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Domain B of *ARS307* Contains Two Functional Elements and Contributes to Chromosomal Replication Origin Function

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***ARS307* is highly active as a replication origin in its native location on chromosome III of *Saccharomyces cerevisiae*. Its ability to confer autonomous replication activity on plasmids requires the presence of an 11-bp autonomously replicating sequence (ARS) consensus sequence (ACS), which is also required for chromosomal origin function, as well as approximately 100 bp of sequence flanking the ACS called domain B. To further define the sequences required for ARS function, a linker substitution mutagenesis of domain B was carried out. The mutations defined two sequences, B1 and B2, that contribute to ARS activity. Therefore, like *ARS1*, domain B of *ARS307* is composed of functional subdomains. Constructs carrying mutations in the B1 element were used to replace the chromosomal copy of *ARS307*. These mutations caused a reduction in chromosomal origin activity, demonstrating that the B1 element is required for efficient chromosomal origin function.**

In general, eukaryotic chromosomal replication origins are poorly characterized. The notable exception is that in the budding yeast *Saccharomyces cerevisiae*, short chromosomal fragments have been identified which promote high-frequency transformation of shuttle vectors and allow these plasmids to replicate as extrachromosomal elements, features expected of replication origins (reviewed in references 11 and 38). These small fragments are called autonomously replicating sequence (ARS) elements. With the development of two-dimensional (2-D) agarose gel electrophoresis techniques for identifying replication intermediates, ARS elements were shown to colocalize with plasmid replication origins (7, 23). However, only a subset of ARS elements, defined by the transformation assay, function as replication origins in their native chromosomal contexts (8, 14, 16, 18, 22, 30, 37, 49).

DNA sequence analysis of several ARS elements revealed two common features, the presence of an 11-bp consensus sequence, (A/T)TTTA(T/C)(A/G)TTT(A/T), and a higher A + T content than in bulk yeast DNA (9, 47). Subsequent studies have shown that the ARS consensus sequence (ACS) is necessary but not sufficient for ARS function (reviewed in references 10 and 38). In addition, the essential ACS is not always a perfect match to the consensus but instead may agree at only 9 or 10 positions (22, 26, 34, 41, 48). Linker substitution mutagenesis suggests that the ACS is only part of a slightly larger essential element extending several base pairs on either side (5, 31, 48, 49). This element is referred to as domain A and is likely to be specified by the DNA-binding characteristics of the origin recognition complex, the putative replication initiator (2, 3, 13, 17, 28, 33).

In addition to domain A, sequences 3′ to the T-rich strand of the ACS are required for ARS function. The extent of these sequences, called domain B, is variable from ARS element to ARS element and also somewhat dependent on vector context (reviewed in references 10 and 38). Two general models, which are not mutually exclusive, have been proposed to explain the requirement for sequences in domain B. The first model suggests that the important feature of domain B is a physical property of the DNA, the energy required to unwind a region of duplex, rather than a specific sequence. Evidence in support of the role of a DNA-unwinding element (DUE) in ARS function includes the observation that external deletions which remove the DUE from domain B of the H4 ARS, abolishing ARS function, can be complemented by the insertion of an easily unwound 147-bp fragment from pBR322 (45). In addition, the effects of deletion mutations at several ARS elements have been correlated with increases in the helical stability of domain B (22, 34, 35). DUEs are a common feature of prokaryotic, yeast, and viral replication origins (29, 36, 50).

The second model emphasizes primary sequence, suggesting that this region contains binding sites for accessory factors. Since there is little or no sequence conservation in the domain B region, either various factors can be utilized by different ARS elements or the binding site for a common factor is highly degenerate. One such accessory factor is the transcription factor ARS-binding factor 1 (Abf1). Abf1 (also called OBF1) has been shown to function as a replication enhancer at *ARS121*. In this case, the binding sites are located in domain C, 5′ to the T-rich strand of the ACS, though the enhancement is still seen when the sites are moved further away or into domain B (48, 49). The Abf1-binding site in domain B of *ARS1* contributes to ARS function and can be functionally replaced by binding sites for two other transcription factors, Rap1 and Gal4 (31). Together, these results imply that many different transcriptional activators may act to enhance replication at various ARS elements and may do so in a relatively position- and distance-independent manner.

The detailed linker substitution analysis of domain B of *ARS1* (31) defined three important elements, called B1, B2, and B3, which together account for the activity of the domain. B3 corresponds to the binding site for the transcription factor Abf1. The roles of the B1 and B2 elements are unknown.

Previous work from our laboratory defined the extent of *ARS307* (39) and explored in detail the sequence requirements for its ACS (47). In this study we extended the analysis of domain B of *ARS307* by examining the DNA sequence requirements of the domain B region. Our results suggest that the organization of domain B in *ARS307* is similar to that in *ARS1*, with two short sequence elements, corresponding to B1 and B2

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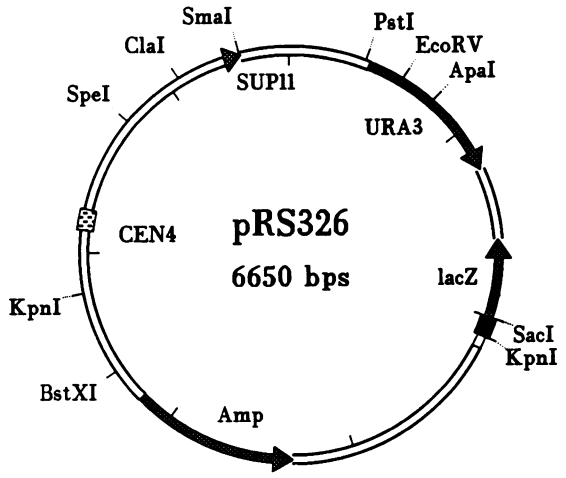

