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Functional Equivalency and Diversity of *cis*-Acting Elements among Yeast Replication Origins

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The DNA replication origins of the yeast *Saccharomyces cerevisiae* require several short functional elements, most of which are not conserved in sequence. To better characterize *ARS305*, a replicator from a chromosomal origin, we swapped functional DNA elements of *ARS305* with defined elements of *ARS1*. *ARS305* contains elements that are functionally exchangeable with *ARS1* A and B1 elements, which are known to bind the origin recognition complex; however, the *ARS1* A element differs in that it does not require a 3′ box adjacent to the essential autonomously replicating sequence consensus. At the position corresponding to *ARS1* B3, *ARS305* has a novel element, B4, that can functionally substitute for every type of short element (B1, B2, and B3) in the B domain. Unexpectedly, the replacement of element B4 by *ARS1* B3, which binds ABF1p and is known as a replication enhancer, inhibited *ARS305* function. *ARS305* has no short functional element at or near positions corresponding to the B2 elements in *ARS1* and *ARS307* but contains an easily unwound region whose functional importance was supported by a broad G+C-rich substitution mutation. Surprisingly, the easily unwound region can functionally substitute for the *ARS1* B2 element, even though *ARS1* B2 was found to possess a distinct DNA sequence requirement. The functionally conserved B2 element in *ARS307* contains a known sequence requirement, and helical stability analysis of linker and minilinker mutations suggested that B2 also contains a DNA unwinding element (DUE). Our findings suggest that yeast replication origins employ a B2 element or a DUE to mediate a common function, DNA unwinding during initiation, although not necessarily through a common mechanism.

To ensure the precise inheritance of genetic information, the replication of chromosomal DNA is tightly controlled at the initiation stage so that it occurs exactly once during the S phase of each cell cycle (reviewed in references 36, 46, and 49). Studies of *Escherichia coli*, bacteriophages, eukaryotic viruses, and the yeast *Saccharomyces cerevisiae* have revealed that DNA replication initiates at distinct sites, replication origins (reviewed in references 9 and 28). The activity of a replication origin is determined by *cis*-acting elements that are generically called replicators. Yeast replicators were initially identified as autonomously replicating sequence (ARS) elements that enable plasmids containing them to be stably maintained (21, 50). ARS elements confer replication origin activity on plasmids, and a subset of ARSs colocalize with natural replication origins in chromosomes (5, 13, 18, 24, 25). DNA mutations that affect ARS activity in plasmids also influence the activity of the corresponding replication origin in the chromosome (10, 22, 23, 33, 45, 51).

Every ARS contains an essential match to an 11-bp ARS consensus sequence (ACS) (reviewed in reference 39). The essential ACS match is sometimes imperfect, containing one or two mismatches to the consensus (22, 27, 35, 48, 55). A multiprotein origin recognition complex (ORC) that specifically binds the ACS element has been found to contribute to the initiation of DNA replication (1, 11, 15, 30). Although they are not conserved, sequences that immediately flank the ACS element are also important for ARS function; together with the ACS, they form the A element (7, 32).

Downstream (3′) to the T-rich strand of the ACS, a broad region is also required for ARS function (7) and chromosomal replication origin function (22). This region, the B domain, spans about 100 bp and shows no apparent sequence similarity among ARSs. A DNA unwinding element (DUE), whose intrinsic helical instability facilitates origin activity, has been detected within the B domains of several ARSs (22, 35, 37, 52). DUEs are composed of easily unwound sequences (38) that can be functionally replaced by dissimilar sequences that are also easily unwound. Mutations that raise the DNA helical stability within the DUE reduce replication origin activity. Analyses of mutations in both *E. coli oriC* and the simian virus 40 (SV40) replication origin in this laboratory have identified a DUE (29, 31) that corresponds to the site which the initiator protein induces to unwind during the initiation of replication (2, 3, 17). In yeast replication origins, the site of initiator protein-induced unwinding is not known. Localization of the DUE will help further define the *cis*-acting elements in yeast replicators and may point to the crucial site where DNA unwinding initiates.

Linker substitution analysis of the B domain of *ARS1* identified short functional elements that are important both for ARS activity and for replication origin activity in yeast chromosomes (32, 33). Three functionally distinct elements were detected. Element B1 contributes to ORC binding and has an unidentified role (44, 47). The functional role of the B2 element is not known. Element B3 contains a binding site for ABF1p, a transcription and replication factor, and can be replaced by the binding sites of other transcription factors (32). ABF1-binding sites serve as replication enhancers in *ARS121* (54), and such sites have been identified in other ARSs (reviewed in reference 46). In one other ARS, *ARS307*, short

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elements at positions similar to those of B1 and B2 were identified (43, 51). The similarly positioned elements in *ARS1* and *ARS307* are functionally conserved despite their dissimilarities in DNA sequence (43). It is uncertain whether the functional conservation between *cis*-acting elements at these two replication origins can be generalized to the approximately 400 replication origins in the yeast genome.

ARS305 (39a) shares *cis*-acting components with a chromosomal replicator (22, 23), and the chromosomal replication origin fires early in S phase, even earlier than the origin associated with *ARS1* (6) (reviewed in reference 14). Linker scan analysis of *ARS305* has revealed short functional elements in the B domain at positions corresponding to *ARS1* B1 and B3 (23). *ARS305* has no short functional element in the region corresponding to the B2 elements of *ARS1* and *ARS307*. In that general region, however, *ARS305* contains a DUE defined by deletion and helical stability analyses (22). The *ARS305* DUE is present in a broad region that is easily unwound in negatively supercoiled DNA (22, 38). Intriguingly, the B2 element of *ARS307* is embedded in a broad easily unwound region that also contains a DUE (37). In vitro studies with *ARS1* show that B2 is responsible for unwinding the B domain in a negatively supercoiled plasmid which interacts with single-stranded binding proteins (34). The functional relationship between B2 elements and DUE regions and the exact function of B2 elements in vivo are unknown.

In this study, we examined the functional equivalency of *cis*-acting elements in yeast replication origins by swapping the functional elements of *ARS305* with elements previously defined in *ARS1* (32), which, except for B3, are functionally conserved in *ARS307* (43). *ARS305* has elements that are functionally exchangeable with *ARS1* elements A and B1, the known ORC binding sites (44, 47); however, unlike *ARS305*, *ARS1* does not rely on a nonconserved sequence in the essential A element. Surprisingly, the *ARS1* B3 element, which binds ABF1p and is known as a replication enhancer (54), inhibited *ARS305* function. At the position corresponding to *ARS1* B3, *ARS305* has a novel element, B4, that can functionally substitute for every type of short element (B1, B2, and B3) in the B domain. The *ARS1* B2 element (32), whose role is not known, can be functionally replaced by an easily unwound region of *ARS305*, but *ARS1* B2 was found to have a sequence requirement that is distinct from a helical instability requirement. The functionally conserved B2 element in *ARS307* contains a known sequence requirement (43), and helical stability analysis of linker and minilinker mutations suggested that it also contains a DUE. Our findings suggest that yeast replication origins employ a B2 element or a DUE to mediate a common function, DNA unwinding during initiation, although not necessarily through a common mechanism.

MATERIALS AND METHODS

Reagents. Restriction enzymes, T4 DNA ligase, and Deep $Vent_R$ DNA polymerase were from New England BioLabs. Other reagents (and their sources) included AmpliTaq DNA polymerase in a PCR reagent kit (Perkin-Elmer Cetus), the fmol DNA sequencing system (Promega), calf intestine phosphatase (Boehringer Mannheim), and α-^{32}P-labeled deoxyribonucleotides (Amersham). The components of cell culture media were from Difco Laboratories. Other chemicals were from Sigma Chemical Co.

Plasmids and DNA. Plasmid pARSWTA, containing wild-type *ARS1*, and its derivatives, including AB1−− and linker substitution mutations of short functional elements, were gifts from Bruce Stillman, Cold Spring Harbor Laboratory (32). Plasmids containing wild-type *ARS305* and derivatives Lin12, Lin22, Lin94, Lin102, and Lin117 were constructed by the method of Huang and Kowalski (23). All the plasmids used carry an ampicillin resistance gene, a bacterial replication origin, a *URA3* gene, and a centromere. Oligonucleotide primers were made by Al Cairo (Biopolymer Facility, Roswell Park Cancer Institute).

Cells and cell culture. *E. coli* DH5α (Bethesda Research Laboratories) was used for transformation and plasmid propagation. Bacteria were grown in Luria-Bertani medium supplemented with ampicillin (50 μg/ml) at 37°C. *S. cerevisiae* YPH98 (haploid; *MAT***a** *ade2-101 lys2-801 ura3-52 trp1-1 leu2-1*), obtained from Philip Hieter (Johns Hopkins University), was used for testing the activities of ARS derivatives. Plasmids were transformed into yeast cells by the lithium acetate-polyethylene glycol procedure (26). Selective growth of yeast cells was carried out in synthetic minimal medium SD minus uracil (0.67% Bacto yeast nitrogen base without amino acids, 0.5% ammonium sulfate, 2% dextrose, supplemented with 20 mg of adenine sulfate per liter, 30 mg of lysine per liter, 20 mg of tryptophan per liter, and 30 mg of leucine per liter). Nonselective growth of yeast was done in complete medium YPD (1% Bacto yeast extract, 2% Bacto Peptone, 2% dextrose). SD and YPD liquid media were added to 2.5 and 2% of Bacto Agar, respectively, to produce selective (minus uracil) and nonselective plates.

