORC binding to *ARS1* remained unaffected (Figure 7B and D). The non-origin region R11 was included as a negative control (Figure 7B and D). Thus, the ability of ORC to bind *ARS603 in vitro* accurately reflected its behavior *in vivo*.

Origin activation parallels ORC binding

So far, we have shown that substitutions within the ACS as well as changes in orientation and spacing between A_1 and A_2 severely affected ORC binding at *ARS603*. To test whether these mutations also affected origin activation, we performed plasmid stability assays. These assays require origin sequences to be cloned into plasmids containing the *URA3* gene and a centromere sequence (40). Once transformed into yeast, the plasmid constructs are stably retained only with the presence of an active and effective yeast origin. Mitotic stability indicates the ability of the origin to support DNA replication initiation and retain the plasmid, thus allowing for survival in selective medium.

Sequences of ~200 bp covering the entire *ARS603* region were used to test origin activity. WT *ARS1* served as a positive control, and *ARS1*/A^- served as a negative control. The pARS1/A^- construct did not produce viable transformants that could be restreaked onto selective medium, as reported earlier (14). The level of origin activity *in vivo* correlated well with ORC binding *in vitro* in all cases examined (Figure 8A). While WT *ARS603* had a mitotic stability similar to that of *ARS1*, the double substitution mutants for A_1 and A_2 at *ARS603* were unable to grow on selective medium (Figure 8A and B). Notably, the deletion of a single base pair or the insertion of a half helical turn also produced only microcolony transformants that were unable to be passaged onto selective medium (Figure 8A and B). We obtained similar results when we deleted a single base pair between the two ACSs in *ARS101* (data not shown). Consistent with our *in vitro* ORC binding studies, the insertion of a full helical turn between the A elements partially rescued growth (Figure 8B). The 38 bp core region of *ARS603* containing WT A_1 and A_2, which was capable of supporting ORC binding (Figure 3, WT) also produced transformants that could be restreaked onto selective medium (Figure 8C, A_1+A_2). At the same time, fragments of the same length containing only either A_1 or A_2 of *ARS603* were unable to grow on selective medium (Figure 8C), as expected from their inability to bind ORC *in vitro* (Figure 4A and B). Thus, in all cases examined, loss of ORC binding corresponded well with loss of origin activity.

