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cis-Acting Components in the Replication Origin from Ribosomal DNA of *Saccharomyces cerevisiae*

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The ribosomal DNA (rDNA) repeats of *Saccharomyces cerevisiae* contain an autonomously replicating sequence (*ARS*) that colocalizes with a chromosomal origin of replication. We show that a minimal sequence necessary for full *ARS* function corresponds to a 107-bp rDNA fragment which contains three 10-of-11-bp matches to the *ARS* consensus sequence. Point mutations in only one of the 10-of-11-bp matches, GTTTAT GTTTT, inactivate the rDNA *ARS*, indicating that this consensus sequence is essential. A perfect match to a revised *ARS* consensus is present but not essential. Sequences up to 9 bp 5′ from the essential consensus are dispensable. A broad DNA region directly 3′ to the essential consensus is required and is easily unwound as indicated by (i) hypersensitivity to nicking of an approximately 100-bp region by mung bean nuclease in a negatively supercoiled plasmid and (ii) helical instability determined by thermodynamic analysis of the nucleotide sequence. A correlation between DNA helical instability and replication efficiency of wild-type and mutated ribosomal *ARS* derivatives suggests that a broad region 3′ to the essential *ARS* consensus functions as a DNA unwinding element. Certain point mutations that do not stabilize the DNA helix in the 3′ region but reduce *ARS* efficiency reveal an element distinct from, but overlapping, the DNA unwinding element. The nucleotide sequence of the functionally important constituents in the *ARS* appears to be conserved among the rDNA repeats in the chromosome.

An autonomously replicating sequence (*ARS*) confers the ability to replicate to an episomal vector in the yeast *Saccharomyces cerevisiae* (19, 21, 25, 44). *ARS* elements colocalize with replication origins on plasmids as shown by the mapping of DNA replication intermediates (5, 22). Some *ARS*s are closely associated with sequences responsible for chromosomal DNA replication (15, 23, 30, 52). However, certain other functional *ARS*s derived from chromosomal DNA clearly do not serve as replication origins within their native context in the chromosome (13; reviewed in reference 14). The factors that determine the activity of *ARS* elements and chromosomal replication origins are not fully understood.

All *ARS* elements require a sequence that matches the *ARS* consensus, (A/T)TTTAT(A/G)TTT(A/T) (1, 3, 7, 9, 24, 50, 52). The essential consensus sequence can be a perfect match (11 bp) or an imperfect match (10 or 9 of 11 bp) to the *ARS* consensus. A revised *ARS* consensus, with a T or C at position 6, has been proposed (50). Several consensus matches are frequently present in an *ARS*; however, it is the sensitivity of the essential consensus sequence to point mutations and linker substitution mutations that distinguishes it from other, nonessential, consensus matches (20, 31, 51). The sensitivity to point mutations suggests that the essential consensus sequence interacts with proteins that participate in the initiation of DNA replication. The replacement of the chromosomal copies of *ARS307* and the HMRE *ARS* with derivatives having inactivating mutations at the essential consensus results in the inactivation of origin function at these loci, conclusively demonstrating that an intact *ARS* consensus element is required for origin activity in the chromosome (10, 39).

In addition to the consensus sequence, efficient replication requires a broad nonconserved region (about 100 bp) immediately 3′ to the T-rich strand of the essential consensus sequence. In all *ARS* elements the DNA in the broad 3′-flanking region is easily unwound (35) and may facilitate origin unwinding during initiation (see below). The broad 3′-flanking region also contains near matches to the *ARS* consensus that are proposed to function in protein binding (36) and that contribute to the ease of unwinding (34). Some *ARS* elements contain binding sites for transcription factors in the 3′-flanking DNA (8, 11, 46). The DNA-binding site for the transcription factor ABF1 (same as GF1 [12] and OBF1 [16]) occurs in the 3′-flanking sequence of *ARS1* and contributes to replication efficiency (31). ABF1 acts as a replication enhancer in that it functions either 5′ or 3′ to the *ARS* consensus sequence and in a distance- and orientation-independent manner (51). In the 3′-flanking sequence of *ARS1*, other transcription factors and their cognate binding sites can substitute for ABF1 and its binding site (31).