PCR-mediated mutagenesis. The procedure involved two rounds of PCR with a mutagenic primer (41). For an *ARS1* substitution mutation, plasmid pARSWTA, containing wild-type *ARS1*, was used as a template in the first-round PCR. A synthetic mutagenic primer and a pUC end primer, 5′-GTAAAACGACGGCCAGT-3′, were used as the primer pair for amplification. Standard 50-μl reaction mixtures (5 ng of template plasmid [0.04 pmol], 10 pmol of each primer, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM [each] deoxynucleoside triphosphates, 2.5 U of AmpliTaq or Deep $Vent_R$ polymerase) were employed. The temperature cycles, controlled by a GeneAmp PCR thermal cycler 9600 (Perkin-Elmer Cetus), for the first-round PCR were initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation (94°C, 15 s), annealing (42°C, 15 s), and extension (72°C, 45 s), completed by final extension (72°C, 2 min), and then held at 4°C. The resultant partial-length (~150-bp) DNA fragment was used as the mutagenic primer (mega primer) in the second-round PCR. Half of the mega primer and an M13/pUC reverse sequencing primer, 5′-AACAGCTATGACCATG-3′, were used as the primer pair for amplification. All other procedures were the same as those described for the first-round PCR. The second-round PCR generated a full-length (290-bp) mutated DNA fragment.

After purification, the full-length mutated fragment was digested with *Eco*RI and *Hin*dIII sequentially. Wild-type plasmid pARSWTA was digested with the same enzymes, and then the large fragment was purified. The large fragment was dephosphorylated and then ligated with the full-length mutated fragment according to the enzyme manufacturer's procedures. The ligation mixture was used to transform *E. coli* DH5α.

The mutations of *ARS305* were carried out by procedures similar to those used to generate *ARS1* mutations. A plasmid containing wild-type *ARS305* in centromere-bearing vector pVHA (53) was used as the initial template. End primers L3 and R3 (23) were used for the first- and second-round PCRs, respectively. *Cla*I and *Sac*I restriction sites were used for cloning mutations.

DNA sequence analysis. Plasmid DNA was obtained from cells lysed by boiling in the presence of lysozyme (20). The fmol DNA sequencing system (Promega) was used for sequencing. The high-temperature nature of the PCR-based sequencing procedure reduced the secondary structure of the template caused by local G+C-rich sequences and eliminated band compression problems. The manufacturer's protocol for sequencing by direct incorporation was used, except that 10 (instead of 30) cycles of PCR were performed to reduce nonspecific products. The DNA sequences of the ARS inserts for wild-type DNA and mutant derivatives were determined to be authentic for at least one strand.

The precise sequence positions for mutations in *ARS305* (hereafter noted as $[\text{positions}]_{305}$ according to the sequence numbering of Huang and Kowalski [23]) and in *ARS1* (hereafter noted as $[\text{positions}]_1$ according to the sequence numbering of Marahrens and Stillman [32]) for *Xho*I linker substitution derivatives are as follows: $B1_{305}$, $[22\text{-}29]_{305}$; $B4_{305}$, $[102\text{-}109]_{305}$; $B1_1$, $[835\text{-}842]_1$; $B2_1$, $[802\text{-}808]_1$; and $B3_1$, $[757\text{-}764]_1$. For element swapping derivatives (the symbol $\leftrightarrow$ indicates the elements swapped), the sequence positions are as follows: A_{305} + flank $[-3\text{-}19]_{305} \leftrightarrow A_1$ + flank $[870\text{-}849]_1$; $B1_{305}$ + flank $[21\text{-}33]_{305} \leftrightarrow B1_1$ $[847\text{-}835]_1$; $B4_{305}$ + flank $[100\text{-}118]_{305} \leftrightarrow B3_1$ $[768\text{-}750]_1$; and $B4_{305}$ + flank $[102\text{-}112]_{305} \leftrightarrow B2_1$ $[808\text{-}798]_1$. In other ARS derivatives (the symbol $\leftarrow$ stands for "replaced by," the symbol $\rightarrow$ stands for "substituted for," and the symbol $\leftrightarrow$ indicates the elements swapped), the sequence positions are as follows: AB1B2/EZX, $[60\text{-}70]_{305} \leftarrow [808\text{-}798]_1$; AB1B2X and AB1EZ−, $[30\text{-}101]_{305} \leftrightarrow [834\text{-}765]_1$; AB1(0/11)X and AB1GCB4, primer sequences given below; AB1(0/11)B4, $[30\text{-}101]_{305} \leftarrow [834\text{-}763]_1$ + the same 0/11 mutation as that in the AB1(0/11)X primer sequence; AB1(6/11)−, $[56\text{-}66]_{305} \rightarrow [808\text{-}798]_1$; and AB1(5/11)−, $[60\text{-}70]_{305} \rightarrow [808\text{-}798]_1$.

Quantitative determination of ARS efficiency. Yeast strain YPH98 was transformed as described above with plasmids carrying individual ARS derivatives, a centromere, and a *URA3* gene. Transformed cells were plated on selective SD plates (minus uracil). Six primary transformants were picked and streaked on sectors of selective plates. Plates were incubated at 30°C for 3 to 4 days (when colonies were approximately 1 mm in diameter). Single colonies from all streaked sectors were inoculated into 3 ml of selective liquid medium and grown for 22 h at 30°C. The optical density at 600 nm (OD_{600}) was determined and multiplied by 3×10^7 cells/ml for an estimation of cell density. Each cell culture was diluted and plated onto two selective SD plates and two nonselective YPD plates, with an estimated 200 cells on each plate. For mutants with weak ARS activities, multiple amounts of cells were plated on selective plates in order to obtain reliable counts of cell numbers and were adjusted later in the calculation. The initial plasmid stability (*I*) under selection was calculated as the ratio of the

total number of colonies formed on selective plates to the total number of colonies on YPD plates.

Cell cultures were also diluted to an OD_{600} of 0.0003 in 3 ml of YPD and grown at 30°C for 30 h. The OD_{600} was measured again, and cell cultures were diluted and plated as described above. The final plasmid stability (F) was determined as described above for the determination of I. The mitotic stability values reported correspond to the F values at approximately 12 generations. The number of generations was calculated based on OD_{600} readings after growth in YPD. Plasmid retention per generation was calculated by the following formula: $PR = [F/I]^{1/n}$ (23), where PR is plasmid retention per generation and n is the number of generations.

Helical stability analysis and computer-aided design of mutations. A computer program, MutEnergy, was developed to facilitate the design and analysis of mutations. To assess the extra free energy ($\Delta\Delta G$) required for unwinding the double helix caused by a substitution mutation, the helical stability difference ($\Delta\Delta G = \Delta G_{mut} - \Delta G_{wt}$) between mutant and wild-type DNA sequences was calculated by this program with nearest-neighbor thermodynamic data and formulas described previously (4, 31). For a linker substitution, input required only the linker DNA sequence plus the two flanking nucleotides and the corresponding wild-type sequence. For linker mutations that contain single-nucleotide deletions or insertions, the sequence downstream of the linker was shifted to accommodate the deletion or insertion. For *ARS307* mutations, similar results were obtained when the upstream sequence was shifted. The relationship between the $\Delta\Delta G$ and known ARS activities of a collection of linker substitutions across the replication origin was examined in scatter diagrams generated by using SlideWrite Plus (Advanced Graphics Software).

MutEnergy was also used to facilitate the design of new mutations within a specified range of $\Delta\Delta G$. This program simulates the mutation of a given nucleotide sequence according to the following two parameters: (i) a condition string composed of standard ambiguous nucleotide codes to indicate the allowed nucleotide changes and (ii) a desired $\Delta\Delta G$ range. All possible sequences allowed by the condition nucleotide string were automatically generated, and their $\Delta\Delta G$ values were calculated as described above. Only those mutations whose $\Delta\Delta G$ values fell within the specified range were reported.

