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Multiple DNA elements in *ARS305* determine replication origin activity in a yeast chromosome

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ABSTRACT

A yeast autonomously replicating sequence, *ARS305*, shares essential components with a chromosome III replicator, *ORI305*. Known components include an ARS consensus sequence (ACS) element, presumed to bind the origin recognition complex (ORC), and a broad 3′-flanking sequence which contains a DNA unwinding element. Here linker substitution mutagenesis of *ARS305* and analysis of plasmid mitotic stability identified three short sequence elements within the broad 3′-flanking sequence. The major functional element resides directly 3′ of the ACS and the two remaining elements reside further downstream, all within non-conserved ARS sequences. To determine the contribution of the elements to replication origin function in the chromosome, selected linker mutations were transplaced into the *ORI305* locus and two-dimensional gel electrophoresis was used to analyze replication bubble formation and fork directions. Mutation of the major functional element identified in the plasmid mitotic stability assay inactivated replication origin function in the chromosome. Mutation of each of the two remaining elements diminished both plasmid ARS and chromosomal origin activities to similar levels. Thus multiple DNA elements identified in the plasmid ARS are determinants of replication origin function in the natural context of the chromosome. Comparison with two other genetically defined chromosomal replicators reveals a conservation of functional elements known to bind ORC, but no two replicators are identical in the arrangement of elements downstream of ORC binding elements or in the extent of functional sequences adjacent to the ACS.

INTRODUCTION

Activation of DNA replication origins within a eukaryotic chromosome is a tightly regulated event, limited to a single occurrence during the S phase of the cell cycle (reviewed in 1). The collection of *cis*-acting DNA elements that determine the activity of an origin are called the replicator, in general usage, and the *ori* or *ORI*, for a particular genetic locus, e.g. *oriλ* (2–4). Among eukaryotic replicators, those in the yeast *Saccharomyces cerevisiae* are currently best defined. Yeast replicators were first identified as chromosomal DNA fragments that, when inserted into plasmids, permitted high frequency transformation of cells without integration (5,6). Such DNA fragments are called autonomously replicating sequence (ARS) elements.

ARS activity requires a specific sequence that matches the ARS consensus sequence or ACS: $^{A}/_{T}TTTA^{T}/_{C}{}^{G}/_{A}TTT^{A}/_{T}$ (7–9). The ACS is the core of a larger functional sequence called element A (10,11). The ACS is sensitive to point mutations and serves as a binding site for the origin recognition complex (ORC), a multiprotein complex that contributes to the initiation of DNA replication (12–15). Sequences in the A element but outside the ACS appear non-conserved (7) and little is known about the precise requirements for such sequences in different ARS elements.

In addition to the A element, a broad region called domain B (10), located downstream (3′) of the T-rich strand of the ACS, is also required for ARS function (reviewed in 8). Domain B spans ~100 bp and exhibits no extended sequence conservation among ARSs (7). In several ARSs, domain B is easily unwound (16). A functional role for a DNA unwinding element (DUE) within domain B is supported by mutations that reduce both the ease of DNA unwinding and replication origin activity and by the functional substitution of domain B sequences with dissimilar sequences that are easily unwound (3,17–19). Linker substitution analysis has identified short sequence elements that contribute to the function of domain B in *ARS1* and *ARS307* (11,20,21). *ARS1* function requires three short sequence elements in domain B. Element B3 binds ABF1, a transcription factor and a replication enhancer protein (22). Element B1 contributes to ORC binding *in vitro* and may have additional roles (23). Element B2 interacts with a single-stranded binding protein (RP-A) in supercoiled DNA and has been suggested to function as a DUE (24). *ARS307* contains functionally conserved elements at positions overlapping *ARS1* B1 and B2 elements (20,21). The *ARS307* B2 element is embedded in a broader region that contains a DUE (17). Analogous to models for protein-induced activation of *Escherichia coli oriC* and the SV40 *ori* (25,26), including a general role for a DUE (27,28), a model for activation of yeast replication origins is that DNA-bound ORC, when appropriately modified or associated with other factors (13,29), facilitates opening of the DNA helix in the DUE and subsequent entry of the replication machinery.

Experiments aimed towards genetically defining replicators within yeast chromosomes have shown that ARS elements share essential components with chromosomal replicators. At four different chromosomal loci a specific sequence that matches the ACS is essential for origin function (3,30–32). Also essential is domain B, including the DUE region in *ARS305* (3), as well as the three B elements of *ARS1* (31). The contribution of element A

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sequences outside the ACS to the chromosomal replicator are not known. Also unknown is why the contribution of certain B elements to plasmid ARS function differs from the contribution of those same elements to chromosomal replicator function (21,31). Definition of the replicator by direct measurement of replication origin activity in the natural context of the chromosome is important, since ARS activity only indirectly reflects replication origin activity (33,34) and can be strongly influenced by plasmid context (35–37).

We have performed a linker substitution analysis of *ARS305* to identify short DNA sequence elements that contribute to plasmid ARS function and to define the contribution of such elements to replication origin function in the natural chromosomal locus. In addition to the conserved ACS, we find that a non-conserved sequence in element A is a major determinant of *ARS305* and chromosomal replicator function. Also, two B elements contribute quantitatively to ARS and chromosomal replication efficiency. Comparison of functional elements in three genetically defined chromosomal replicators shows that an element A–B1 array, the bipartite ORC binding site (23), is conserved, but no two replicators are identical in the extent of the A element outside the ACS or in the arrangement of B elements downstream of B1.

MATERIALS AND METHODS

Bacteria and yeast: strains and plasmid isolation

The *E.coli* strain used for plasmid propagation was DH5 (BRL). *Saccharomyces cerevisiae* strain YPH98 (haploid, MAT**a**, *ade2-101*, *lys2-801*, *ura3-52*, *trp1-1*, *leu2-1*) was obtained from Philip Hieter (Johns Hopkins University). Bacteria and yeast cells were propagated and plasmid DNA was isolated as described in Huang and Kowalski (3).