FIG. 1. Map of pRS326. The plasmid was constructed as described in Materials and Methods. It carries Amp^r for bacterial selection, the *lacZ* α peptide for bacterial colony color screens, *URA3* for selection in *S. cerevisiae*, *CEN4* for proper plasmid segregation in *S. cerevisiae*, and *SUP11-1*, an ochre suppressor, for yeast colony color screens. The polylinker region is identical to that of pRS306 (39) and indicated by the solid box between the *Sac*I and *Kpn*I sites at positions 2001 and 2103, respectively. The other restriction sites shown are those which cut both within the polylinker and elsewhere in the plasmid.

of *ARS1*, contributing to ARS function. The B1 element is shown to play an important role in chromosomal origin function.

MATERIALS AND METHODS

Strains. *Escherichia coli* DG101 (12) was used for plasmid propagation. The diploid yeast strain (1C6) used for plasmid stability assays was constructed by the following crosses. H243-13-2a (*MAT***a** *ura1 trp1* [6]) was crossed to YP500 (*MATα ura3-52 trp1Δ63 leu2Δ1 lys2-801 his3Δ200 ade2-101* [42]), and a spore (1C) of genotype *MATα ura3-52 trp1Δ63 leu2Δ1 his3Δ200 ade2-101* was identified by complementation tests and Southern hybridization. In parallel, YNN215 (*MATα ura3-52 lys2-801 ade2-101* [42]) was crossed to FY23 (*MAT***a** *ura3-52 trp1Δ63 leu2Δ1* [51]), and a spore (6B) of genotype *MAT***a** *ura3-52 leu2Δ1 lys2-801 ade2-101* was identified by complementation tests. Spores 1C and 6B were crossed to yield a diploid with the desired genotype, *MAT***a**/*MATα ura3-52/ura3-52 TRP1/trp1Δ63 leu2Δ1/leu2Δ1 LYS2/lys2-801 HIS3/his3Δ200 ade2-101/ade2-101*. Yeast strain YP45 (42) was the recipient strain for the two-step gene replacements used to introduce linker mutations back into the chromosome.

Plasmids. A new vector, pRS326, was constructed for plasmid stability assays. pRS326 (Fig. 1) is a derivative of the *URA3* yeast shuttle vector pRS306 (42), in which a *CEN4–SUP11-1* cassette, CENSUPII (44), was cloned into the *Aat*II site of pRS306. The inclusion of a centromere and the ochre suppressor *SUP11-1* allows the use of a colony color assay to determine the presence of the plasmid (20). The *SUP11-1* fragment contained in this cassette lacks *ARS3*, eliminating any complications which might be caused by the presence of a weak ARS in the vector itself. The suitability of this vector was examined by determining the mitotic stability of two ARS elements, the 0.5-kb *Eco*RI-*Cla*I fragment containing *ARS307*, which is a weak ARS, and the 0.8-kb *Eco*RI-*Hin*dIII fragment containing *ARS1*, a strong ARS. These plasmids gave mitotic stabilities of 30 and 87%, respectively, in good agreement with previous studies (39, 47).

To construct 3VH402, the 210-bp *Bam*HI-*Sal*I fragment of pVH402, containing a modified version of *ARS307* (47), was cloned into pRS326 cut with *Bam*HI and *Xho*I. Similarly, 3VH411 was constructed from pVH411, which is identical to pVH402 except for a T-to-C transition at position 3 of the ACS; in this case the *Bam*HI-*Sal*I fragment was cloned into pRS326 digested with *Bam*HI and *Sal*I. p411-306 was derived by cloning the 260-bp *Kpn*I-*Sac*I fragment of 3VH411 into pRS306 cut with *Kpn*I and *Sac*I.

Construction of linker substitution mutations. 3VH402 was digested with *Bam*HI and *Sac*I, and p411-306 was digested with *Kpn*I and *Xho*I. Each doubly digested DNA was then treated with exonuclease III (New England Biolabs) followed by mung bean nuclease (New England Biolabs) to generate unidirectional deletions (19). Eight-base-pair phosphorylated *Bgl*II linkers (New England Biolabs) were ligated to the deletion endpoints. Plasmid was purified away from linkers by electrophoresis in low-gelling-temperature agarose (SeaPlaque; FMC). The plasmid-containing band was excised and digested with β-agarase (New England Biolabs), and DNA was precipitated with isopropanol. The DNA was then digested with *Bgl*II and repurified by electrophoresis as described above prior to self-ligation. Strain DG101 was transformed by electroporation with the ligation mixes. It was noted that a substantial fraction of deletion plasmids harbored deletions in both directions. This appeared not to be the result of exonuclease III digesting through the 3′ overhang but rather a result of a recombination event occurring after transformation. To identify those clones with minimal retrograde deletions, colony screens were performed by using a γ-^{32}P-end-labeled M13 −20 primer for 3VH402 transformants and a reverse primer for p411-306 transformants. Clones were crudely sized by amplification of the inserts between the M13 −20 and reverse primers via PCR, digestion with *Bgl*II, and electrophoresis on polyacrylamide gels. Candidate PCR products were then sequenced by using a γ-^{32}P-end-labeled primer (T7 primer [Bethesda Research Laboratories {BRL}] for 3VH402 clones; T3 primer [BRL] for p411-306 clones) with the a double-stranded DNA cycle sequencing kit (BRL). The linker substitution constructs were made by ligating the 5.0-kb *Bgl*II-*Pst*I fragments from the 3VH402 deletions with the appropriate 1.6-kb *Bgl*II-*Pst*I fragments from the 411–306 deletions.