Candidate sequence mutations were analyzed by IBI Pustell sequence analysis programs (International Biotechnologies) to check for single and rare cutting restriction enzyme sites that could be used later to screen for clones. The Oligo 4.0 program (National Biosciences) was used to analyze and optimize mutagenic primers for PCR amplification. The primer used to generate AB1(0/11)X was 5′-GCAGGCAAGTGCAattatttattcattcatttataTACTCAGTAATAAC-3′, where uppercase and lowercase letters indicate wild-type and mutant nucleotides, respectively. The sequence chosen for the G+C-rich primer used to generate AB1GCB4, 5′-TTTTATTTcCTcgaggtcctcccgcagcgccaccacacgtacgttagtccctgctcag cccgcggtcgcccactggatccAAATAAACAATACATAACAAAAC-3′, was also analyzed with the Mfold and StemLoop programs of the Genetic Computer Group sequence analysis software package to confirm that possible secondary structures were minimized. Additionally, primers were selected so that a T was present in the template immediately next to the 5′ end of the primer in order to match the extra A that is frequently added by *Taq* polymerase at the end of polymerization. This special arrangement was waived when amplification was done with a DNA polymerase that possesses 3′ exonuclease activity, such as Deep $Vent_R$.

RESULTS

Swapping of functional elements between *ARS305* and *ARS1*. A comparison among the functional components of *ARS305*, *ARS1*, and *ARS307* identified by linker scan analysis (23, 32, 43, 51) is shown in Fig. 1. These ARS elements were aligned at the essential match to the ACS. *ARS1* elements A, B1, and B2 are functionally interchangeable with the elements at corresponding positions in *ARS307* despite their dissimilarities in sequence (43). Boxes 1 and 2 of *ARS305* are located at positions similar to those of *ARS1* elements B1 and B3, respectively. Although no sequence similarity was found (not shown), these similarly positioned elements may also be equivalent to each other in terms of the capability to augment the function of an ARS. The swapping experiments shown below examined the functional interchangeability between components of *ARS1* and *ARS305* and, for *ARS305*, defined the ACS and 3′ box as element A, box 1 as B1, and box 2 as B4 (or A_{305}, $B1_{305}$, and $B4_{305}$).

Elements of *ARS305* were interchanged with elements at corresponding positions in *ARS1* (Fig. 2). The essential ACS and the critical 3′ box of *ARS305* were interchanged as a unit with the *ARS1* A element (or element A_1), which also contains an essential ACS. For the $B2_1$ element, which has no short

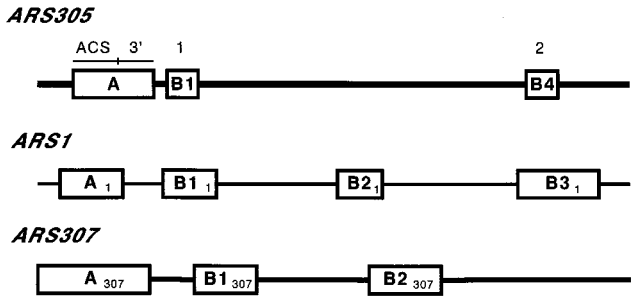

FIG. 1. Comparison of short functional elements among *ARS305*, *ARS1*, and *ARS307*. Short functional elements defined by linker scan analysis in *ARS305* (23), *ARS*1 (32), and *ARS307* (43, 51) are shown. The ARSs are aligned at the essential ACS. The functional elements of *ARS305* are represented by open boxes, and those of *ARS1* and *ARS307* are represented by shaded boxes. Flanking sequences are represented by lines of different thicknesses for different ARSs. All DNA lengths have been drawn to scale. Previous work showed that *ARS305* contains an ACS and short functional elements, temporarily designated boxes 3′, 1, and 2 (23). Element swapping experiments (Fig. 2) examined the functional equivalency between *ARS305* and *ARS1* and, for *ARS305*, defined ACS and box 3′ as element A, box 1 as element B1, and box 2 as element B4. A DUE (not shown) has also been detected in the B domains of *ARS305* and *ARS307* (22, 37).

element counterpart at the corresponding position in *ARS305*, an extra test was carried out to switch it with element $B4_{305}$. Some of the elements are very different in size, e.g., the size of $B3_1$ is more than double that of $B4_{305}$. Moving a smaller foreign element alone into the corresponding position does not substitute for the entire sequence of the original element. The residual sequence of the original element could still be functional and interfere with the function of the new element (43). To resolve the differences in size between elements at corresponding locations in two ARSs, the swapping was carried out in such a way that the residual sequences of the original elements were eliminated by the flanking sequences brought along with the foreign elements.

The biological activity of each chimeric derivative was examined by measuring the mitotic stability of the plasmid (or plasmid stability), which is defined as the percentage of yeast cells containing the plasmid with the ARS derivative after growth for about 12 generations in a nonselective medium (YPD). The vector sequence carried a centromere to ensure proper segregation of plasmids. A plasmid containing an ARS derivative defective in the initiation of DNA replication is expected to show reduced mitotic stability compared to that of a plasmid containing the wild-type ARS.

Element A_1 efficiently substituted for element A_{305} in the context of the *ARS305* sequence (Fig. 2A). The reciprocal experiment, replacing A_1 with element A_{305}, gave similar results (Fig. 2B). A G+C-rich linker substitution in either A element eliminated ARS function (23, 32). $B1_{305}$ and $B1_1$ appeared to be functionally conserved. Introduction of the foreign B1 element significantly restored ARS function from a linker mutation at the B1 location. Element $B4_{305}$, along with some of its flanking sequence, efficiently substituted for the $B3_1$ element. However, placing $B3_1$ in the position of $B4_{305}$ caused a severe reduction in ARS activity, more severe than that caused by the linker mutation of element $B1_{305}$ or $B4_{305}$. Similarly severe reductions in ARS activity were seen in three independently isolated plasmid clones containing the authentic DNA sequence of *ARS305* but with $B3_1$ in the position of $B4_{305}$. These results show that $B3_1$ does not work as a replication enhancer in *ARS305*, as it does in *ARS121* (54) and has been suggested to do in *ARS1* (39). The finding that the func-

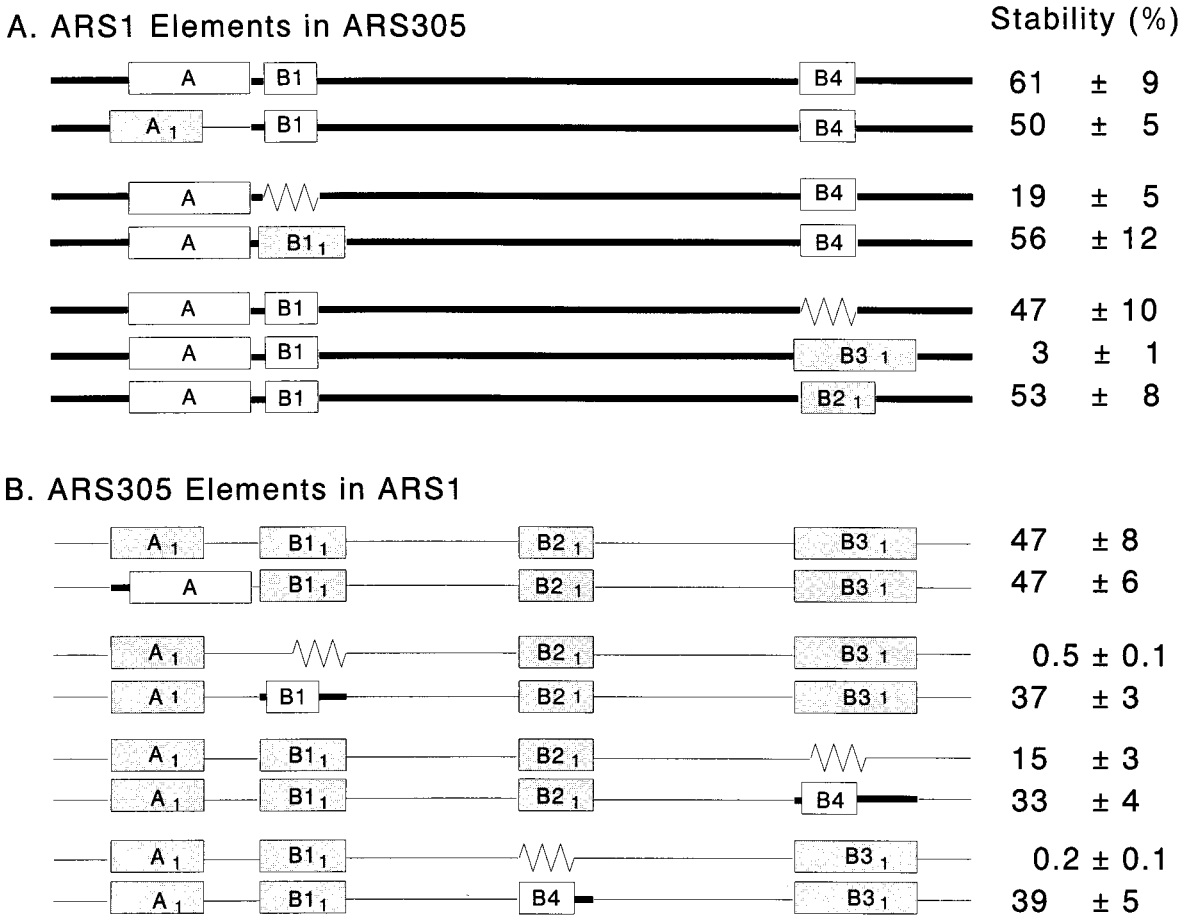

FIG. 2. Examination of the functional equivalency between *ARS305* and *ARS1* elements. (A) Short functional elements of *ARS305* were replaced with elements and sequences from *ARS1*. (B) Reciprocal experiments of those in panel A. The functional elements of *ARS305* are represented by open boxes, and flanking sequences are represented by thick lines. The functional elements of *ARS1* are represented by shaded boxes, and flanking sequences are represented by thin lines. The ARSs are aligned at the essential ACS. Zigzags indicate *Xho*I linker substitutions. All DNA lengths have been drawn to scale. To accommodate the size and position differences between elements at similar locations in the two ARSs, the swapping was designed in such a way that the residual sequences of the original elements were replaced by the flanking sequences brought along with the foreign elements. Mitotic stability was assayed as the percentage of cells retaining the plasmid after the release of selection and growth for 30 h (~12 generations) in YPD medium (see Materials and Methods). The average and standard deviation of the mitotic stability measurements for at least six independent transformants of each derivative are listed.

tional substitution of B4 for B3 goes only one way is surprising, given the functional conservation of similarly positioned elements in *ARS1* and *ARS307* (43).