Enzymes and reagents

AmpliTaq polymerase was obtained from Perkin Elmer Cetus. Restriction enzymes and T4 DNA ligase were obtained from New England BioLabs Inc. α-^{32}P-Labeled deoxyribonucleotides were purchased from Amersham Corp. Media components were from Difco Laboratories. DNA sequencing gel solutions (Sequagel) were from National Diagnostics. Other chemicals were purchased from Sigma Chemical Co.

Linker substitution mutations and point mutations by the polymerase chain reaction (PCR)

Mutations in *ARS305* were generated by the method of Perrin and Gilliland (38). The p305BP plasmid (3) containing the 2 kb *Bam*HI–*Pst*I fragment derived from the A6C region of chromosome III (39) was used as a template in the PCR. Oligonucleotides were synthesized by Al Cairo (Biopolymer Facility, Roswell Park Cancer Institute). The mutagenesis procedure involved two rounds of PCR and was performed as described previously (3) except for the following modifications. In the first amplification a double-stranded fragment with the mutation of interest was prepared using 2.5 ng p305BP DNA along with the *ARS305* mutant primer (10 pmol) at the 3′-end and a wild-type primer 305L3 (10 pmol) at the 5′-end. The DNA fragments were separated by gel electrophoresis (1.5% Nusieve/1.5% SeaPlaque LM agarose; FMC) and isolated using the PCR Magic Prep kit (Promega). The second amplification to generate a full-length mutant fragment was performed using a wild-type primer 305R3 (10 pmol) as 3′-flanking primer and the first PCR product (estimated 10 pmol) as the source of 5′-primer. The mutant primers for linker substitutions contain an 8 bp *Xho*I linker sequence flanked by 16 or 17 bp wild-type sequences on each side. For some of the point mutations degenerate primers containing the three alternative nucleotides at the specific position of interest were used as mutant primers. The sequences of the primers are:

305L3 5′-CGTGTAAGCTGGGGTGACTTTTGAGCTATTCGC AC-3′;
305R3 5′-TGATCTTAGTTGGTAGCACTTTGATGAGGTCTCTAG-3′.

Each of the PCR products was cleaved with *Sac*I and *Cla*I and the electrophoretically purified 336 bp *Sac*I–*Cla*I fragments were then ligated with purified vector DNA (p305BP without the corresponding wild-type *Sac*I–*Cla*I region of *ARS305*) and transformed into DH5α cells. Plasmids with a *Xho*I linker substitution were identified by their susceptibility to cleavage by *Xho*I restriction enzyme. The DNA sequence of each mutant derivative was verified by the dideoxy chain termination method using Sequenase (US Biochemicals) and the 305R1 primer (3).

High frequency transformation (HFT) assay

The *Sph*I–*Eco*RI fragments from p305BP derivatives were subcloned into the *Sph*I and *Eco*RI sites of a centromere-containing vector, pVHA (40), a gift from Carol Newlon (UMD–New Jersey Medical School). Plasmids were transfected into the yeast cells using the lithium acetate procedure (41). After transfection, cells were plated on synthetic minimal medium minus uracil (SMM/U$^-$) at 30°C for 2–4 days and scored for HFT (>200 transformants/g DNA). Transformants were scored as Ars$^+$ if they could be passaged in SMM/U$^-$ liquid culture.

Mitotic stability, plasmid retention and plasmid replication efficiency

The mitotic stability assays were performed as described (42) with the following modifications. Single transformants from the SMM/U$^-$ plates were each inoculated in 3 ml selective medium and grown for 22–24 h at 30°C. Cultures were plated on non-selective (YPD) and selective plates (SMM/U$^-$) at dilutions that gave rise to ~200 colonies on non-selective plates to determine the initial percentage of plasmid-containing cells under selection (*I*). Cultures were also diluted (1×10^4 cells/ml) and grown in 3 ml non-selective medium for 12 generations at 30°C. Cultures were plated on non-selective and selective media as above to determine the final percentage of plasmid-containing cells after growth in the absence of selection (*F*). For transformants with extremely low growth rates, twice and 200 times the amount of cells were plated on the selective plates to determine the *I* and *F* values respectively. Mitotic stabilities (percentage of plasmid-containing cells) before release of selection and after growth in non-selective medium for a specific number of generations (*n*) correspond to *I* and *F* respectively. The value of *n* is 12 generations in this study and the mitotic stability values reported correspond to F_{12}. Plasmid retention per generation (*PR*, %) is expressed as $[F/I]^{1/n}$ (intuitively $F_n = I[PR]^n$). The plasmid replication efficiency was calculated by normalizing *PR* for the centromere (CEN)-containing plasmid retention assumed to occur in the absence of any plasmid replication (50% per generation) and is expressed as $2[PR - 50]$.

Mutation of *ORI305* in chromosome III and analysis of replication origin activity

Linker substitution mutations were introduced into the *ORI305* locus by transplacement using procedures previously described (3). The desired homologous recombinants, each containing a *Xho*I linker substitution in the *ORI305* locus, were identified after restriction digestion of genomic DNA with *Xho*I and *Eco*RI and detection of the appropriate sized fragment following gel electrophoresis and Southern blotting. DNA replication intermediates isolated from high molecular weight yeast genomic DNA were isolated, enriched and separated using two-dimensional gel electrophoresis and the signals analyzed using a phosphorimager (Molecular Dynamics), as previously described (3).

A modified two-dimensional agarose gel electrophoresis method was used to determine the direction of replication fork movement adjacent to the mutant *ORI305* loci. The method involves an 'in gel' digestion with a second restriction enzyme between the first and second dimensions of electrophoresis and is essentially as described by Brewer *et al*. (43). After electrophoresis, DNA was transferred to a nylon membrane and hybridized with a ^{32}P-labeled DNA probe. The radioactive signals were detected by scanning in a phosphorimager and were quantified using ImageQuant software (Molecular Dynamics) by analyzing parallel, non-overlapping regions of the two Y arcs.