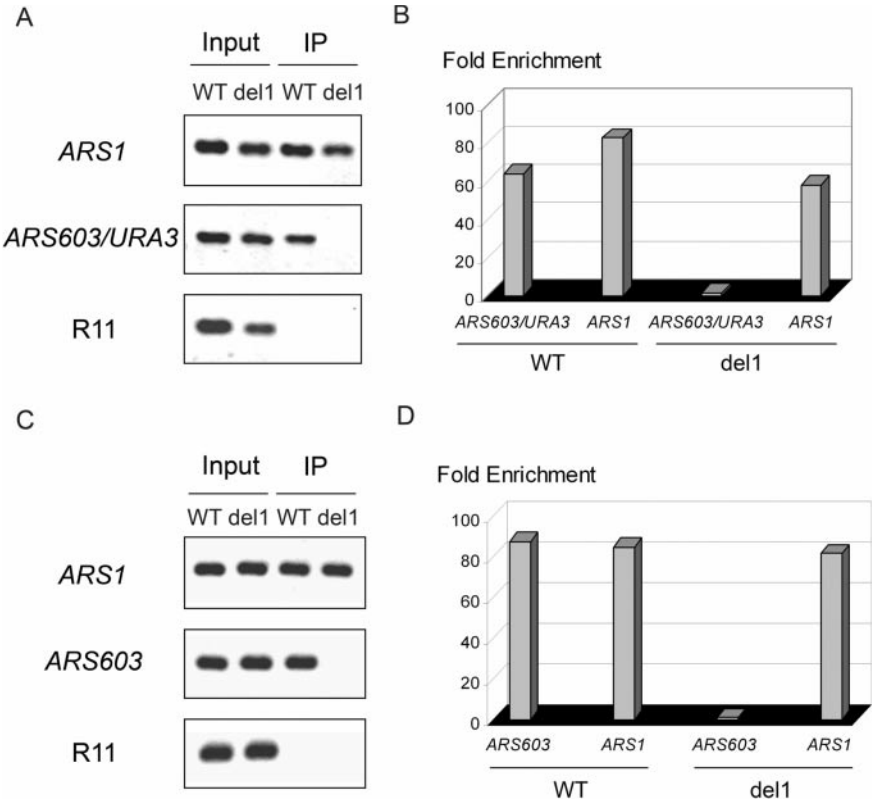

Figure 7. A single bp deletion inhibits ORC binding to chromosomal *ARS603*. *ARS603* carrying a single bp deletion (del1) between A_1 and A_2 was inserted at the *URA3* locus (**A** and **B**) or replaced WT *ARS603* at the endogenous locus (**C** and **D**). Control strains harbored wild-type *ARS603* sequences (WT) at both chromosomal locations. (A and C) ChIPs were performed using ORC2-specific antibodies. PCR reactions contained *ARS1*-, *ARS603*-, *ARS603*/*URA3*- or R11-specific primers and immunoprecipitated (IP) or input DNA (A and C). (B and D) Quantification of PCR fragments was performed using *ImageJ* (NIH) as described (34).

DISCUSSION

Analysis of *ARS603* provides a new perspective on the contribution of both DNA sequence and structure to origin function. Because no two origins of DNA replication are identical, it has been difficult to understand what distinguishes functional from non-functional ACSs. Examination of the structural framework necessary for ORC binding in budding yeast provides clues to the complex nature of its interaction with DNA. In this study, we demonstrate that while two ACSs at *ARS603* contribute equally to ORC binding, they cannot function independently. The ACSs at *ARS603* require a restricted alignment to form a functional ORC binding site, and it is reasonable to assume that this may apply to other loci besides *ARS603* in the yeast genome.

Two classes of origins in budding yeast

In the course of defining the parameters that allow for the efficient binding of ORC to *ARS603*, we discovered that the interdomain spacing between A_1 and A_2 is critically important. This phenomenon was not found to be unique to *ARS603* since binding of ORC to *ARS101* was also responsive to single bp interdomain deletions. At the same time, not all of the origins we tested were affected by such changes, as *ARS1* and *ARS307* did not show any significant loss of ORC binding. Therefore, we propose that two classes of origins exist in yeast: those that require exact interdomain spacing and those that are tolerant of spacing manipulation. These differences directly reflect two different modes of ORC binding. In *ARS603*, the two ACSs that constitute the bipartite ORC binding site appear to contribute equally to ORC binding, whereas the A element in *ARS1* appears to be the predominant site of ORC interaction (41). Furthermore, it is possible that other ORC-interacting proteins modulate the affinity of ORC for certain sites in the genome. Indeed, previous studies have found that another replication initiation protein, Cdc6, regulates the sequence-specific binding of ORC at *ARS1* (42,43). It will be interesting to see whether Cdc6-dependent regulation is characteristic for this class of origins or also applies to *ARS603* and *ARS101*.

ORC binding at bipartite sites

Previous studies suggested that ORC could bind to a bipartite site comprised of A and B1 elements (*ARS1* and *307*) (14,15,18,26). However, prior evaluations of origin function also described the discovery of origins with multiple ACSs with redundant function (26). These origins were named compound if they contained redundant ACSs that were proposed to function as individual ORC binding sites (26). Our study on *ARS603* reconciles these two origin models.

Although *ARS603* contains two ACSs (A_1 and A_2), we concluded from our results that these two elements are not acting as binding sites for separate ORCs but rather as a bipartite binding site for a single initiator complex. This may also apply to *ARS101* because the deletion of a single bp