The ease of unwinding the broad region that is directly 3′ to the T-rich strand of the consensus sequence is a determinant of *ARS* function (34, 48, 49). The intrinsic ease of DNA unwinding in the 3′-flanking DNA was first detected in negatively supercoiled plasmids as the region that displays hypersensitivity to nucleases specific for single-stranded DNA (47, 48). Certain deletion mutations in the easily unwound sequence increase the stability of the DNA helix and are accompanied by a reduction in *ARS* activity (34, 48). Mutations that raise the helical stability to a sufficient degree completely inactivate the *ARS*. The portion of the easily unwound region that is sensitive to mutations which raise the helical stability defines a DNA unwinding element (DUE). The energetics of unwinding the DUE are important in vivo, since thermal energy, which facilitates DNA unwinding at the DUE in vitro, can suppress replication-defective mutations that raise the helical stability of the DUE (49).

A DUE is proposed to facilitate localized DNA unwinding in response to the binding of initiation factors that recognize

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the *ARS* consensus element (48, 49). The plausibility of this model is supported by a mutational analysis of the *Escherichia coli* origin, which demonstrates a requirement for a DUE (27). In *E. coli*, the DUE corresponds to the same site that is induced to unwind in response to the binding of the initiator protein to nearby consensus sequences (2). Since unwinding the double helix is essential for the replication machinery to gain access to the individual DNA strands, it is likely that a DUE will prove to be a conserved part of the *cis*-acting sequences that participate in origin function in other yeast *ARS* elements as well as other organisms.

The ribosomal DNA (rDNA) of *S. cerevisiae* contains multiple copies of a head-to-tail repeat that includes an *ARS* element and encodes the polymerase (Pol) III (5S) and Pol I (35S) transcripts of rRNA (54). The rDNA repeats are located on chromosome XII (37). An rDNA *ARS* is presumed to be present in each of the 100 to 200 tandem rDNA repeats and is a potential replication origin in the chromosome. However, not every *ARS* in the rDNA repeats serves as a chromosomal replication origin. Fewer than one-third of the rDNA *ARS*s are actually used as replication origins in a given S phase (6, 30, 41, 53). The factors that determine the frequency of origin usage in the rDNA repeats are not known. The *ARS* activity of fragments from the rDNA has been partially characterized. The portion of the rDNA with *ARS* activity is located between the divergent Pol I and Pol III promoters (43). Plasmids that utilize an rDNA *ARS* as an origin of replication are inefficiently maintained relative to plasmids containing most other *ARS* elements derived from yeast chromosomal DNA (26). The molecular basis for the weak origin activity of the rDNA *ARS* on a plasmid is not known.

The *cis*-acting components of the rDNA *ARS* have not been defined. Here we report a mutational analysis of the rDNA *ARS* as a first step towards identifying the genetic components that determine the activity of the rDNA replication origin.

MATERIALS AND METHODS

Enzymes. Restriction enzymes and T4 phage DNA ligase were purchased from New England Biolabs. Mung bean nuclease and snake venom phosphodiesterase were obtained from Pharmacia and Boehringer Mannheim Biochemicals, respectively. The DNA polymerases, Klenow fragment and Sequenase, were purchased from United States Biochemical, and *Taq* polymerase was obtained from Perkin-Elmer Cetus.

Plasmids and DNA. Three vectors were used in the experiments presented here. YIp5 and pVHA are pBR322-based vectors that have been described previously (45, 50). YIp5 and the centromere-containing pVHA vector were used to determine the transforming ability and mitotic stability, respectively, of the rDNA *ARS* derivatives described below. Another vector, used in the creation of rDNA *ARS* deletions, is called pWE. This vector was derived from pBR322 by deletion of portions of the β-lactamase and repressor of primer genes (27), and it includes an insertion of the *Pst*I-to-*Eco*RI polylinker sequence from pUC12 (33). pWE was chosen for experiments involving DNA unwinding because the pBR322 sequences that compete with the unwinding of the rDNA *ARS* are deleted from this vector.

The 769-bp *Hae*III-*Eco*RI fragment containing the rDNA *ARS* was a gift from the laboratory of J. Huberman and is referred to here as r1. The rDNA used to isolate this *ARS* was derived from a genomic clone obtained through T. Petes.

Cells and cell culture. Circular *ARS* plasmids were propagated in the HB101 and DH5α strains of *E. coli* by standard protocols as described previously (48). The diploid YPH3 strain of *S. cerevisiae* (*ade2-101 lys2-801 ura3-52*, provided by P. Hieter) was used for the transformation and mitotic-stability analysis of the *ARS* plasmids. Yeast cells were transformed by a modification of the lithium acetate-polyethylene glycol method (17). Transformation assays were carried out under selection for the *URA3* gene of the YIp5 or pVHA vector. Ars^+ transformants are defined as cells containing the episomal vector that could form colonies after being restreaked on selective medium.