Element $B4_{305}$ is important for replication origin efficiency in the natural location of the *ARS305* replicator in chromosome III (23). $B4_{305}$ shows moderate sensitivity to the linker substitution mutation in the plasmid, but $B4_{305}$ stands out as a functional element compared to the flanking sequences in *ARS305*, which are much less sensitive (23). The effect of $B4_{305}$ replacement by $B2_1$ (Fig. 2B) and $B1_{305}$ (see below) is not readily distinguishable from the moderate negative effect of linker substitution in $B4_{305}$. However, with some of its flanking sequence, $B4_{305}$ can clearly functionally replace elements $B2_1$ (Fig. 2B) and $B1_{305}$ (see below). This is surprising since these elements are not similarly positioned and earlier studies showed that B elements at different positions in ARSs cannot functionally substitute for each other (32, 43). Based on these distinct properties of $B4_{305}$ as well as on its newly discovered one-way substitution behavior with $B3_1$ mentioned above, element $B4_{305}$ is a novel functional element of an ARS.

***ARS1* does not rely on a 3′ box.** Element A_{305}, containing an ACS and a 3′ box, was found to be functionally equivalent to A_1 (Fig. 2), which is composed of an ACS and some flanking nucleotides. In *ARS305*, the 3′ box is extremely important for activity and spans several nucleotide positions in an 8-bp region, as revealed by different linker and point mutations (23). However, detection of the *ARS305* 3′ box by linker scan analysis was highly dependent on the precise position of the linker substitution since just a 2-bp shift of the linker 3′ to the ACS T-rich strand had little or no effect on activity (23). *ARS307* is also extremely sensitive to linker substitution directly 3′ to the ACS T-rich strand (43, 51). The short sequence in *ARS1* at the precise position of the 3′ box was not replaced in a random linker scan (32). To test whether that precise sequence in *ARS1* has similar functional importance, we constructed a *Xho*I linker substitution mutation, 849-856, directly adjacent to the ACS.

As shown in Fig. 3, the same *Xho*I linker substitutions in *ARS1* and *ARS305* had extremely different effects on ARS activity, as evidenced by mitotic stability and by plasmid retention per generation. The linker mutation next to the ACS of *ARS305*, Lin12, essentially inactivated the ARS, whereas the one next to the ACS of *ARS1*, 849-856, had little or no effect. This direct comparison with identical linkers at the same po-

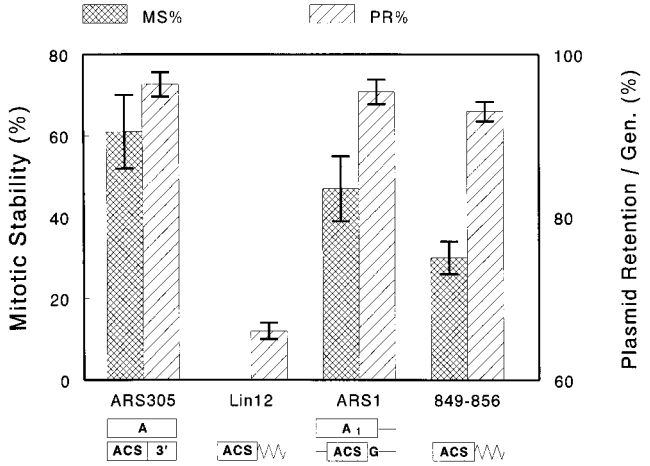

FIG. 3. Differential effects of the same linker substitution on two ARSs. Below the graph, the 8-bp sequences directly 3′ to the T-rich strands of the ACSs of both *ARS305* and *ARS1* replaced by a *Xho*I linker (zigzag) are illustrated. ARS activity is reflected by mitotic stability (MS) and plasmid retention (PR) per generation (Gen.). The standard deviations for measurements of at least six independent transformants for all plasmid ARS derivatives are shown by error bars.

sitions in the two ARSs shows that *ARS1* does not rely on a 3′ box that is crucial to *ARS305*. Thus, with respect to a crucial 3′ sequence, *ARS1* differs substantially from both *ARS305* (23) and *ARS307* (43, 51) in the functional composition of the essential A element. The results also indicate that the single nucleotide 3′ to the ACS T-rich strand in the A element, a G residue, is not essential for *ARS1* function.

B4 can functionally substitute for the B1 element in *ARS305*. To examine whether the short elements in the B domain of *ARS305* are functionally distinct, we performed a swapping experiment between elements $B1_{305}$ and $B4_{305}$ (Fig. 4). The *Xho*I linker substitution of $B1_{305}$ reduced the plasmid mitotic stability by 3-fold, but replacing the linker with $B4_{305}$ sequence restored efficient ARS function, by 2.5-fold, showing that B4 can functionally substitute for the B1 element in *ARS305*. In the reciprocal experiment, the replacement of $B4_{305}$ with $B1_{305}$ yielded ARS activity similar to that of a linker mutation in $B4_{305}$ (Fig. 4). Thus, $B1_{305}$ does not functionally substitute for $B4_{305}$. It has been shown that the B elements within *ARS1* and *ARS307* are functionally distinct and cannot substitute for one another (32, 43). The observation that $B4_{305}$ can functionally substitute for the $B1_{305}$ element in *ARS305* may suggest a novel property of *ARS305*. Two contiguous T nucleotides within element B1, around positions 28 to 30 (numbering starts at the first nucleotide of the T-rich strand of the ACS), were found to be functionally important for the ribosomal DNA ARS and for *ARS1* (35, 43). Here, TT was changed to AA when $B1_{305}$ was replaced with $B4_{305}$ and ARS305 activity was largely restored relative to that seen for the linker substitution in $B1_{305}$. The results suggest that the requirement for TT in the B1 element of *ARS305* either may not be as stringent as the requirement in other ARSs or can be compensated for by changes elsewhere in $B1_{305}$.

A broad G+C-rich substitution in an easily unwound region, in which no short functional element was detected by linker scan analysis, reduces *ARS305* activity. The B domain of *ARS305* is easily unwound in negatively supercoiled DNA, and the functional effects of deletion mutations that alter the stability of the DNA helix suggested the presence of a DUE in this broad region (22). The activity lost after deleting easily unwound sequences can be restored by introducing dissimilar sequences that are easily unwound, but not by introducing other sequences that are G+C rich. Linker scan mutagenesis, which introduced 8-bp G+C-rich substitutions between B1 and B4, had little or no effect on *ARS305* activity (23). To further investigate the possible function of the broad easily unwound sequence in *ARS305*, we introduced a broad G+C-rich substitution, replacing the entire 72-bp sequence between B1 and B4. Unlike previous internal deletions (23), this substitution did not alter the spacing between B1 and B4.

G+C-rich substitution in the easily unwound sequence between elements B1 and B4 of *ARS305* significantly reduced ARS activity (Fig. 5A). The broad G+C-rich substitution showed an even stronger effect, a sixfold reduction in plasmid stability, in a context without a $B4_{305}$ element and the 15-bp downstream sequence (Fig. 5B). These effects, combined with the lack of effect of 8-bp G+C-rich linker substitutions in the region between $B1_{305}$ and $B4_{305}$ (23), are consistent with the suggestions of previous studies that the easily unwound sequence contains a DUE (22) and that the DUE is longer than the 8-bp linker.