RESULTS

Linker substitution analysis of *ARS305*

To identify short sequence elements that are essential or otherwise important for ARS function we constructed a series of linker substitution mutations in *ARS305*. Oligonucleotide-directed mutagenesis via PCR was used to substitute an 8 bp G+C-rich sequence (*Xho*I linker) in place of the wild-type sequence at a variety of positions in *ARS305*. The ARS was present in a vector containing the *URA3* selectable marker and a CEN for proper plasmid segregation.

The mutant ARS derivatives present in these plasmids were first examined for autonomous replication function as indicated by their competence for HFT of a yeast strain that requires the plasmid *URA3* gene for growth in selective medium. Linker substitution mutations in only one specific region of *ARS305* led to complete loss of ARS activity (Fig. 1, Lin1 and Lin4). The region spans a sequence that is a near match to the ACS and was previously found by analysis of deletion mutations and a limited number of point mutations to be essential for *ARS305* function (3). Despite the occurrence of other near matches to an ACS in *ARS305* (44), linker substitution analysis detected no other short sequences that are essential for ARS function. The results presented here confirm and extend our earlier finding that sequences in one specific near match to the ACS are essential for autonomous replication activity mediated by *ARS305*. The essential ACS match is called the ACS element (Fig. 1).

None of the remaining linker substitutions in *ARS305* affected the ability of plasmids to transform yeast cells at high frequency (Fig. 1, HFT$^+$), however, slow colony growth rates were observed for transformants containing derivative Lin12. The activity of each mutant ARS plasmid was evaluated by measuring the mitotic stability: the fraction of plasmid-containing cells after growth for a fixed number of generations in non-selective medium. The results are shown in Figure 2. The high mitotic stability of wild-type *ARS305* reflects efficient ARS function. The mitotic stability of derivative Lin–8, which contains a linker substitution in the 8 bp sequence directly 5′ of the ACS element, was similar to that of wild-type *ARS305*, consistent with the lack of a requirement for 5′-flanking sequences shown by deletion and point mutation analysis (3).

Derivative Lin12 contains a linker mutation in the 8 bp sequence directly 3′ of the essential ACS match. The Lin12 plasmid was rapidly lost from cells, as indicated by an extremely low mitotic stability (Fig. 2, arrow). In contrast, the Lin14 mutation, which skips over the 2 nt immediately 3′ of the essential ACS match, had very little effect on mitotic stability; however, further mutational analysis indicates that nucleotide residues within the region of Lin12 that overlap with Lin14 are functionally important (see below).

Two other linker mutations, Lin22 and Lin102, reduced the mitotic stability of *ARS305* significantly below the wild-type level, although the extent of reduction was not nearly as great as

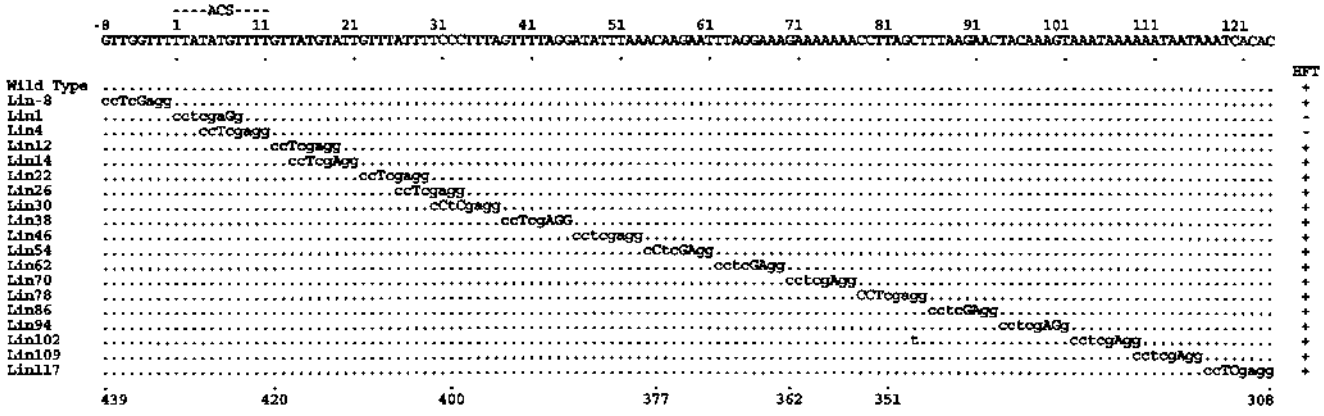

Figure 1. Effects of linker substitutions in the *ARS305* sequence on activity in the HFT assay. The wild-type *ARS305* sequence is shown at the top and the essential ACS match is indicated. The linker substitution derivatives (left) are named according to the first position substituted by the 8 bp linker sequence, relative to ACS position 1. The linker sequence present in each derivative is shown, with the lower case letters indicating bases that differ from those present in the wild-type *ARS305*. ARS activity (+) or inactivity (–) in the HFT assay are indicated. All of the linker substitution derivatives had the expected DNA sequence except for one, Lin102, which contained a single point substitution in a functionally unimportant sequence (Lin78; see also Fig. 2). The numbers at the bottom of the figure are reference points for deletion mutations that were previously described (3).