For three constructs, 147-156, 148-156, and 155-164, the 3VH402 deletions were found in the exonuclease III-generated set, but the corresponding 411–306 deletions were not. The appropriate 1.6-kb fragments were generated by PCR, using primers synthesized on an Applied Biosystems model 380A oligonucleotide synthesizer. One primer created a *Bgl*II site at the proper position in the *ARS307* sequence (147, CGCGCA GATCTGGAAGAGAGAGGATCAATA; 148, CGCGCAG ATCTGAGAAGAGAGAGGATCAAT; 155, CGCGCAGAT CTGAAAATAAAGAAGAGAG), and the second primer was distal to the *Pst*I site in pRS326 (CCAACTGCACAGAAC). The amplified 1.6-kb fragments were digested with *Bgl*II plus *Pst*I and ligated to the 5.0-kb *Bgl*II-*Pst*I fragment of 3VH402 to generate the final linker substitution plasmids.

Replacement of *ARS307* with linker substitution mutants. Because the *Sal*I site present in the ARS-containing fragment of pVH402 was lost in the construction of 3VH402, a two-step cloning procedure was used to introduce some of the linker substitution mutations back into the chromosome. Three B1 mutations which caused decreased plasmid stability, 162-172, 147-156, and 148-156, were chosen for analysis. In each case, a 175-bp *Eco*RI-*Sph*I fragment containing the mutation was

cloned into pVH402 cut with *Eco*RI and *Sph*I. The 201-bp *Eco*RI-*Sal*I fragments of the resulting constructions were inserted into pAC502 cut with *Eco*RI and *Sal*I. pAC502 contains a several kilobases of flanking DNA on both sides of the 201-bp fragment and was used to introduce point mutations in the ACS back into the chromosome (11). The resulting plasmids were linearized with *Cla*I to direct integration at *ARS307* and used to transform strain YP45. Integrants were confirmed by Southern analysis. Popouts were selected by streaking on 5-fluoro-orotic acid plates (4). Colonies carrying the linker substitution mutations were identified by the presence of a *Bgl*II site at *ARS307*.

Yeast transformations. Yeast strains were transformed by the lithium acetate method (24) or by electroporation (1).

Mitotic stability assays. Mitotic stability is the fraction of plasmid-bearing cells in cultures grown under selection for the plasmid. Plasmid stability assays were performed as described by Palzkill and Newlon (39).

2-D gel analysis. The procedures described by Deshpande and Newlon (11) for 2-D gels (7) and for fork direction analysis (15) were used, with the following minor modifications. Cells were harvested and DNA was prepared as described, except that cells in toluene stop solution were harvested by centrifugation, then resuspended in water, and washed by filtration (MAGNA nylon membranes, 142-mm diameter, 1.2-μm pores). Restriction digestion and electrophoresis were performed as described, except that 30 to 80 μg of genomic DNA was used per gel. The in situ digestions with *Eco*RV used for fork direction analysis were performed in 2 ml of buffer, so that the gel slice was completely submerged. Hybridization buffer was 5× SSC (1× SSC is 0.15 m NaCl plus 0.015 M sodium citrate), 5 mM sodium pyrophosphate (pH 7.0), 10× Denhardt's solution, 0.5% sodium dodecyl sulfate, and 150 μg of sonicated calf thymus DNA per ml.

Quantitation of origin usage. Densitometric analysis of autoradiograms of the fork direction gels was performed on a Molecular Dynamics model 300B computing densitometer. Lines parallel to the first dimension were drawn across the arcs at three positions, and the signals arising from the leftward- and rightward-moving forks were determined by integrating the densities along the lines. Additionally, by using a lighter exposure than shown in Fig. 5, the densities of the signals at the top of the arcs were determined. The different determinations gave similar values, which were averaged.

Helical stability calculations. Calculations were performed by the THERMODYN program (35) on the basis of the sequences of the mutations.

RESULTS

Linker substitution mutations. The 201-bp *Eco*RI-*Sal*I *ARS307*-containing fragment from pVH402 was selected for linker substitution mutagenesis for three reasons. First, in previous studies it showed the same mitotic stability as the 522-bp *Eco*RI-*Cla*I fragment from which it is derived (39, 47). Second, this fragment was used previously in a systematic analysis of the core consensus sequence (47). Third, it contains a number of base changes relative to the wild-type *ARS307* sequence, the effect of which is to abolish a 10-of-11 match to the ACS which overlaps the perfect match. In the wild-type *ARS307*, a double-point mutation in the perfect match to the ACS (position 5, A to T; position 6, T to C) does not eliminate ARS function, though it does reduce plasmid stability, presumably because the overlapping 10-of-11 match can partially substitute (39). The same double-point mutation is Ars$^-$ in the pVH402 construct lacking the overlapping 10-of-11 match (47). As shown in Fig. 2, there are two regions of difference between the pVH402 and wild-type *ARS307* sequences. The first includes positions 170 to 176, where there are four changes within the 7-bp interval which result in the creation of a *Sph*I site. The second region includes positions 196 to 201, where 5 of 6 bps are changed in order to eliminate the 10-of-11 match to the ACS and create a *Sal*I site.