Another possibility is that the effects are explained by the presence of multiple short elements that are redundant in function and thus not detected by individual 8-bp linker substitutions. One candidate for such a short functional element is B2 (32, 43, 51), whose position in *ARS1* and *ARS307* corresponds to the easily unwound sequence between elements B1 and B4 in *ARS305* (Fig. 1). However, insertion of $B2_1$ into the broad G+C-rich substitution between $B1_{305}$ and $B4_{305}$ did not restore ARS activity to the levels of parental *ARS305* derivatives containing the easily unwound region (Fig. 5). Thus, the basis for the functional contribution of the easily unwound region appears to be inconsistent with the presence of multiple redundant B2-like elements. The formal possibility of multiple

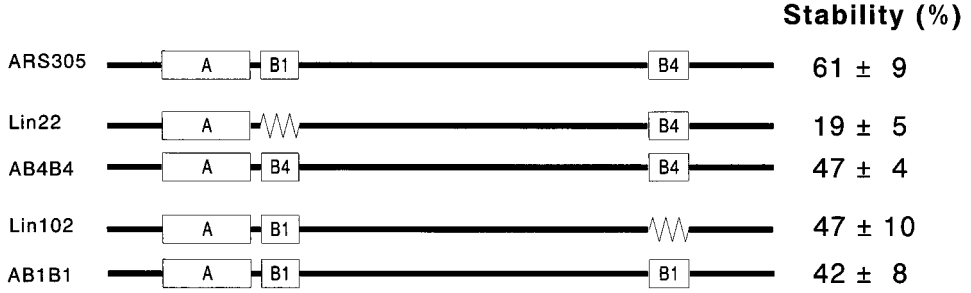

FIG. 4. Effects of interchanging the B1 and B4 elements of *ARS305*. Linker mutations of elements $B1_{305}$ and $B4_{305}$ were included for comparison. The symbols and descriptions are the same as those used in Fig. 2.

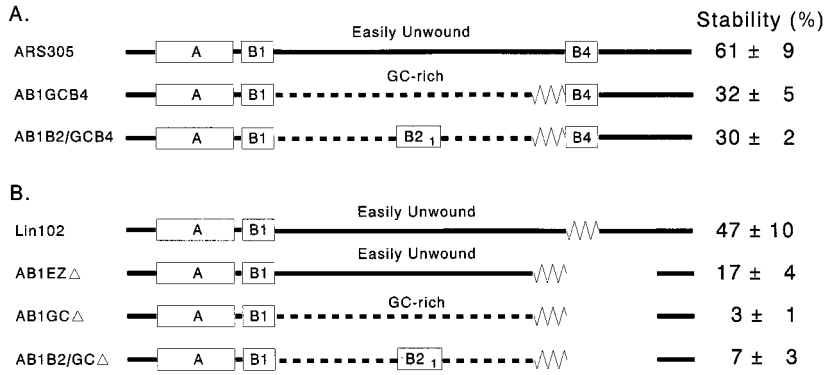

FIG. 5. Effect of G+C-rich substitution of an easily unwound sequence in *ARS305*. A broad G+C-rich substitution (dashed lines) was introduced to test the function of an easily unwound region in which no short functional element was detected by linker scan analysis (23). The *Xho*I linker (zigzag) was considered part of the G+C-rich mutation. The linker mutation alone (Lin94; positions 94 to 101 [23]) does not affect *ARS305* activity (23). The position of $B2_1$ in *ARS305* (positions 60 to 70) corresponds to its original location in *ARS1* (positions 808 to 798) relative to the essential ACS. Both AB1EZΔ and AB1GCΔ have $B4_{305}$ (positions 102 to 109) and 15 bp of its downstream flanking sequence (positions 110 to 124) deleted. The other symbols and descriptions are the same as those used in Fig. 2.

occurrences of other types of short sequence elements yet to be discovered cannot be ruled out, although this possibility is not necessarily mutually exclusive with the presence of a DUE.

The effect of the G+C-rich substitution shows that the easily unwound region between B1 and B4 of *ARS305* possesses a property that is not detected by linker scanning analysis but is important for ARS function. The low plasmid stability of the linker deletion derivative AB1EZΔ raises the possibility that $B4_{305}$-flanking sequences, whose functional importance was also not detectable by 8-bp linker scanning analysis (23), contribute to *ARS305* function.

An easily unwound region of *ARS305* can functionally replace the B2 element of *ARS1*. The position of the *ARS1* B2 element between $B1_1$ and $B3_1$ corresponds to that of the *ARS305* easily unwound region between $B1_{305}$ and $B4_{305}$ (Fig. 1). To examine whether these corresponding regions of DNA sequence are equivalent in function, we swapped the *ARS1* B2 element plus its flanking sequences with the *ARS305* easily unwound region between $B1_{305}$ and $B4_{305}$ (Fig. 6). Substitution analysis was done on the background of a $B3_1$ or $B4_{305}$ mutation to increase the stringency and render the effect of the replacement more readily detectable. This arrangement is especially helpful for substitutions in the context of *ARS305*, given the apparent functional redundancy between $B4_{305}$ and the easily unwound region (see below). Replacement of the *ARS305* easily unwound region between $B1_{305}$ and $B4_{305}$ by $B2_1$ without or with its flanking sequence (Fig. 6A) maintained an ARS function similar to that of the parental derivative, Lin102. Elimination of $B2_1$ and the surrounding partial match to $B2_1$ in AB1B2X maintained the same helical stability but caused a great reduction in ARS activity [AB1(0/11)X], clearly demonstrating that the $B2_1$ sequence functioned in place of the easily unwound region in maintaining *ARS305* activity in derivative AB1B2X. The B2 element in this derivative is shifted by 4 bp relative to its native position in *ARS1*, showing that B2 can tolerate a small relocation in a foreign context.

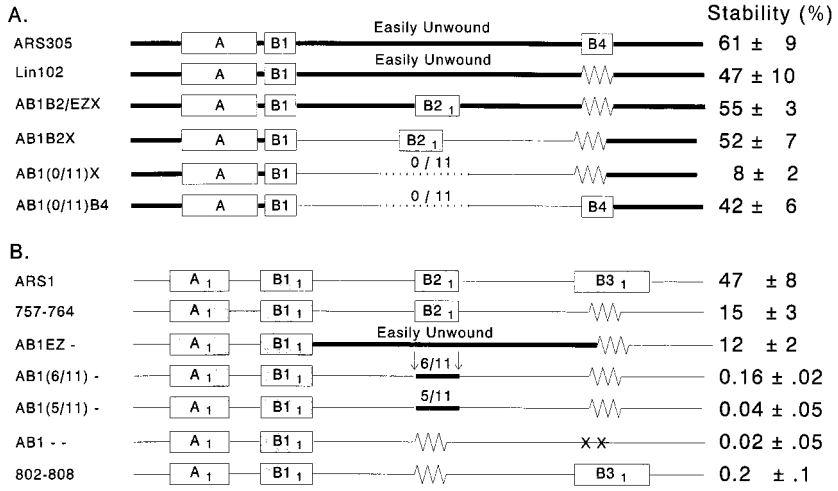

FIG. 6. Functional substitution of an easily unwound region of *ARS305* for the B2-containing sequence of *ARS1*. (A) Replacement of *ARS305* sequences (heavy lines and open boxes) by *ARS1* sequences (thin lines and shaded boxes). (B) Replacement of *ARS1* sequences by *ARS305* sequences. Derivatives AB1(0/11)X and AB1(0/11)B4 have most of the *ARS305* sequences between B1 and B4 replaced. Additionally, AB1(0/11)X was designed with the assistance of the MutEnergy computer program (see Materials and Methods) to have a helical stability identical to that of AB1B2X. Arrows mark the sequences transplanted to the next derivative, and each fraction indicates the extent of partial match to the B2 sequence of *ARS1* (see text). Derivative AB1−− (also known as −−B1A when it is presented in reverse orientation) inactivated $B2_1$ and $B3_1$ by a *Xho*I linker mutation and two point mutations, respectively (32). The other symbols and descriptions are the same as those used in Fig. 2.

Conversely, the *ARS305* easily unwound region between $B1_{305}$ and $B4_{305}$ can functionally substitute for the $B2_1$ element and the flanking sequence in *ARS1* (Fig. 6B). A chimeric derivative, AB1EZ−, that contains the *ARS305* easily unwound region in the context of *ARS1* was constructed. Although both its $B2_1$ and $B3_1$ elements were changed, AB1EZ− functioned much more efficiently than did the double-element mutant AB1−−. AB1EZ− showed a mitotic stability similar to that of its parental derivative, 757-764, which contains only a $B3_1$ mutation. The match (6 of 11) to the $B2_1$ sequence brought in by the easily unwound sequence of *ARS305* cannot account for the success of substitution [AB1(6/11)−]. Another match (5 of 11) of $B2_1$ sequence in *ARS305*, located at the position corresponding to *ARS1* B2 when the ACSs of the two ARS elements are aligned, also cannot provide the same activity supplied by the $B2_1$ element [AB1(5/11)−]. Instead, these two derivatives, each of which contains a partial match to $B2_1$, had activities close to that of the doubly mutated derivative AB1−−. These results confirm the finding (Fig. 5) that the easily unwound region between B1 and B4 of *ARS305* possesses a property that is not detected by linker scanning analysis but is important for ARS function. Contrary to the inability of $B2_1$ to functionally substitute for the easily unwound region of *ARS305* in a G+C-rich context (Fig. 5), $B2_1$ can functionally substitute for the easily unwound region of *ARS305* in the context of the flanking sequences from *ARS1* (Fig. 6), which are more easily unwound (38).