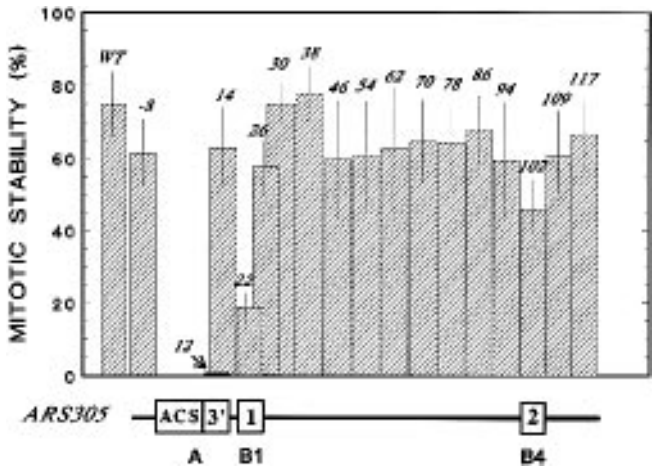

Figure 2. Linker substitution analysis identifies DNA elements important for *ARS305* function. The bar graph shows mitotic stabilities of wild-type and linker substitution mutations of *ARS305* in CEN-containing plasmids. Mitotic stability is the percentage of plasmid-containing cells remaining after 12 generations of growth in the absence of selection. The thin vertical lines overlapping each bar indicate the standard deviation in assays performed using at least six independent transformants. The name of each Lin derivative is indicated above each bar. Below the graph is a schematic diagram of *ARS305* showing the ACS (boxed) and the downstream elements revealed by linker substitution (boxes 3′, 1 and 2). The ACS and box 3′ (3′ to the ACS T-rich strand) define element A and boxes 1 and 2 are in domain B. Element swapping experiments between *ARS305* and *ARS1* define box 1 as B1 and box 2 as B4 (S.Lin and D.Kowalski, unpublished data).

that seen for Lin12 (Fig. 2). Relative to the first nucleotide in the essential ACS match (position 1), the Lin22 mutation occurs at positions 22–29 and the Lin102 mutation occurs at positions 102–109 (Fig. 1). Other linker substitutions downstream of the ACS had little or no effect on mitotic stability (Fig. 2).

Our results show that in addition to the essential ACS, *ARS305* has three other functional elements detectable by linker substitution analysis (Fig. 2). The major determinant, revealed by Lin12, resides directly 3′ of the ACS element (box 3′). Because box 3′ is contiguous with the ACS and because linker substitution of box 3′ essentially inactivates *ARS305*, box 3′ is included as part of element A. Separate elements in the B domain are revealed by Lin22 (box 1) and Lin102 (box 2). Based on results of element swapping experiments with B elements of *ARS1* (S.Lin and D.Kowalski, unpublished data), boxes 1 and 2 are designated as elements B1 and B4 respectively (see Discussion).

The level of mitotic stability of an ARS–CEN plasmid depends upon several experimental parameters, including the presence or absence of selection, as well as the number of generations of cell growth in the absence of selection. All of these parameters are accounted for by calculating the plasmid retention per generation (see Materials and Methods), permitting comparisons of ARS activities between studies that grow cells for different numbers of generations. Plasmid retention per generation was 98 ± 1% for the wild-type ARS, 66 ± 1% for Lin12 (box 3′ in element A), 90 ± 2% for Lin22 (element B1) and 96 ± 1% for Lin102 (element B4). The wild-type ARS exhibits the highest plasmid retention per generation and mutations in B4 and B1 lead to significant decreases. For the mutation in box 3′ plasmid retention is extremely low, approaching the theoretical retention in the absence of any plasmid replication (50% per cell generation).

Effects of different linker and point mutations in box 3′

In contrast to Lin12, which caused a severe reduction in ARS mitotic stability, Lin14, the same linker sequence introduced only 2 nt further downstream, had little or no effect (Table 1). Introduction of a different linker sequence at positions 14–21 (Lin14a) also had little effect compared with that seen for the Lin12 mutation (Table 1). One of the possible interpretations of these results is that one or two residues adjacent to the 11 bp ACS are crucial for replication origin function. The residue 3′ of the ACS (position 12) is known to be important but not essential in both *ARS307* (40) and the H0 *ARS* (45). As seen in Table 1, point mutations at position 12 in *ARS305* reduce mitotic stability while all point mutations at position 13 had little or no influence. These results indicate that a G at position 12 is important for *ARS305* function and that point substitutions lead to decreased function in the following order G ≥ T > C > A; however, none of the single point mutations at position 12 or 13 led to the extremely low mitotic stability seen for Lin12, which substitutes positions 12–19.

A double point mutation that matches the first two residues of Lin12 was introduced at positions 12 and 13 to test whether the extremely low mitotic stability seen for Lin12 was due solely to an effect of simultaneous mutations at positions 12 and 13. As shown in Table 1, this was not the case, since the mitotic stability of the double point mutant C12C13 was much greater than that of Lin12.

Since separate mutations in positions 12–13 (C12C13) or 14–19 (Lin14 and Lin14a) did not severely reduce mitotic stability, the extremely low mitotic stability of derivative Lin12 appeared to result from a synergistic effect of mutations in two regions: positions 12–13 and positions 14–19. Three different linker sequences at positions 14–19 in combination with the C12C13 mutation led to an extremely low mitotic stability (Table 1, Lin12, Lin12a and Lin12b), confirming the synergistic effect and supporting the functional importance of the region containing positions 14–19.

Reversion of the C mutation at position 13 in Lin12b to a wild-type T in an otherwise constant mutant background in derivative Lin12c led to a high level of mitotic stability (Table 1, Lin12c). Thus the T at position 13 is functionally important when substitutions are present simultaneously at position 12 and in the region containing positions 14–19. This observation is surprising, since position 13 appeared relatively unimportant when point mutated in the wild-type context; however, this observation is consistent with the synergistic effects seen for other box 3′ mutations. Overall, the results show that *ARS305* function is influenced by several nucleotide residues at positions 12–19 which comprise box 3′ and that simultaneous mutations in these residues can act synergistically to essentially inactivate ARS function.