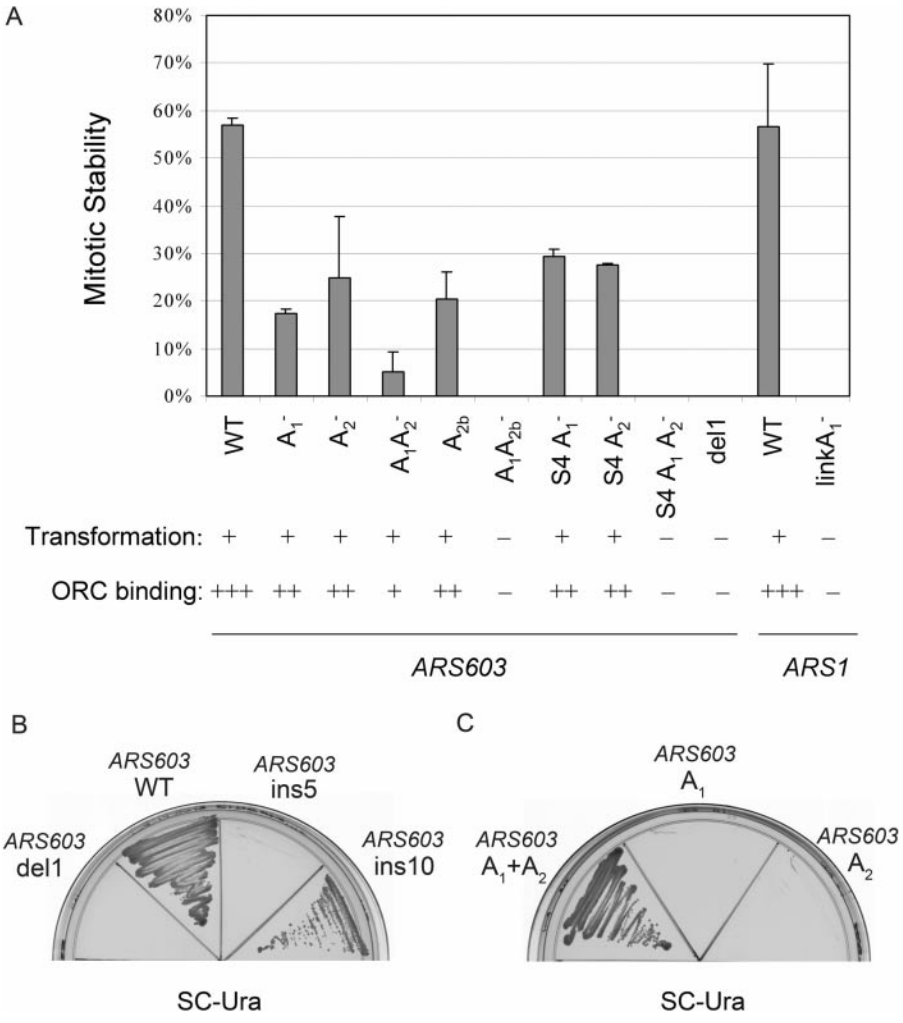

Figure 8. ORC binding *in vitro* mimics ARS activity *in vivo*. (**A**) The mitotic stability of different *ARS603* constructs was tested. Transformants that yielded viable colonies surviving passage onto selective medium were designated with a plus sign (+). ORC binding was designated as (+++) for wild-type *ARS603* and *ARS1* and (++) or (+) for decreasing levels of binding. Lack of ORC binding was indicated by a minus sign (−). The error bars indicate the standard deviation for the ARS assay on two separate transformants performed in duplicate. (**B**) Plasmids carrying wild-type (WT), single bp deletion (del1), and 5 or 10 bp (ins5, ins10) insertions within the 206 bp of *ARS603* were streaked onto selective medium (SC–Ura) to assess growth. (**C**) The same experiment as described in (B) was performed with the following constructs: 38 bp wild-type *ARS603* core region (A_1+A_2), element A_1 alone (A_1) and element A_2 alone (A_2).

between the ACSs, as well as changes in their orientation, abolished ORC binding *in vitro*. In addition, *ARS101* activity was markedly reduced when one ACS was mutated, arguing that ORC strongly prefers to bind to both ACSs (26). At first glance, our results seem to contradict the finding by Theis and Newlon that the 10/11 match in *ARS101* can retain ARS activity by itself (26). However, further inspection of the *ARS101* sequence reveals two additional 8/11 matches downstream of the 10/11 ACS element (data not shown). It is conceivable that one of these 8/11 matches cooperated with the 10/11 match to form a bipartite ORC binding site. Consistent with this notion, we show that a single A element in *ARS603*, another compound origin, cannot sustain ORC binding. These data are in agreement with early reports that the 11 bp ACS is necessary but not sufficient for ARS activity (44). This is further supported by the fact that substitution mutations at *ARS101* showed the gain of new hypersensitive sites with the loss of one ACS element (26), indicating that ORC contacted a second site in close proximity to the remaining A element.

At *ARS1* as well, footprinting studies showed that mutation of the A element resulted in protection over a 9/11 ACS at an additional B element (24,35), consistent with the recovery of a bipartite site. Indeed, *in vitro* studies determined that two ORCs cannot bind to two inverted ACSs at *ARS1* simultaneously (24,45), and other studies have determined that the stoichiometry of ORC to the *ARS1* origin is 1:1 (46). It follows that the recognition of a bipartite binding site with two ACSs by a single ORC likely applies to the vast majority of origins in the yeast genome.