A collection of 38 linker substitution mutations was constructed. These result in the introduction of an 8-bp linker carrying a *Bgl*II site, CAGATCTG. As shown in Fig. 2, 17 of the constructs contain simple substitutions of an 8-bp *ARS307* sequence with the linker sequence, while the remainder contain a 1-bp insertion or deletion in addition to the linker substitution. In two direct comparisons of a simple substitution with a 1-bp insertion or deletion (constructs 96-105, 97-105, 118-128, and 119-128), the small insertion or deletion had an effect similar to that of a simple substitution. Two of the constructs (100-109 and 163-174) carry a mutation in a base adjacent to the linker. There are several small gaps in the collection. Further mutations in the first region, from positions 181 to 187, were not sought because it is within the 12-bp region containing the ACS previously examined at single-base resolution (47). The other gaps are in the region from 40 to 52. Additional mutations in this region were not isolated because external deletions in this region do not affect plasmid stability (39) and because linker substitution mutations with associated 2-bp deletions in this region had no effect on plasmid stability (43).

Effects of linker substitution mutations on *ARS* function. Plasmids carrying the linker substitution mutations were tested first for ARS activity by assaying their abilities to transform at high frequency (Fig. 2). Four of the 38 mutant plasmids failed to yield yeast transformants that could be streaked to give colonies on selective medium in three independent transformations. These mutants were therefore classified as Ars$^-$ and are shown in Fig. 3 as having a plasmid stability of zero. The remaining mutant plasmids were assayed for mitotic stability, the fraction of plasmid-bearing cells in cultures grown under selection, which reflects plasmid loss rate (39). Since the vector contains a centromere, which promotes efficient plasmid segregation, mitotic stabilities provide a measure of ARS function, with high stabilities reflecting efficient function.

The starting plasmid, 3VH402, had a mitotic stability of 7.3%. This value is significantly lower than previously reported for this 201-bp *ARS307*-containing fragment in a different vector (47) and lower than that for the *ARS307* plasmid analyzed in the accompanying report (40). One explanation for these differences is that the lower mitotic stability of 3VH402 reflects the interaction of vector context with one or both of the linker substitutions present in this plasmid (see Discussion). Alternatively, slight differences in experimental technique cannot be excluded as the reason for these differences. Despite the lower starting mitotic stabilities, 12 of the mutations caused a significant decrease in plasmid stability, and a single mutation (87-96) caused a small increase in plasmid stability (Fig. 3). Approximately half of the mutations had no effect on plasmid stability. The mutations which reduce or abolish ARS function fall into three regions of *ARS307*.

Three of the four mutations which abolish ARS function fall into domain A, the essential region containing the ACS. In the case of *ARS307*, the sequence changes between pVH402 and the wild-type sequence (47) placed limits on the extent of the domain A element (Fig. 2). On the 5′ side of the T-rich strand of the ACS, domain A appeared not to extend beyond position 195 because the substitution of a *Sal*I linker starting at position 196 had no effect on ARS function. Similarly, the extent of

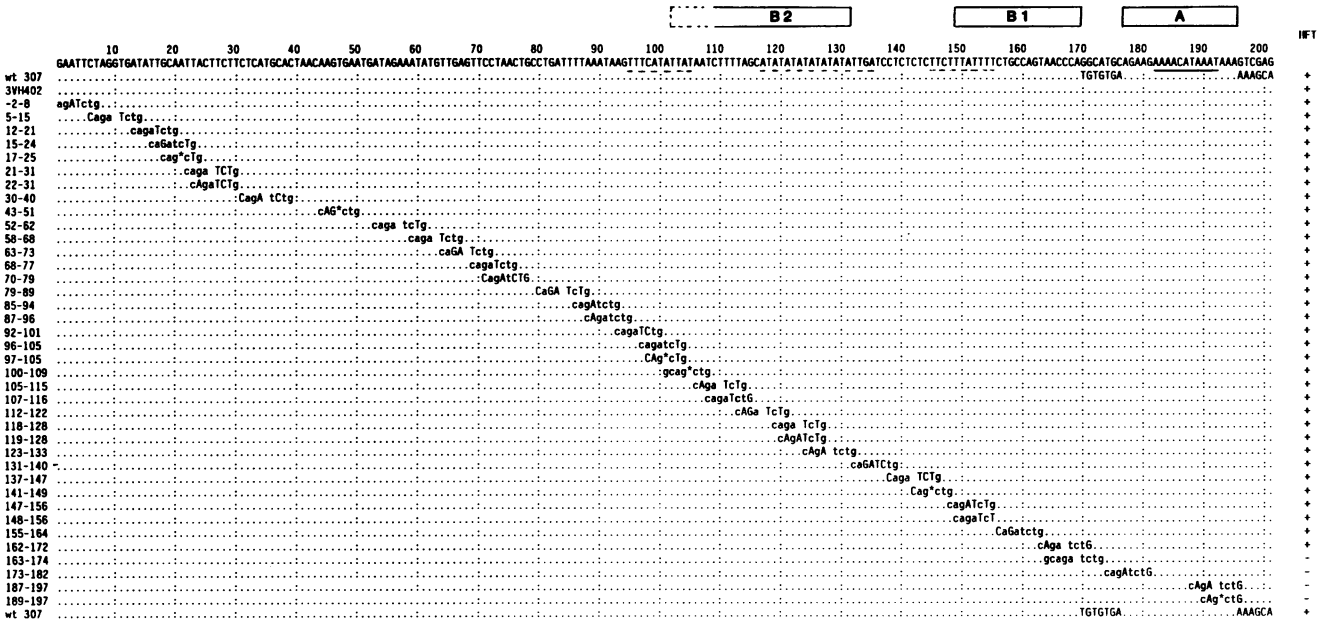

FIG. 2. Linker substitution mutations. The sequence of the 201-bp *Eco*RI-*Sal*I-*Xho*I fragment of 3VH402 is shown in the top line, and linker substitution mutants derived from it are shown below. Dots and colons represent positions of sequence identity. wt 307 shows the sequence differences between the 3VH402 sequence and that of the wild-type *ARS307*. Numbers in the plasmid names are derived from the wild-type bases flanking the substitutions. Uppercase letters indicate positions where the linker sequence is the same as the 3VH402 sequence. A space is shown in the center of the *Bgl*II linker sequence for those plasmids which result in a single-base deletion. An asterisk appears instead of the central AT of the linker sequence for those plasmids with a single-base insertion. The perfect match to the ACS is underlined with a solid line, and 9-of-11 matches are underlined with dotted lines. HFT + or − denotes whether or not the plasmid was able to transform *S. cerevisiae* at high frequency.