Box 3′ is a major determinant of replication origin function in the chromosome

To determine the contribution that elements revealed by linker substitution of *ARS305* make to replication origin activity in the chromosome we first replaced the natural sequence at the *ORI305* locus in a haploid yeast strain with mutated sequences containing linker substitutions by using gene transplacement techniques. Then we analyzed replication origin activity around the mutated *ORI305* locus by two-dimensional gel electrophoresis (46). Chromosomal DNA was cleaved at specific restriction enzyme sites and the fragments were separated by mass in the first dimension gel electrophoresis and by shape and mass in the second dimension. DNA replication intermediates with the sequence of

interest were detected after Southern transfer by hybridization using a specific ^{32}P-labeled DNA probe. If the specific DNA segment in the chromosome contains an active replication origin a high rising arc, termed a bubble arc, is detected, resulting from the progressively retarded mobility of intermediates as the two replication forks move away from the centrally located origin (Fig. 3F). If the replication origin in the specific DNA segment is inactivated as a result of a mutation then the segment is passively replicated from an external origin. In this case the bubble arc is diminished or not detected, depending on the severity of the mutation, and intermediates with a single replication fork give rise to a distinct arc, called a Y arc (Fig. 3F). The relative intensities of the bubble and Y arcs reflect the relative frequencies of replication origin activity and passive replication.

Figure 3 shows two-dimensional gel analysis of chromosomal origin function at the wild-type *ORI305* locus and at mutant *ORI305* loci in the yeast strains we constructed. Analysis of the wild-type *ORI305* locus shows a prominent bubble arc with little or no early Y arc, indicative of efficient replication origin function (Fig. 3A). A strong late Y arc signal is seen, indicating that one of the replication forks from the active origin reaches an end of the specific restriction fragment before the other fork, converting the bubble-shaped intermediates to Y-shaped.

In strain YRHLin12 the *ORI305* locus contains the Lin12 mutation. Lin12 identified box 3′ as extremely important for plasmid ARS efficiency (Fig. 2 and Table 1, Lin12). Two-dimensional gel analysis of replication intermediates from YRHLin12 shows only a strong complete Y arc, indicating that the replication origin is not detectably active in the chromosome (Fig. 3B). Thus the Lin12 mutation identifies box 3′ as extremely important for replication origin function mediated by *ORI305* in chromosome III.

Table 1. Linker and point mutations in box 3′ of *ARS305*

Derivative	Sequence[a]	Mitotic stability (%)[b]
	12 19	
	. .	
WT	GTTATGTATT	74 ± 9
Lin12	**cc**T**cgagg**TT	0.03 ± 0.01
Lin14	GT**ccTcgAgg**	63 ± 12
Lin14a	GT**ggaGctcc**	55 ± 4
A12	**a**TTATGTATT	20 ± 7
C12	**c**TTATGTATT	40 ± 7
T12	**t**TTATGTATT	56 ± 10
A13	G**a**TATGTATT	61 ± 8
C13	G**c**TATGTATT	69 ± 10
G13	G**g**TATGTATT	62 ± 13
C12C13	**cc**TATGTATT	35 ± 6
Lin12a	**ccatacat**TT	0.04 ± 0.04
Lin12b	**ccaAaGTt**TT	0.02 ± 0.01
Lin12c	**cTaAaGTt**TT	47 ± 11

[a]The 10 bp sequence immediately 3′ of the ACS element (positions 1–11, not shown), beginning with position 12 and extending to position 21. Box 3′ spans positions 12–19. Bold lower case letters represent nucleotide residues that differ from those in the wild-type *ARS305*. Upper case letters represent nucleotide residues that are identical to those in wild-type *ARS305*.

[b]The percentage of plasmid-containing cells after growth in non-selective medium for 12 generations. Mitotic stability values represent an average of measurements obtained using at least six individual transformants.

Strains YRHLin22 (Fig. 3C), YRHLin–8 (Fig. 3D) and YRHLin102x–8 (Fig. 3E) contain Lin22, Lin–8 and Lin102 plus Lin–8 mutations respectively. The Lin–8 mutation substitutes the 8 bp immediately 5′ of the ACS and has little effect on plasmid mitotic stability (Fig. 2). The Lin22 and Lin102 mutations define the B1 and B4 elements respectively and significantly decrease plasmid mitotic stability (Fig. 2). In all three strains two-dimensional gel analysis shows prominent bubble arcs, indicating that the mutant *ORI305* loci mediate a high level of replication origin activity (Fig. 3C–E); however, all three strains also show evidence of early Y arc-like signals, raising the possibility that origin activity may be diminished (see quantitative analysis below).

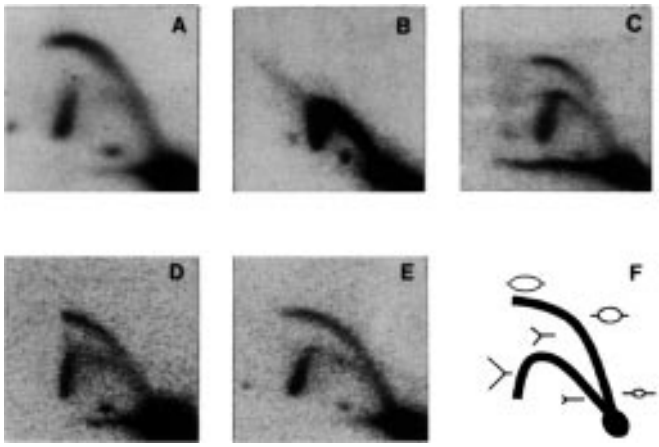

Figure 3. Two-dimensional gel analysis of chromosomal replication origin activity at wild-type and mutated *ORI305* loci. The yeast strains analyzed are: (**A**) YPH98, the parental strain containing wild-type *ORI305*; (**B**) YRHLin12, the Lin12 mutation in box 3′; (**C**) YRHLin22, the Lin22 mutation in the B1 element; (**D**) YRHLin–8, the Lin–8 mutation at positions –8 to –1; (**E**) YRHLin102x–8, both the Lin102 mutation in element B4 and the Lin–8 mutation. (**F**) A schematic diagram of the migration pattern seen for bubble- and Y-shaped replication intermediates in the two-dimensional gel electrophoresis. The first dimension electrophoresis (size separation) is from left to right and the second dimension (shape and size separation) is from top to bottom.