In addition, fluorescence anisotropy studies showed that ORC is capable of binding to ACS matches associated with various elements of *ARS1* (47); however, only with the presence of the A element was ORC binding dependent on ATP, a key factor in the sequence-specific recognition of ORC at origins (24,35,36). Therefore, it seems likely that certain ORC conformations dictated by the spatial arrangements of ACSs may either allow or restrict ATP-hydrolysis by ORC (48). It is conceivable that the single bp deletion between A_1 and

A_2 in *ARS603*, or the reversal of A elements in *ARS1*, *ARS101* and *ARS603*, induced an ORC conformation that failed to repress ATP-hydrolysis by ORC and did not facilitate stable binding to DNA (36,49).

As a heteromultimer protein complex (24), ORC subunits possess the potential to make contact at an origin in a variety of ways. Modification-interference and missing-contact assays at *ARS1* and *ARS305* provided the first look at the diverse nature of the ORC-origin interaction (41). While ORC contacts in *ARS305* were discerned within a ~50 bp region, ORC coverage at *ARS1* extended to a ~92 bp range (41). Footprinting and mobility shift studies also support the ability of DNA sequences to wrap around ORC, enabling it to contact multiple regions at wide ranges (14,18). Thus, it is possible for ORC to make contact with sequences over a long distance. However, ORC may possess limited adaptability, resulting in the selection of certain bipartite sites and providing one explanation for why the yeast genome harbors only ~430 ORC binding sites, but has more than 10 000 ACS matches (19).

In a recent model for the prediction of functional origins in budding yeast, Breier *et al*. devised an Oriscan algorithm based on the alignment of a subset of known origins to the extended 17 bp ACS (20,21). This algorithm was based on the assumption that a single extended ACS (20) in conjunction with certain flanking regions could function as an ORC binding site and did not fully take into account the bipartite structure of replication origins in yeast (18). We propose a revised algorithm definition to include two matches to the ACS as a bipartite binding site for ORC, as previous studies have also shown that synthetic constructs with two copies of the ACS are sufficient for ARS activity (6). Moreover, it is known that the presence of additional ACSs further improve origin function (6,45). This definition does not preclude the use of the extended 17 bp ACS (20).

Prior analyses of the region between the A and B1 elements (and thus between the corresponding ACSs) of *ARS1* indicated that an insertion of 195 bp or greater essentially inactivated ARS activity (38). Inspection of nine origins on chromosome VI with identified, essential ACSs (27,50) revealed that a second ACS (with a 9/11 match or better) resides in close proximity, at a distance of at most 119 bp (data not shown). On average, we calculated the distance to be 38 ± 35 bp (data not shown). We suggest that these considerations might provide a framework for the definition of a functional ORC binding site. Clearly, further studies are needed to test the validity of this hypothesis. Phylogenetic comparisons of conserved sequences may contribute to these endeavors (51).

Bipartite structures for origin recognition in other organisms

Our studies demonstrate that the spacing of the bipartite ORC binding site affects the origin activity of *ARS603*. Replication mechanisms in a number of prokaryotes and viruses show similarity to mechanisms employed at the eukaryotic origins of budding yeast (1). In fact, the recognition of a bipartite binding site for the initiation of bi-directional replication is a familiar theme that is seen in bacterial and viral systems, such as at *oriP* of Epstein-Barr virus and the core origin of SV40 (2). In *Escherichia coli*, ATP-dependent origin activation relies on the presence of two out of the multiple 9-mers that comprise *oriC*, consistent with the recognition of a bipartite site (52). Precise spacing of sequences at the dyad symmetry element DS within *oriP* is required for interaction with EBNA1 in Epstein-Barr virus where origin activity is abolished with the deletion of 1 to 2 bp (3). Likewise, spacing affects binding of the SV40 initiator, large T-antigen, at site II that requires a minimum of 10 bp between two pairs of pentanucleotides with inward orientation (4). According to our results, the A elements at *ARS603* appear to function similarly with a requirement for defined spacing and helical phasing.

Some evidence exists to suggest the conservation of a bipartite site in other eukaryotes as well. For instance, footprinting analyses of a metazoan replication origin, ori II/9A, in *Sciara coprophila* using nuclear extract and purified ORC, show two separate regions of protection for ORC binding (53). Furthermore, recent ChIP studies at the human *c-myc* replicator suggest that ORC binds to two distinct AT-rich regions, resulting in a bipartite binding pattern (54). Thus, the use of a bipartite binding site for the initiator appears to be another connection in the mechanism for the initiation of DNA replication in multiple organisms.

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Conflict of interest statement. None declared.

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