domain A on the 3′ side was limited by the substitution of a *Sph*I linker for the sequence between positions 170 and 176. This boundary for domain A is supported by the linker substitution analysis reported in the accompanying paper (40), which describes a *Xho*I linker at positions 167 to 174 that has no effect on plasmid stability. The Ars$^-$ phenotypes of mutations 187-197 and 189-197, both of which interrupt the ACS, were expected on the basis of the previous analysis of the ACS (47). Mutation 173-182 introduces a linker adjacent to the ACS, and its resulting Ars$^-$ phenotype indicates that the essential domain A sequence extends beyond the ACS on the domain B side. Our previous observation that a G → T change

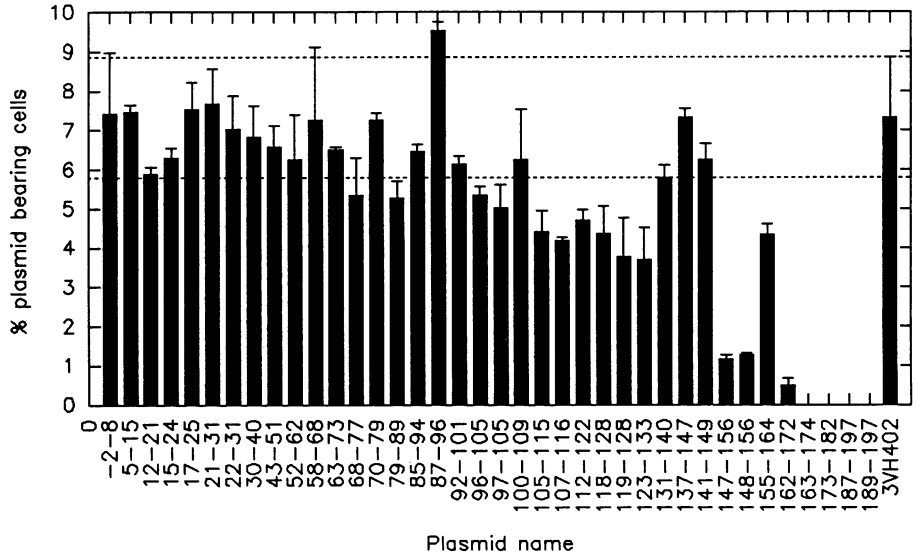

FIG. 3. Effects of mutations on plasmid stability. Mitotic stability assays were performed on the plasmids carrying the linker substitutions shown in Fig. 2. Three independent transformants were assayed for each plasmid. Means and standard errors are plotted. The dashed lines delineate the standard error in the measurements of 3VH402. The four plasmids which failed to transform at high frequency, 163-174, 173-182, 187-197, and 189-197, are shown on the graph as having 0% plasmid-bearing cells.

at position 181 reduced plasmid stability (47) is consistent with the extension of domain A in this direction.

The collection of linker substitution mutations defines two additional regions in domain B that are important for ARS function. The fourth Ars$^-$ mutation (163-174) and three mutations that severely reduce mitotic stability (147-156, 148-156, and 162-172) define one of these regions. Transformants carrying these mutant plasmids grow very slowly under selection, forming very small, nibbled colonies, a result of the high loss rate of these plasmids. These mutations, which severely impair ARS function, are separated by a linker substitution, 155-164, which has only a modest effect on mitotic stability. The boundaries of this region, nucleotides 149 and 176, are defined by the flanking linker substitutions that result in wild-type ARS function, 141-149, and the *Sph*I linker that separates this domain from domain A. These linker substitution mutations define a region of *ARS307* that corresponds to the B1 element of *ARS1* (see Discussion).

The second domain B region important for ARS function is identified by the six linker substitutions from 105-115 through 123-133. These mutations cause modest but significant reductions in mitotic stability. The small reductions in plasmid stability caused by substitutions 96-105 and 97-105 suggest that this region may extend a few nucleotides to the left. As for the B1 element noted above, the boundaries of this region, nucleotides 101 and 131, are established by the flanking linker substitutions that result in wild-type plasmid stabilities. The region defined by these mutations corresponds to element B2 of *ARS1* (see Discussion).

Effects of linker substitution mutations on chromosomal origin function. We have shown previously that point mutations in the ACS which abolish or reduce ARS function on plasmids similarly abolish or reduce origin function when introduced into the *ARS307* locus on chromosome III (11). It was of interest to extend these observations to mutations in domain B of *ARS307*. The three B1 mutations that caused dramatically decreased plasmid stability, 147-156, 148-156, and 162-172, were used to replace the chromosomal copy of *ARS307* via a two-step gene transplacement (4).