B1 and B4 elements contribute to chromosomal origin efficiency

Standard two-dimensional gel analysis of replication intermediates provides mostly qualitative information. To quantify the effects of mutations on origin activity we used a modified two-dimensional gel method that assesses the direction of replication fork movement in the chromosome. Unlike the standard two-dimensional gel method, the modified method is free of possible interference from Y arc-like signals that result from breakage of replication bubbles (47,48). After the first dimension electrophoresis of *Xho*I-cleaved genomic DNA, the DNA was digested in the gel with *Hin*dIII, subjected to second dimension gel electrophoresis and, after blotting, probed for the large *Xho*I–*Hin*dIII fragment (Fig. 4A). Rightward moving forks first enter the large *Xho*I–*Hin*dIII fragment, forming an arc of Y-shaped intermediates which arises from the intense spot containing non-replicating DNA (Fig. 4B, R). Leftward moving forks first enter the small *Xho*I–*Hin*dIII fragment that is removed (Fig. 4A, L_1). Consequently, leftward moving forks form an arc of simple-Y intermediates that is displaced from the intense spot (Fig. 4B, L). If *ORI305* is active, replication forks are expected to move rightward through the probed fragment. If *ORI305* is inactivated by mutation, replication forks are expected to move

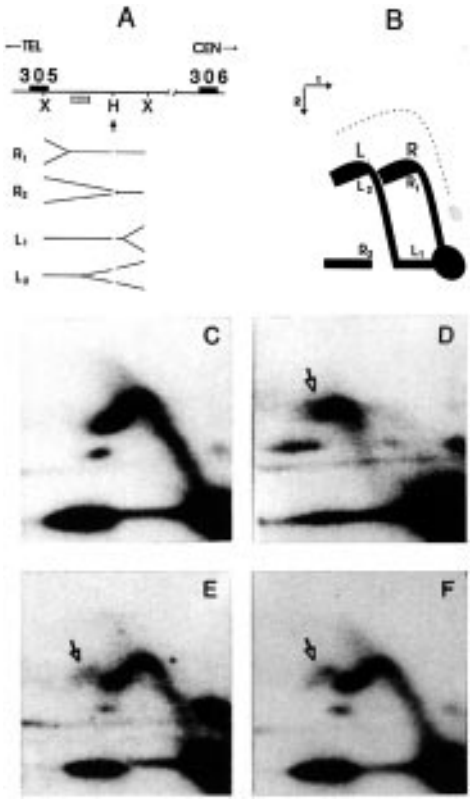

Figure 4. Direction of replication fork movement as an assay for chromosomal origin activity at mutated *ORI305* loci. (**A**) A map of the left arm of chromosome III that includes two chromosomal replicators (black boxes): *ORI305* (telomere-proximal) and that associated with *ARS306* (centromere-proximal). The fragment analyzed and the restriction sites used in the fork direction gels are depicted below the map. Genomic DNA was cleaved with *Xho*I (X), generating a fragment of 3.5–3.6 kb, depending on the position of the *Xho*I linker substitution in *ORI305*. Following the first dimension gel electrophoresis the *Xho*I fragments were digested in the gel with *Hin*dIII (arrow) and electrophoresed in the second dimension. The large *Xho*I–*Hin*dIII fragment was detected by hybridization with a ^{32}P-labeled probe (gray box). R_1, R_2 and L_1, L_2 denote rightward and leftward replication fork movement respectively to two different extents. (**B**) The diagram illustrates the expected signal patterns for various DNA intermediates in the modified two-dimensional gel method. The directions of electrophoresis in the first and second dimensions are indicated by the arrows. The dashed line arc and the gray spot indicate the positions of replicating *Xho*I fragments and non-replicating *Xho*I fragments respectively when the fragments are not cut with *Hin*dIII in the gel. The dark arcs below represent the shortened Y intermediates after cleavage of the *Xho*I fragment with *Hin*dIII in the gel prior to the second dimension gel electrophoresis. The large R and L indicate the simple-Y arcs containing rightward and leftward moving forks respectively. R_1, R_2 and L_1, L_2 indicate the positions for the different extents of fork movement shown in (A). (**C**–**F**) Results obtained using the modified two-dimensional gel method to assess the direction of replication fork movement. The yeast strains analyzed and the mutations they contained were: (C) YRHLin–8, the Lin–8 mutation at positions –8 to –1; (D) YRHLin12, the Lin12 mutation in box 3′; (E) YRHLin22, the Lin22 mutation in the B1 element; (F) YRHLin102x–8, both the Lin102 mutation in element B4 and the Lin–8 mutation.

leftward, since the 306 origin (Fig. 4A) and other centromere-proximal origins are active and since there are no active origins on the telomere side of *ORI305* (49,50). The proportion of rightward moving forks reflects the replication origin efficiency mediated by *ORI305*.

Wild-type *ORI305* mediates efficient origin function in the parental strain (3; Fig. 3A) and, consistent with this, only rightward moving forks are detected (data not shown). The Lin–8 mutation, which has wild-type ARS activity (Fig. 2), also exhibits wild-type *ORI305* activity, since only signals indicative of rightward moving forks are seen (Fig. 4C). In strain YRHLin12, which contains a linker mutation in box 3′, signals indicative of leftward moving forks are primarily detected (Fig. 4D, arrow). Phosphorimager analysis of the signal intensities for the rightward and leftward moving forks shows that replication origin efficiency is 94% in the strain with wild-type *ORI305* activity, but only 12% in the mutant strain. The replication origin efficiency in the mutant strain is a maximal estimate and may actually be lower, since no clear arc indicative of rightward moving forks is visible (Fig. 4D). These result demonstrate that *ORI305* is largely, if not completely, inactivated by a linker mutation in the portion of element A in box 3′ and that the mutated chromosomal locus is passively replicated by leftward moving forks.