Synergistic effect of combined mutations in the easily unwound region and the B4 element in *ARS305*. *ARS1* B2 and B3 are known to be functionally distinct, and separate mutations of either element greatly reduce ARS activity (32). In contrast, separate mutations of the *ARS305* B4 element or the easily unwound region between $B1_{305}$ and $B4_{305}$ reduced ARS activity only moderately (Fig. 6A). However, the combined mutant AB1(0/11)X caused a much greater reduction in ARS activity. The effect of mutating both $B4_{305}$ and the easily unwound region on ARS activity (mitotic stability = 8%) is far greater than the mathematical combination of the effects of the individual mutations calculated on the assumption that they exert their effects independently [calculated mitotic stability, $61\% \times (47/61 \times 42/61) = 32\%$]. A similar phenomenon was observed earlier (Fig. 5), where the introduction of G+C-rich sequence into the easily unwound region of *ARS305* had a far greater effect (sixfold instead of twofold reduction) on mitotic stability when $B4_{305}$ and its flanking sequence were mutated. Our analysis of the results in Fig. 6 shows a synergistic effect of combined mutations in the easily unwound region and the $B4_{305}$ element. The fact that *ARS305* can function relatively efficiently with either of these two elements suggests that the easily unwound region and the $B4_{305}$ element are at least to some extent functionally redundant.

Mutational and helical stability analyses of the *ARS1* B2 element. The fact that the easily unwound region of *ARS305* can functionally replace the B2-containing region of *ARS1* may suggest that B2 and/or its flanking sequence functions as a DUE. We constructed several G+C-rich substitutions flanking B2 and found that they had no effect on *ARS1* activity. In the most extreme substitution (V3V4/769; see Materials and Methods), raising the helical stability of the B2-flanking sequence by up to 42 kcal/mol, which is sufficient to inactivate other ARSs that contain a DUE (38), had no negative effect on *ARS1* activity in the mitotic stability assay, minimizing the possibility that the B2-flanking sequence functions as a DUE. Whether B2 by itself can serve as a compact DUE, similar to the DUE of SV40 (31), remains to be elucidated. We therefore constructed a set of B2 substitution mutations to test how different changes in helical stability and DNA sequence would affect *ARS1* activity.

TABLE 1. Effects of B2 mutations on DNA helical stability and *ARS1* activity

ARS1 derivative	B2 element sequence[a]	Helical stability difference (kcal/mol)[b]	Mitotic stability (%)[c]	Plasmid retention/generation (%)[d]
Wild type	ATTTAAGTATT	0.0	46.9 ± 8.0	95.4 ± 1.5
802–808	ATTTcctcgag	5.6	0.2 ± 0.06	79.2 ± 2.1
B2/AT	AaTatAtataa	−2.7	20.0 ± 5.6	94.2 ± 2.0
B2/EQ	tacattactaat	0.0	0.6 ± 0.1	82.0 ± 0.6
B2/GC	ccgcggcgcgc	18.2	0.1 ± 0.06	77.8 ± 2.0

[a] Mutated nucleotides of the *ARS1* B2 element are shown in lowercase letters, and nonmutated nucleotides are capitalized.
[b] Helical stability differences between mutated and wild-type B2 elements were calculated by using the MutEnergy program.
[c] Mitotic stability was measured as the percentage of cells retaining the derivative plasmid after 30 h of growth in the absence of selection in YPD medium.
[d] Plasmid retention per generation was calculated from the mitotic stabilities before and after release from selection. The standard deviation of the measurements for at least six independent transformants of each mutation is also listed.

The introduction of point mutations into a central portion (5′-TAAGTA-3′) of the B2 element has no effect on *ARS1* activity (43). However, linker substitution 802-808, which largely overlaps the point-mutated region within B2, is known to greatly reduce *ARS1* activity (32). The linker mutation is G+C rich and raises the helical stability of $B2_1$ (Table 1). The reduction in ARS activity was evident from the decline in both mitotic stability (after 12 generations) and plasmid retention per generation. Another substitution mutation, B2/GC, which introduced high helical stability in addition to changing all the nucleotides in B2 also greatly reduced *ARS1* activity. If helical instability alone is responsible for the function of B2, we would expect a substitution by an easily unwound sequence to either elevate or maintain a wild-type level of *ARS1* activity. However, mutation B2/AT reduced ARS activity, albeit not as severely as did the G+C-rich linker substitution. These results suggest that helical instability is not solely responsible for $B2_1$ function. Furthermore, mutation B2/EQ, which changes all the nucleotides of $B2_1$ without affecting the helical stability, reduced ARS activity nearly as much as did B2/GC, suggesting that *ARS1* B2 possesses an important sequence requirement that is distinct from a requirement for DNA helical instability.

In the absence of higher-resolution mutations that can identify specific nucleotides which are functionally important in *ARS1* B2, the separate contribution of helical instability to B2 function cannot be evaluated. However, for the functionally conserved B2 element in *ARS307*, higher-resolution substitution mutations have been constructed by Rao et al. (43) and have revealed specific nucleotides important for B2 function. The availability of this important information and the ability of the easily unwound region of *ARS305* to substitute for a functionally conserved B2 element prompted us to analyze the relationship between *ARS307* activity (43) and helical stability for these substitution mutations.

Helical instability of element B2 facilitates *ARS307* activity. We performed a helical stability analysis of a collection of linker and minilinker substitution mutations across *ARS307* (43) to determine whether there is any relationship between ARS activity and the helical stability changes caused by substitution mutations. Helical stability analysis of substitution mutations across the SV40 DNA replication origin localized a unique region containing a DUE (31); that region coincides with the site of unwinding induced by the initiator protein (2)

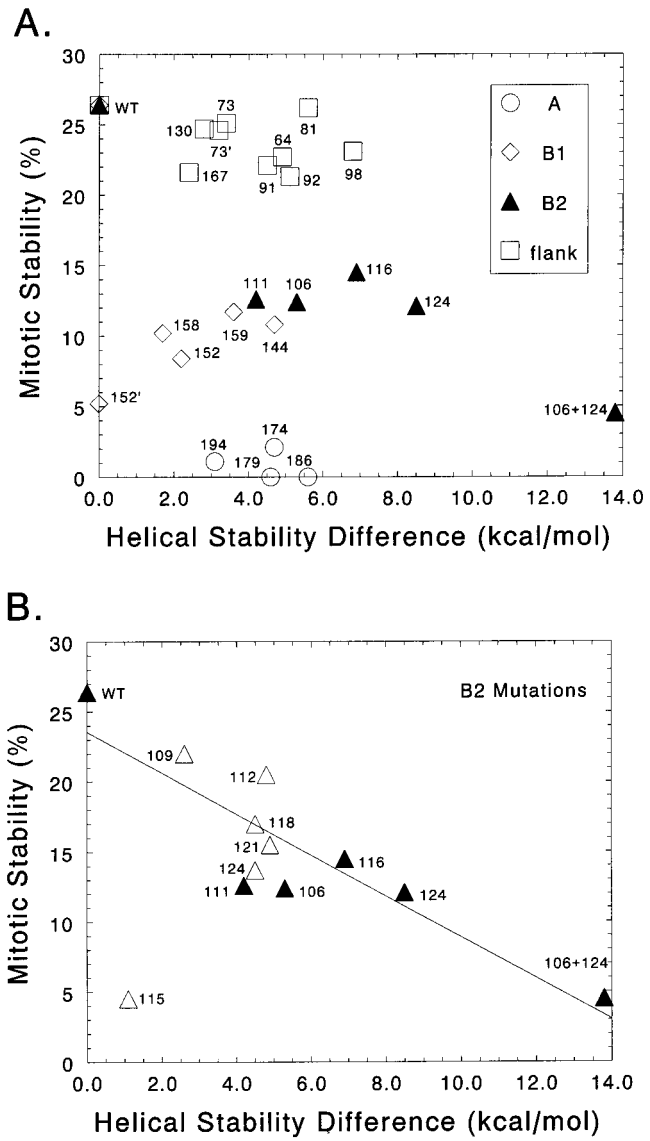

FIG. 7. Relationship between ARS activity and helical stability difference of *ARS307* substitution mutations. The mitotic stability of each *ARS307* derivative was plotted versus the difference between the DNA helical stability of each derivative sequence and that of the wild-type (WT) *ARS307* sequence. (A) Effects of 8-bp substitution mutations across entire *ARS307*. (B) Effects of 8- and 3-bp substitution mutations across the B2 element. Each mutation is designated by the starting position of the substitution, e.g, 124 is mutation 124-131. For mutations that start at the same position but end at different positions, the one that extends further is indicated with a prime after the starting position. Filled and open triangles represent 8- and 3-bp substitution mutations, respectively. Mutations were constructed and their plasmid mitotic stabilities were measured by Rao et al. (43). The helical stability difference of each mutation was calculated by using a novel computer program, MutEnergy, as described in Materials and Methods.

and the origin of bidirectional replication (19). Figure 7A shows a scatter diagram in which the mitotic stability of each linker substitution derivative was plotted against the helical stability difference ($\Delta\Delta G$) from the wild-type *ARS307*. Neither element A nor element B1 showed a clear dependence of ARS activity on the helical stability difference introduced by mutations. Elements A and B1 have known functional roles which are distinct from that of a DUE. Mutations within element A, which contains an ACS, interfere with the binding of ORC (43). Even point mutations, which have a much smaller effect on helical stability than that of 8-bp linker mutations, interfere with ORC binding. Mutations within element B1 interfere with ORC binding and possibly with the binding of another protein (44, 47). Mutations in the flanking sequences have little or no effect on ARS activity and thus contain no detectable element (43). In contrast to mutations in elements A and B1, however, the activities of B2 linker mutations appeared to display an inverse dependence on helical stability compared to the activity of the wild-type ARS (Fig. 7A).