Origin activity was examined by 2-D agarose gel electrophoresis (7). Figure 4 shows a restriction map of the region containing *ARS307*. The ARS element is located asymmetrically within the 3.3-kb *Eco*RV fragment. Because wild-type *ARS307* functions as an origin in nearly all cells (11, 18), this geometry results in the early replication intermediates being predominantly bubble shaped, while the late replication intermediates are Y shaped (Fig. 4A; see Fig. 4D for a diagram of 2-D gel patterns). This pattern is altered in the mutants (Fig. 4B and C). The 148-156 and 162-172 mutations both result in an enhancement of the signal from early Y-shaped molecules at the expense of the signal from bubble-shaped molecules. This finding indicates that origin function is less efficient in these mutants; i.e., in a substantial fraction of cells, the 3.3-kb *Eco*RV fragment is replicated by a fork emanating from either *ARS308* or *ARS309*, which lie 3 and 21 kb, respectively, to the right of the *Eco*RV fragment shown in Fig. 4. As predicted by their relative plasmid stabilities, the signal from bubble-shaped molecules is less intense in the 162-172 mutant than in 148-156. Consistent with its plasmid stability, the 147-156 substitution mutant gave results similar to those for the 148-156 mutant (43). Similar results were seen in three independent DNA preparations.

While the 2-D gels shown in Fig. 4 clearly show that origin function is impaired by these mutations, it is difficult to estimate the extent of the inhibition from this analysis for reasons discussed thoroughly by Fangman and Brewer (15). A better comparison can be made by performing fork direction analysis. This technique is a modification of the 2-D gel procedure wherein a second restriction digestion is performed in situ between the running of the first- and second-dimension gels (15). The informative region lies to the right of *ARS307* (11, 18) and can be examined as the 3.4-kb *Eco*RI fragment digested in situ with *Eco*RV (Fig. 4). When *ARS307* is active as an origin, a fork traverses this fragment from left to right. The origin is so close to the left end of this fragment that the small bubble-containing molecules are not seen, and the fragment appears to be replicated by a rightward-moving fork. When initiation fails to occur at *ARS307*, this fragment is replicated by a leftward-moving fork emanating from either *ARS308* or *ARS309* (11, 18). Figure 5D diagrams the patterns expected when the 2.2-kb *Eco*RI-*Eco*RV fragment is used as a probe for fork direction analysis. The arc labeled R arises from a rightward-moving fork emanating from *ARS307*, and the arc labeled L results from a leftward-moving fork coming from either *ARS308* or *ARS309*.

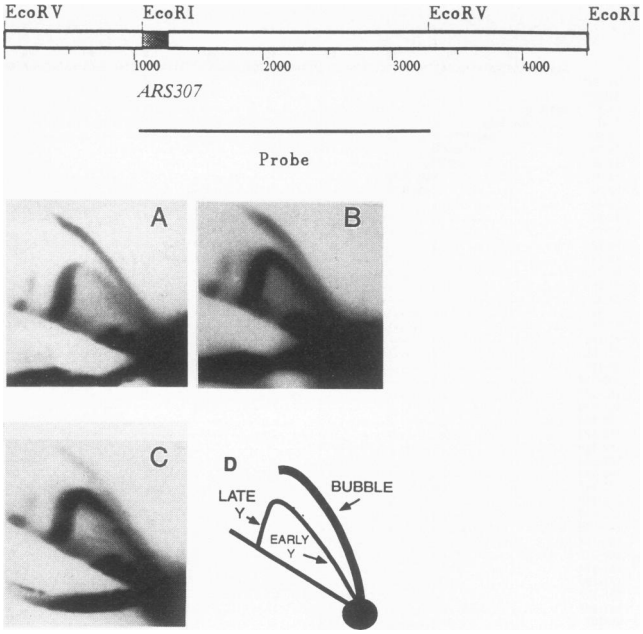

FIG. 4. Analysis of chromosomal replication origin function of B1 mutants. The restriction map of the region surrounding *ARS307* is shown at the top. The 201-bp *ARS307* fragment is shown by the shaded box, and the 2.2-kb *Eco*RI-*Eco*RV fragment used as a probe is indicated below the restriction map. (A to C) Patterns obtained from analysis of the 3.3-kb *Eco*RV chromosomal DNA fragment containing wild-type *ARS307* (A), mutant 147-156 (B), and mutant 162-172 (C). (D) Diagram of the patterns produced by Y-shaped replication intermediates and bubble-containing replication intermediates. Electrophoresis in the first dimension was from left to right, and electrophoresis in the second dimension was from top to bottom.

Consistent with previous results (11, 18), the predominant signal from wild-type *ARS307* is from rightward-moving forks (Fig. 5A). In the two mutants, however, the signal from leftward-moving forks is at least as intense as the signal from rightward-moving forks (Fig. 5B and C). Quantitation of the relative intensities of the signals revealed that when wild-type *ARS307* was present, 95% of the forks were rightward moving, demonstrating that the origin is active in nearly every S phase. In mutants 148-156 and 162-172, 55 and 67%, respectively, of the forks were leftward moving, demonstrating that origin