In strain YRHLin22, which contains a linker mutation in the B1 element, most of the forks move rightward, indicative of *ORI305* activity (Fig. 4E); however, leftward moving forks are also seen (Fig. 4E, arrow), demonstrating a reduction in origin efficiency. Similar results are seen for strain YRHLin102x–8 (Fig. 4F, arrow), which contains linker mutations in the B4 element and in positions –8 to –1. Phosphorimager analysis of the signal intensities of the rightward and leftward moving forks shows that replication origin efficiency is 71% and 75% in strains YRHLin22 and YRHLin102x–8 respectively. In contrast, replication origin efficiency is 94% in strain YRHLin–8 (Fig. 4C), identical to that of wild-type *ORI305*. Thus linker mutations in either the B1 element or in the B4 element together with positions –8 to –1 result in a significant reduction in replication origin efficiency mediated by *ORI305* in chromosome III.

We compared the effects that the B1 and B4 mutations have on chromosomal origin activity with their effects on plasmid ARS activity. Chromosomal origin activity reflects events occuring during a single S phase. We calculated the plasmid replication efficiency in a single S phase from the plasmid retention per cell generation. The calculation assumes that a failure of the ARS to initiate replication in a given cell cycle leads to retention of the CEN-containing plasmid in one of the two cells produced after division (see Materials and Methods). For the B1 mutation, chromosomal origin efficiency is 71% and the plasmid replication efficiency is 80%. For the B4 mutation together with Lin–8 the chromosomal origin efficiency is 75% and the plasmid replication efficiency is 86%. Thus the contributions that the B elements make to plasmid ARS reflect the quantitative contributions that they make to the replication origin in the chromosome.

DISCUSSION

Linker substitution analysis of DNA is useful in identifying short sequences that serve as functional elements, since the substitution alters the sequence of an individual element but not the potentially important spacing between elements (51). Linker substitutions in *ARS305* and analysis of plasmid mitotic stability revealed three elements downstream of the ACS. The major functional element is box 3′, which resides directly 3′ of the essential ACS and is part of element A. Two additional elements, B1 and B4, were detected further downstream in sequences in the B domain. All three elements reside in sequences that are not conserved among ARSs (7; data not shown). Our results show that *ARS305* function

requires multiple short sequence elements in non-conserved ARS sequences downstream of the ACS.

Since plasmid ARS activity only indirectly reflects replication origin activity and can be influenced by plasmid context, we examined whether and to what extent each element that affects plasmid *ARS305* function also contributes to authentic replication origin function at the *ORI305* locus within chromosome III. Box 3′ in element A is a major determinant, since linker substitution essentially inactivates the replication origin in the chromosome. This is the first identification of a short sequence element outside the ACS that is crucial for replication origin function in a yeast chromosome.

Previous studies suggested that B elements contribute differently to plasmid and chromosomal replicator function (21,31). For *ARS1* the B1 and B2 elements appeared to make different relative contributions to plasmid stability and origin activity (31), although origin activity was not analyzed quantitatively. For *ARS307* B1 mutations affected quantitative origin activity less dramatically than they affected plasmid stability over multiple generations (21). Here the contribution that the B1 and B4 elements make to chromosomal origin efficiency was demonstrated by modified two-dimensional gel analysis, which permits quantitation of replication fork directions. Furthermore, we compared the plasmid replication efficiency in a single S phase, as opposed to the plasmid stability over multiple generations. Our findings show that the contributions the B elements make to plasmid *ARS305* function reflect the quantitative contributions that they make to replication origin function in the chromosome. Thus B elements detected in the ARS plasmid likely serve the same function at the natural replicator locus within the chromosome.

The overall organization of genetic elements in *ARS305* is compared with that in *ARS1* (11) and *ARS307* (20,21) in Figure 5A. In addition to an ACS in element A, all three ARSs contain a B1 element and one or two additional B elements. No element was detected by linker substitution in *ARS305* at positions corresponding to B2 elements in *ARS1* and *ARS307*. The B4 element in *ARS305* overlaps the position of the B3 element in *ARS1*; however, B3 corresponds to an ABF1 binding site, but the sequence of B4 does not match an ABF1 consensus and cannot be functionally substituted by B3, B2 or B1 (S.Lin and D.Kowalski, unpublished data). At the B3/B4 position no element is present in *ARS307*. All three ARSs differ in the extent of their sequence requirements directly 3′ and 5′ of the ACS. Only in *ARS305* and *ARS307* does element A extend significantly 3′ of the ACS element, corresponding to the box 3′ region of *ARS305*. The comparison reveals that an element A–B1 array, the bipartate ORC binding site (23), is conserved among the three replicators, however, no two replicators are identical in the extent of element A outside the ACS or in the arrangement of B elements downstream of B1.

Box 3′ resides in between the ACS and the B1 elements which bind ORC (12,23). DNase I footprinting shows that ORC protects the ACS, the B1 region and the region corresponding to the box 3′ sequence in a variety of ARSs (12,52). We find that mutation of multiple nucleotide residues are required to inactivate box 3′ function. It is possible that ORC, or another origin binding protein, interacts directly with box 3′ sequences at multiple nucleotide positions. Alternatively, the DNA conformation of box 3′ may be important to facilitate protein recognition or other events that lead to origin activation.