The inclusion of high-resolution mutations in this analysis is important since such mutations can help distinguish specific sequence or nucleotide contributions from helical instability contributions. The effects of 3-bp minilinker mutations (43) in addition to 8-bp linker mutations on the helical stability of the B2 element (nucleotides 109 to 126) are shown in Fig. 7B. Except for mutation 115, the 3-bp minilinker mutations strengthened the inverse correlation between helical stability and ARS activity first seen for the B2 element in Fig. 7A. The inverse relationship suggests that the intrinsic helical instability within the B2 element facilitates the activation of *ARS307*. Mutation 115 exhibited a distinct effect on *ARS307* activity that cannot be explained solely by an increase in helical stability. The 3-bp mutation (positions 115 to 117) hits specific nucleotides which are important for a $B2_{307}$ function (43), such as a target for a protein interaction, that is distinct from the helix-destabilizing function of a DUE. Our analysis, together with the findings of Rao et al. (43), suggests that $B2_{307}$ is comprised of two distinct but overlapping components, a specific nucleotide component within positions 115 to 117 and a DUE within positions 106 to 131.

Mutations that change the function of the specific nucleotide component at positions 115 to 117 and the helical stability outside that 3-bp region would be expected to interfere with a correlation between ARS activity and the helical stability difference. Mutation 111, an 8-bp substitution at positions 111 to 118, changed two of the three specific nucleotides in the region from 115 to 117 and was the poorest correlated mutation aside from mutation 115 (Fig. 7B). On the other hand, linker mutation 116, which spans positions 116 to 123, changed only one of the specific nucleotides in the important region from 115 to 117 and the mitotic stability decreased in proportion to the increase in helical stability (Fig. 7B). Consistent with the absence of effect of the latter mutation on the function of the specific nucleotide component, point mutations in the B2 element of *ARS1* do not affect ARS function (32).

Helical stability analysis of an independent set of linker mutations in a different *ARS307* derivative was inconclusive (51) (data not shown), likely because of the absence of high-resolution mutations to distinguish specific sequence contributions and because of the limited range of $\Delta\Delta G$ achieved by 8-bp linkers that were 50% A+T. In the linker mutations analyzed here (43), the range of $\Delta\Delta G$ achieved by 8-bp G+C-rich linkers was greater and was further expanded by including high-resolution 3-bp minilinkers and a double 8-bp linker substitution mutation (Fig. 7) which spanned 16 nucleotides at positions 106 to 113 and 124 to 131.

The borders of the B2 element, as defined by 3-bp minilinker mutations, are positions 109 and 126 (43), whereas the borders of the DUE are positions 106 and 131. The slight discrepancy between the borders of the B2 element and the borders of the DUE may suggest that near the edges of the DUE, the effect of a helical stability increase on ARS activity can be detected by 8-bp but not 3-bp substitutions.

DISCUSSION

To further define the genetic determinants of DNA replication origins in the yeast *S. cerevisiae*, we swapped *cis*-acting elements of *ARS305* with those of the well-defined *ARS1*. Element swapping and functional analysis are necessary to identify common features among ARSs since the DNA sequences of most of the known functional elements are not conserved. Short functional elements A, B1, and B2 in *ARS1* were previously found to be functionally conserved in one other ARS tested, *ARS307* (43). Here we report that the *ARS1* A and B1 elements, known to bind ORC (1, 44, 47), are functionally conserved in *ARS305* (Fig. 2), in support of elements A and B1 as common determinants of yeast replicators.

However, *ARS305* differs from both *ARS1* (32) and *ARS307* (43, 51) in that it has no short functional element that is detectable by linker scan analysis at positions corresponding to those of the B2 elements (23). Instead, previous deletion analyses suggested that *ARS305* contains a DUE in a broad region that is easily unwound in negatively supercoiled DNA (22). A broad G+C-rich substitution indicates that the easily unwound region of *ARS305* is functional (Fig. 5). The results are consistent with a DUE in the easily unwound region, although the formal possibility that the easily unwound region contains multiple redundant elements neither can be ruled out nor is necessarily mutually exclusive with a DUE. Surprisingly, though it is devoid of detectable short sequence elements (23), the easily unwound region can functionally substitute for the B2 element of *ARS1* (Fig. 6). Conversely, $B2_1$ can functionally substitute for the easily unwound region of *ARS305* in some contexts (Fig. 6), although not in all contexts (Fig. 5). Additional mutations in $B2_1$ revealed that an important sequence property which is distinct from DNA helical instability contributes to its biological function (Table 1). For the functionally conserved B2 element of *ARS307*, the important sequence property is known to consist of at most only a few specific nucleotides (43), although the basis of the requirement for the bulk of the $B2_{307}$ element is not known. Our helical stability analysis of *ARS307* substitution mutations (43) suggests that in addition to the known specific sequence component, the B2 element contains a DUE (Fig. 7B). The presence and location of the DUE in *ARS307* are consistent with earlier studies which showed that the B domain is easily unwound in negatively supercoiled DNA and that the activities of deletion mutations (39b) correlate inversely with the helical stability of the domain (37). Our findings for *ARS305*, *ARS1*, and *ARS307* suggest that yeast replication origins employ a B2 element or a DUE to mediate a common function, although not necessarily through a common mechanism (see below).

Analyses of *ARS305*, *ARS1*, and *ARS307* in several laboratories yield a model for the basic genetic determinants for a DNA replication origin in the yeast *S. cerevisiae* (Fig. 8). General determinants of the replicator include an A element that contains an essential match to the ACS, a B1 element, and a B2 element or DUE (10, 22, 23, 32, 33, 43, 51, 53) (this study). Individual ARSs may have additional B elements with redundant (e.g., $B4_{305}$) or distinct (e.g., $B3_1$) functions (32) (this study). Element A binds ORC, an essential component of the initiator protein complex (1, 11, 15, 30). B1 also contributes to ORC binding and may have an additional function (44, 47). We propose that the common function of the B2 element or DUE is to facilitate the initial unwinding of the DNA replication origin. Differences in the properties of the B2 element or DUE among ARSs suggest that the common function does not necessarily occur through a common mechanism. In *ARS1*, the B2 element has an important sequence property (Table 1) and

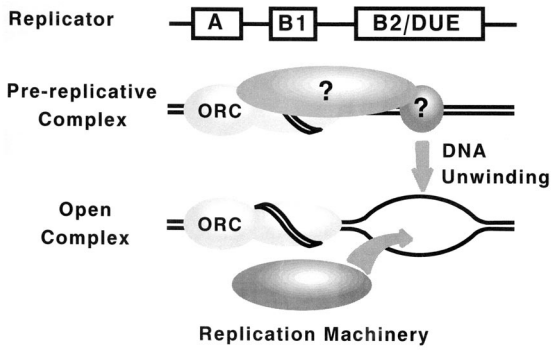

FIG. 8. Model for the initiation of DNA unwinding at yeast replication origins. Yeast replicators are composed of three functionally conserved elements, A and B1, which contribute to ORC binding (1, 44, 47), and a B2 element or DUE, which serves to facilitate DNA unwinding (this study), although not necessarily through a single mechanism for all ARSs. Particular ARSs may employ additional ARS-specific elements to augment or regulate replication function. ORC bound to the replication origin is activated in a prereplicative complex (11) that is characterized by extended nuclease protection which may be caused by the binding of other protein factors (question marks), such as Cdc6 (8). The requirement for specific nucleotides or sequences within B2 elements (43) and other genetic evidence (12) suggest that B2 interacts with a protein. Analogous to the correspondence of the DUE and the site of initiator-induced unwinding in *E. coli oriC* and SV40 *ori* (29, 31), the yeast B2 or DUE element is proposed to be the site of initial DNA unwinding in the replicator (open complex). Localized DNA unwinding would serve to facilitate entry of the helicase and additional replication machinery that primes and initiates DNA synthesis.