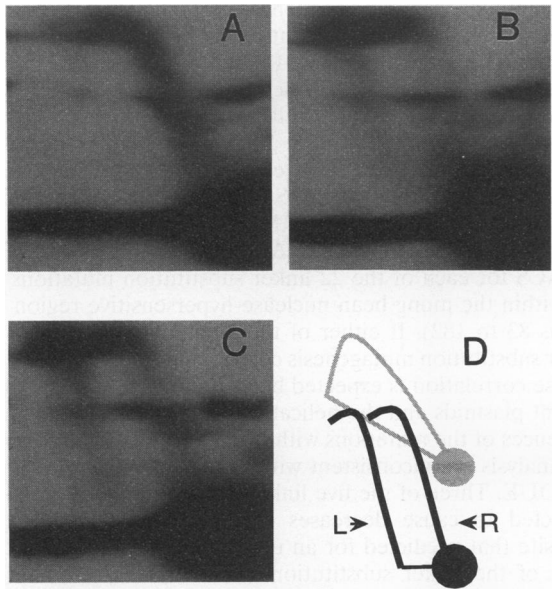

FIG. 5. Fork direction analysis of B1 mutants. (A to C) Patterns obtained from analysis of the 3.4-kb *Eco*RI chromosomal DNA fragment digested in situ with *Eco*RV after running the first dimension (see Fig. 4 for restriction map). Panel A shows wild-type *ARS307*, while panels B and C show mutants 147-156 and 162-172, respectively. (D) Diagram of the expected patterns of replication intermediates. The shaded arc at the top indicates the migration of replication intermediates from the 3.4-kb *Eco*RI fragment in the absence of subsequent digestion by *Eco*RV. The arc labeled R arises from forks moving rightward through the 2.2-kb *Eco*RI-*Eco*RV fragment and is indicative of origin activity at *ARS307*. Conversely, the arc labeled L arises from leftward-moving forks, indicating that *ARS307* is inactive and the fragment is replicated by a fork emanating from either *ARS308* or *ARS309*.

activity is dramatically reduced by the substitutions in the B1 element. Therefore, we conclude that the B1 element of *ARS307* contributes to chromosomal origin function.

DISCUSSION

The linker substitution analysis described here was undertaken primarily to further define important sequences in domain B of *ARS307* and to determine the extent to which the organization of this domain resembles the modular structure of domain B of *ARS1*. The mutations that affect ARS function, as measured by plasmid stability, fall within a 97-bp segment that corresponds closely to the sequences defined by our previous external deletion analysis (39) and define two regions of domain B that contribute to ARS function. The effects of previously studied internal deletions of domain B are also broadly consistent with the present analysis. Deletions that removed all or part of element B2, or encroached on element B1, caused reductions in plasmid stability, while a deletion that removed only sequences distal to the two elements had no effect on plasmid stability (39). The complete loss of ARS activity caused by deletions that remove both of these elements (39) and double linker substitution mutations (40) demonstrates that these two elements account for domain B function. Thus, it appears that *ARS307* conforms to the modular design described for *ARS1* (31) and also apparent, though less precisely detailed, in *ARS121* (49).

The rather modest effects on plasmid stability of the B2 mutations isolated in this study compared with the mutations made with a different linker by Rao et al. (40) are most easily explained by the relative efficiency of the linkers used as mutators of the B2 sequence. The *Bgl*II linker used in this study is an inefficient mutator of the AT-rich B2 sequence compared with the *Xho*I linker used in the other study. The extreme example is shown by mutation 119-128, in which the *Bgl*II linker changes the wild-type sequence at only four of eight positions, while a *Xho*I linker changes six positions.

Our demonstration that mutations in the B1 element impair chromosomal origin function extends the correspondence between sequences required for ARS function on plasmids and for chromosomal origin activity. The finding from both standard 2-D gel analysis and fork direction analysis that three B1 mutations decrease the frequency of replication initiation at *ARS307* confirms and extends the observations of Huang and Kowalski (22), who showed that a complete deletion of domain B of *ARS305* abolished chromosomal origin activity. Together with our previous demonstration that point mutations in the ACS have parallel effects on plasmid ARS function and chromosomal origin activity (11), these results strongly suggest that the dissection of ARS elements by using the plasmid assay provides meaningful information about chromosomal origins.

The reduction in chromosomal origin activity caused by the B1 mutations analyzed (35 to 45% of wild-type activity) is somewhat less dramatic than the reduction in plasmid stabilities caused by the same mutations (5 to 10% of wild-type activity). A likely explanation for this discrepancy is the presence of sequences on the chromosome, but not present in the plasmid constructions, which enhance chromosomal origin function. The relative stabilities of plasmids carrying *ARS307* subclones suggests that a replication enhancer is located in the domain C region between 0.3 and 1.8 kb away from the fragment used in these studies (43, 46).

In our analysis, the B1 element is defined by five linker substitution mutations, 147-156, 148-156, 155-164, 162-172, and 163-174. Both the results presented here and the mini-linker scan mutagenesis of Rao et al. (40) show that the element is not uniformly sensitive to mutation but has two regions where substitutions cause a large decrease in plasmid stability flanking a third region where mutations have a smaller effect. The analysis of point mutations in the B1 element of *ARS1* identified two essential A · T base pairs 18 and 19 bp away from the ACS (40). The B1 element of *ARS307* contains two A · T base pairs with similar spacing from the ACS (17 and 18 bp, positions 164 and 165). Changes in one or both of these residues may account for the phenotypes of substitutions 162-172 and 163-174. These residues are unaffected by the other substitutions in the B1 element, demonstrating that these two nucleotides cannot account for all of B1 function. Moreover, of the 17 ARS elements for which the essential ACS has been identified (38, 43), only 12 have two A · T base pairs at positions relative to the ACS that correspond to the important A · T base pairs in *ARS1* B1. These observations and the finding that the B1 elements of *ARS307* and *ARS1*, which share little sequence identity, can substitute for each other (40) are consistent with the failure of sequence comparisons to reveal a conserved sequence in the domain B region of *ARS* elements and suggest that at least several different sequences are able to supply the B1 element function.