Neither the entire box 3′ sequence nor individual nucleotides in box 3′ are conserved among 21 ARS elements (data not shown), some of which do not function as replication origins in their

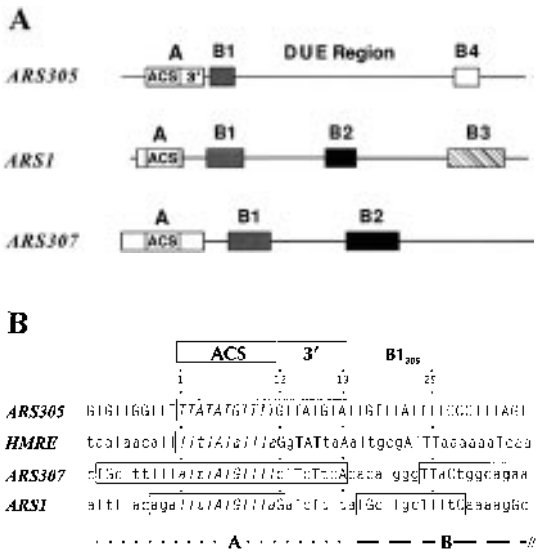

Figure 5. Organization and sequence of genetic elements in ARSs derived from yeast chromosomal replicators. (**A**) Overall organization of functional elements (boxes) detected by linker scan analysis of *ARS305* (this study), *ARS1* (11) and *ARS307* (20,21). The three ARSs are aligned at the essential match to the ARS consensus sequence (ACS box). Box 3′ in *ARS305* and sequence elements (open boxes) adjacent to the ACS in the other ARSs are parts of element A. Multiple B elements (labeled boxes, drawn to scale) were identified by linker substitution in all three ARSs. B elements identified in *ARS305* overlap a DUE-containing region identified by deletion analysis (3). (**B**) Chromosomal replicators share certain identities and similarities at nucleotide residues in non-conserved sequences outside the ACS. The *ARS305* sequence in and around the ACS box, box 3′ and the B1 element are compared with sequences in chromosomal replicators from which the ARS elements *HMRE ARS, ARS307* and *ARS1* (30–32) were derived. The essential ACS is shown in italics. Matches to bases in the *ARS305* sequence are indicated by upper case letters. Matches present in all four replicator sequences outside the ACS are in bold type. Elements A and B1 of the chromosomal replicators associated with *ARS305, ARS1* and *ARS307* (only part of B1 is shown) are indicated by the boxed sequences (20,31; this study). For the *HMRE* ARS the ACS (boxed) is the only known functional element (32). Domain A (dotted line) and an ACS-proximal portion of domain B (dashed line) are indicated.

chromosomal locations (50). The crucial role of box 3′ in chromosomal origin function prompted us to compare its DNA sequence with the corresponding sequence in other replicators for which the ACS element had been genetically defined in the chromosome. As seen in Figure 5B, the box 3′ sequence as a whole is not conserved in the four chromosomal replicators; however, all four replicators have either a G at position 12, a T at position 13 or both, the same residues that are functionally important within the different sequence contexts substituted in *ARS305*. In *ARS307* the C that occurs at position 12 is functionally important, but can be substituted by a G without loss of activity (40). The sequence comparison also reveals that base identities exist among all four chromosomal replicators at positions 14 and 16 and among three of four at position 19 (A), corresponding to *ARS305* region 14–19, where one or more residues are important. Thus while the entire box 3′ sequence is not conserved among chromosomal replicators, the sequence exhibits limited nucleotide identities and similarities in functionally important subsequences at a fixed spacing from the ACS.

A B1 element appears to be a common feature in yeast replicators (Fig. 5A). At the DNA sequence level (Fig. 5B) the B1 element of

ARS305 maps at positions within the B1 element identified in *ARS1* (11) and overlaps the first two bases, T-28 and T-29, in the B1 element of *ARS307* (20). A TT subsequence is present in the B1 regions at the same position relative to the ACS in all four genetically defined chromosomal replicators (Fig. 5B). Although the positions of the B1 elements as a whole appear to differ, the occurrence of a TT subsequence at a fixed distance from the ACS may be an important component of the B1 elements. Higher resolution mutational analysis indicates that T residues at position 28, 29 or 30 are functionally important in several ARS elements, including the rDNA *ARS*, *ARS1* and *ARS307* (18,20). In *ARS1* the T at position 29 contributes to binding ORC (23). The T residue at position 29 may be generally important for ARS function and ORC binding, since among 21 ARSs in which the ACS element has been defined T-29 shows the highest occurrence (71%) for any individual residue outside and near the ACS (data not shown).

The B domain of *ARS305* is easily unwound and a DUE (Fig. 5A) was previously assigned to this domain by deletion analysis (3). External deletion mutations within the region between B1 and B4 and including B4 stabilize the DNA helix and reduce ARS efficiency. External deletion of the broad region downstream of B1 and including B4 resulted in a much lower mitotic stability (6%, derivative 80/400 in ref. 3) than did individual linker substitutions across that region (60%) or within B4 (Fig. 2). Detection of the DUE by large deletions but not by linker substitution suggests that the DUE is longer than the 8 bp linker, i.e. the G+C-rich linker increases the local helical stability but does not reduce the ease of unwinding in the remainder of a broader sequence containing the DUE. Consistent with this interpretation, large deletions, but not 10 bp G+C-rich linker substitutions, reduce the ease of DNA unwinding detected by single-strand-specific nuclease in the DUE region of the H4 *ARS* (19). Our previous results (3), when combined with those presented here, are consistent with DUE function in a broad sequence downstream from element B1. Interestingly, the B2 element detected in this region of *ARS1* (Fig. 5A) has been suggested to function as a DUE and may have other functions (20,21,24,29). Also, the B2 element of *ARS307* (Fig. 5A) is embedded within a broader functional region identified by deletion (53) and that region contains a DUE (17).

The results presented here, together with our earlier findings (3), reveal that DNA elements essential or important to the function of *ARS305* contribute to replication origin activity at *ORI305* in chromosome III. It remains to be determined whether the DNA elements sufficient for ARS function are also sufficient for chromosomal replicator function or whether additional elements are required in the chromosome.

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