the B domain is not as easily unwound as it is in *ARS305* and *ARS307* (34, 38). In vitro, the single-stranded DNA-binding protein RPA (reviewed in reference 56) interacts specifically with *ARS1* B2 and facilitates localized DNA unwinding (34). While *ARS1* B2 functionally substitutes for *ARS307* B2, we note that *ARS307* B2 substitutes only weakly for *ARS1* B2 (43); therefore, these functionally conserved elements may not be mechanistically equivalent. *ARS307* B2 is considerably broader than the *ARS1* B2 element is (Fig. 1), and $B2_{307}$ contains a DUE (Fig. 7B) as well as a specific nucleotide requirement which may be the target for a protein (12, 43). *ARS305* contains a DUE (22) which may be even broader than is the DUE in *ARS307* (see below), and the easily unwound region has either no or a redundant specific nucleotide requirement (Fig. 5) (23). DUEs identified by our analysis of mutations in *E. coli oriC* and the SV40 *ori* (29, 31) comprise easily unwound DNA sequences which melt when the initiator protein binds to specific sequences of the replicator in vitro (2, 3). Analogous to models for initiation at the *E. coli* and SV40 replication origins, a model for the initiation of yeast DNA replication is that activation of origin-bound ORC in a prereplication complex (11, 12, 42) leads to unwinding of the double helix at the DUE or B2 element (Fig. 8). The mechanistic details of the unwinding process are unknown, and as mentioned above, there may be more than one mechanism, depending on the ARS-specific properties of B2 or DUE. Localized DNA unwinding in the replicator would permit entry of the replication machinery that catalyzes extensive unwinding and DNA synthesis.

Despite the functional equivalency between the A elements of *ARS305* and *ARS1*, substantial differences in functional constitution were disclosed by the fact that the two ARSs responded extremely differently to the same linker mutation directly 3′ to the ACS T-rich strand (Fig. 3). In that region of *ARS305* is a major functional element, the 3′ box. Detection of the 3′ box is extremely sensitive to the precise position linker substitution (23), making the 3′ box difficult to detect by random linker scan analysis, as was done for *ARS1* (32). Our re-

sults provide a direct comparison between *ARS305* and *ARS1*, and no 3′ box counterpart in *ARS1* has been found. In this regard, the A element of *ARS305* appears to be more similar to that of *ARS307*, which also has a major functional determinant that is detectable by linker substitution directly 3′ to the ACS in the A element (43, 51). ORC binding to the A and B1 elements protects the DNA region corresponding to the 3′ box in many ARSs (1, 44, 47). Whether the 3′ box interacts directly with ORC or serves some conformational role is not known. Our findings indicate that yeast replicators can utilize A elements which differ in a requirement for an extremely important component, the 3′ box, to accomplish a common function.

In the foreign context of *ARS305*, the $B3_1$ element, an ABF1p-binding site, clearly does not serve as a replication enhancer as it does in *ARS121* (54). The $B3_1$ element is inhibitory in *ARS305*, since it impaired ARS activity more severely than did either a $B4_{305}$ linker mutation or a $B4_{305}$ deletion mutation that altered more nucleotides than did the substitution of $B3_1$ (Fig. 2A and 5B). The $B3_1$ element in *ARS305* reduced activity to the same low level seen in an *ARS305* derivative, AB1GCΔ, in which $B4_{305}$ and flanking sequences were deleted and the easily unwound region was replaced by a broad G+C-rich sequence (Fig. 5B). A functional B3 element was not found in *ARS307* (43, 51), and no match to the consensus for the ABF1p-binding site was observed in the DNA sequences of other ARSs, including the ribosomal DNA ARS (35), chromosome VI ARSs (48), and chromosome XIV ARSs (16). Element B3 thus appears to be a special feature of certain ARSs rather than a universally required element. ABF1p, which binds element B3, is also a transcription factor that acts positively or negatively (6a, 31a), depending on the context of the binding site. Our studies indicate that in addition to the known role of element B3 in positively affecting the DNA replication activities of certain ARS elements (32, 54), element B3 can actually inhibit replication function in a particular ARS context.

The newly defined B4 element of *ARS305* is different from all other short functional B elements identified to date. $B4_{305}$ can functionally substitute for every type of short element in the B domain; however, replacement of $B4_{305}$ by $B3_1$, $B2_1$, or $B1_{305}$ did not detectably restore *ARS305* function in comparison to a *Xho*I linker mutation of $B4_{305}$ (Fig. 2 and 4). Such a one-way functional substitution is most dramatically demonstrated by the swapping experiment between B4 of *ARS305* and B3 of *ARS1* (Fig. 2). How $B4_{305}$ can functionally substitute for different types of B elements which are functionally distinct in *ARS1* (32) is unknown. There is little sequence requirement for $B1_1$ function (32), only two nucleotides, and we cannot rule out the possibility that $B4_{305}$ coincidentally fulfills the sequence requirement of $B1_{305}$. In addition, moving an element into a foreign location or context may in certain cases have a compensatory effect rather than a mechanistically equivalent effect on function. $B4_{305}$ appears to be functionally redundant with the easily unwound region since either $B4_{305}$ or the easily unwound region can functionally substitute for a common element, $B2_1$ (Fig. 2 and 6). The synergistic effect of combined mutations in $B4_{305}$ and the easily unwound region of *ARS305* (Fig. 5 and 6) is consistent with the functional redundancy of these elements. $B4_{305}$ contains an easily unwound sequence, but its sensitivity to linker substitution distinguishes it from the easily unwound region between B1 and B4. It is possible that $B4_{305}$ is a critical part of a DUE or that it interacts with an unidentified protein that promotes DNA unwinding.

DUEs in different replication origins can vary in breadth, and the broader the DUE, the broader the G+C-rich substitution that must be introduced to significantly reduce replication origin activity. The SV40 DUE spans only 9 bp, and its function is significantly affected by point mutations that increase helical stability (31). The *ARS307* DUE spans 26 bp, and its function was weakly affected by 3-bp G+C-rich substitutions, more strongly affected by 8-bp G+C-rich linker substitutions, and most strongly affected by a 16-bp G+C-rich substitution (Fig. 7B). The function of the easily unwound region in *ARS305* was not significantly affected by 8-bp G+C-rich linker substitutions (23) but was reduced by a broad G+C-rich substitution between $B1_{305}$ and $B4_{305}$ (Fig. 5). These results suggest that the DUE of *ARS305* is even broader than the 26-bp DUE of *ARS307*. *ARS307* has no B4 element, which appears to be functionally redundant with the easily unwound region in *ARS305* (see above). It is possible that the B4 element, as well as some B4-flanking sequences that appear to be sensitive to deletion (Fig. 5), contributes to the breadth of the DUE in *ARS305*.

Our finding of a DUE in the B2 element of *ARS307* may provide a clue to the role of the unusual type of nucleotide specificity in the $B2_{307}$ element (43). The nucleotide specificity is extremely limited, including at most three positions (115 to 117), and does not appear to be conserved among ARSs. In both *E. coli oriC* and SV40 *ori*, within which either a repeated sequence motif or a specific nucleotide is embedded in the DUE (29, 31), weak interactions between the DUE regions and the respective initiator proteins were observed (40, 57). By analogy, one hypothesis is that the specific nucleotides in $B2_{307}$ serve to interact with an active domain of the yeast initiator, which, together with the surrounding helical instability of the DUE, facilitates DNA unwinding at the replication origin. The absence or variation of these specific nucleotides may be compensated for in part by a broad DUE, such as the easily unwound region of *ARS305*, which appears to be less dependent on a specific nucleotide sequence. Various combinations of helical instability and/or nucleotide specificity may be employed in different ARSs to mediate a common function, localized DNA unwinding. The fact that the specific nucleotides within $B2_{307}$ are not conserved in $B2_1$ and that the B2 elements and/or DUEs of the ARSs studied here and elsewhere (43) are functionally exchangeable in certain contexts, even though they possess no apparent sequence similarities, may be explained by this model.

Our findings help to solidify the identities of short *cis*-acting determinants that are common to yeast replicators, specifically elements A and B1, and the diversity in functional compositions of equivalent A elements, which differ in their dependence on a 3′ box (Fig. 3) (23, 32, 43, 51). Our findings reveal for the first time the functional substitution of an easily unwound region (22) for a B2 element ($B2_1$) (32) (Fig. 5) and the presence of a DUE in a different but functionally conserved B2 element ($B2_{307}$) (43, 51) (Fig. 7). Our findings also reveal diversity in the functional compositions of the easily unwound region of *ARS305* (22) and B2 elements (32, 43, 51), which appear to employ different contributions from nucleotide specificity and helical instability but are interchangeable in certain contexts. Surprising functional diversity was discovered between elements $B3_1$ and $B4_{305}$; although they occupy common positions in different replicators (23, 32), when they were swapped, B4 activated and B3, a known replication enhancer (54), inhibited (Fig. 2). A comprehension of the existence of diversity as well as similarity among *cis*-acting determinants of yeast replication origins should be helpful in understanding the regulation of DNA replication and in deciphering the complexities of other eukaryotic replicators.

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