The results of the linker substitution analysis demonstrate that domain A of *ARS307* extends beyond the ACS. The boundary between domain A and the B1 element is identified by a *Sph*I linker present between positions 170 and 176 in the construct that we analyzed and by a *Xho*I linker at a similar site (positions 167 to 174) in the analysis of Rao et al. (40). Linker

substitutions at identical positions between this boundary and the 3′ end of the ACS were isolated in both studies and were found to severely impair ARS function. Our mutant 173-182 abolished high-frequency transformation, while their mutant 174-181 drastically reduced plasmid stability. The differing results probably reflect the higher starting plasmid stability seen by Rao et al. and/or the fact that our substitution changes three of the last four positions whereas theirs changes only one of four. Thus, domain A extends between 4 and 8 bp toward domain B from the ACS.

Domain A also extends for several base pairs on the domain C side of the ACS. Previous work had shown that a deletion to position 192 of the fragment used in this analysis caused a twofold increase in plasmid loss rate compared with the deletion to position 195 used in pVH402 (46). Therefore, we place the boundary of domain A at position 195. Rao et al. (40) isolated a mutation (194-201) which alters positions between the previous deletion endpoints and decreases plasmid stability. However, we cannot eliminate the possibility that changes in sequences beyond position 195 are responsible for the lower stability of our *ARS307* construct compared with the construct of Rao et al. (40).

The results presented in this study allow a further assessment of two proposed models for domain B function, the presence of near matches to the ACS (39) and the presence of a DUE (35, 45). It appears that neither can fully account for the new observations. The effects of domain B deletions and the ability of synthetic oligomers carrying the ACS to restore ARS activity were the basis for the proposal that near matches to the ACS play an important role in the function of domain B of *ARS307* (39). However, the introduction of point mutations into all of the near matches in domain B of the histone H4 ARS had no effect on ARS activity (21), and a similar analysis of *ARS121* (49) was also inconsistent with near matches to the ACS having an important role in ARS function. Both the B1 and B2 elements of *ARS307* defined by this study overlap 9-of-11 matches to the ACS (Fig. 2). However, there are two mutations (137-147 and 141-149) which disrupt the 9-of-11 match partially contained in B1 but have no effect on plasmid stability, indicating that the 9-of-11 match is not the important feature of this element. Similarly, the series of near matches contained within the B2 element cannot solely account for B2 function because there are mutations in this region (105-115 and 107-116) that affect plasmid stability but do not disrupt the near matches. The 9-of-11 match from positions 95 to 105 appears to be dispensable, since three of the five mutations that disrupt it show wild-type plasmid stability, and the other two (96-105 and 97-105) cause only marginal decreases in stability.

In well-studied origins of replication, a critical event in the initiation of replication is the unwinding of a small region of DNA adjacent to the initiator protein binding site(s) where the rest of the replication apparatus is assembled and the initial primers for DNA synthesis are laid down. Analyses of mutations in *E. coli oriC* (27) and the simian virus 40 origin (29) have suggested that a critical feature of this DUE is its helical instability. It has also been suggested that domain B of *ARS307* is simply a DUE (35). In supercoiled plasmids containing *ARS307*, mung bean nuclease preferentially nicks domain B DNA, suggesting that *ARS307* contains easily unwound DNA within domain B (35). This hypersensitive region starts immediately 3′ to the T-rich strand of the ACS and continues for about 100 bp. A cluster of hypersensitive sites on each strand maps to the alternating AT track, positions 117 to 132, which comprises much of the B2 element.

A computer program (THERMODYN) that calculates helical stabilities from base sequences correctly predicts both the positions and hierarchies of nuclease-hypersensitive sites in supercoiled plasmids (35, 36). Reductions in plasmid stability caused by previously studied deletion mutations in domain B of *ARS307* (39) correlate well with predicted increases in helical stability of the 100-bp 3′-flanking region, suggesting that the nuclease-hypersensitive region of *ARS307* contains a DUE that is important for ARS function (35). Because of this correlation, we used the THERMODYN program to calculate the free energy of unwinding (ΔG) of the 100 bp region 3′ to the ACS for each of the 22 linker substitution mutations that fall within the mung bean nuclease-hypersensitive region (positions 83 to 182). If either of the elements identified by the linker substitution mutagenesis corresponds to a DUE, then an inverse correlation is expected between mitotic stability of the mutant plasmids and the helical stabilities predicted from the sequences of the mutations within that element. The results of this analysis are inconsistent with the B1 element functioning as a DUE. Three of the five linker substitutions within B1 are predicted to cause decreases in helical stability, an effect opposite that predicted for an unwinding element.

All of the linker substitutions within the B2 element are predicted to increase the helical stability of the 100-bp region, as expected if B2 were a DUE. However, there is not a strong correlation between the magnitude of the increase in helical stability and the corresponding decrease in plasmid stability. A role for B2 as a DUE or some other structural element remains attractive for several reasons. First, the relative insensitivity of B2 to mutagenesis is consistent with this proposal. Saturation mutagenesis of the *ARS1* B2 element failed to identify point mutations with a plasmid stability phenotype (40), suggesting that B2 is not a binding site for a sequence-specific DNA-binding protein. Second, the hypersensitivity of B2 to nicking by mung bean nuclease in supercoiled plasmids suggests that B2 DNA is intrinsically easy to unwind (32). Third, a substantial fraction of leading strands appear to initiate within or near the B2 element of *ARS307*, supporting the idea that B2 unwinds to allow assembly of the replication apparatus (25). Finally, the incubation of a supercoiled *ARS1* plasmid with human single-stranded-DNA-binding protein RP-A, which is required for the initiation of simian virus 40 DNA replication in vitro, greatly enhances the sensitivity of *ARS1* to nicking by mung bean nuclease in a manner dependent upon the B2 element, suggesting that RP-A binds B2 sequences preferentially (32).

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