Generation of deletion mutations. Mutations in the rDNA *ARS* sequence were created by a variety of methods. Initial deletions were created by isolating the r1 fragment, cutting at restriction sites (see Fig. 1), and cloning the fragments into the *Sma*I site of the pWE vector by blunt-end ligation. Internal deletions in pWEr3 were created by using the mutagenesis method outlined in Fig. 4. Supercoiled pWEr3 plasmid (10 μg) was incubated for 30 min at 37°C in a volume of 145 μl of TE (10 mM Tris HCl, 1 mM EDTA) at a pH of 7.0. Mung bean nuclease (7.75 U) was added, and digestion proceeded for 30 min. The solution was placed on ice, and 5 μl of sample was separated on an agarose gel to assess the completion of the nicking reaction. The solution was adjusted to 100 mM NaCl and 1 mM magnesium acetate, and an equal volume of TMTX buffer (40 mM Tris HCl, 2 mM magnesium acetate, 0.01% Triton X-100, pH 8.5) was added. Snake venom phosphodiesterase (3 mU) was added, and aliquots were incubated for 1 to 10 min at 37°C. The snake venom phosphodiesterase converts the nicked form of the plasmid to a linear form by its 3′-to-5′ exonuclease activity in conjunction with its single-strand-specific endonuclease activity. It subsequently creates deletions at the ends of both DNA strands by the action of its 3′-to-5′ exonuclease (38). Samples were placed on ice, and aliquots were run on an agarose gel to detect linearization and deletion as indicated by the electrophoretic mobility of the DNA. The populations with small deletions were selected from the samples that showed linearization without extensive deletions. These aliquots were subjected to organic extraction and precipitated with 70% ethanol. All deletion samples were treated with Klenow polymerase and 200 μM each deoxynucleoside triphosphate to create blunt-ended structures. The DNA ends were joined with T4 ligase and then transformed into *E. coli*. Additional short deletions in the vicinity of consensus 3 of the pWEr3 plasmid were created by a standard method of BAL 31 exonuclease digestion (42). The modified rDNA *ARS* plasmids were digested with *Bam*HI and *Eco*RI, and the rDNA *ARS* fragments were subcloned between the *Bam*HI and *Eco*RI sites of the YIp5 or pVHA vector. Deletion endpoints within the rDNA region were determined by DNA sequence analysis.

Cloning of the *ARS* region from rDNA repeats. Fragments of 2.7 kb from rDNA (see Fig. 1) were cloned from a *Hin*dIII digest of yeast genomic DNA that was separated on a 1.2% low-melting-point agarose gel. The 2.7-kb fragments were visualized by ethidium bromide staining, excised, and ligated into the *Hin*dIII site of the pVHA vector by a protocol for cloning fragments isolated from low-melting-point agarose gels (42). The ligation mixture was transformed into *E. coli*. Four of eight plasmids isolated from transformed *E. coli* contained 2.7-kb rDNA fragments. The plasmids containing

rDNA were then transformed into *S. cerevisiae* to assess *ARS* function.

Sequence analysis. DNA sequencing was performed by the Maxam-Gilbert protocol (32) for the sequence level analysis of nicking by mung bean nuclease (48). The sequence of mutated rDNA *ARS* derivatives was determined by the Sequenase protocol from United States Biochemical. During sequencing of the rDNA *ARS* derivatives, we noted that the sequence of the r1 fragment differs from the published sequence (43) at positions 1924 and 1939 by a replacement of a G with an A and an A with a G, respectively. The latter sequence heterogeneity occurs at the seventh position of consensus 3 described in this report. It does not affect the extent of the near match to the *ARS* consensus, since an A or G may occur there. The r1 derivative is also missing a restriction site for *Nde*I at position 2058 of the published sequence (43).

PCR. Polymerase chain reactions (PCRs) were performed with various synthetic primers to invert the 9th and 10th positions in each of the three near matches to the *ARS* consensus element. Oligonucleotide primers were made by Al Cairo (Biopolymer Facility, Roswell Park Cancer Institute), using a model 394 Applied Biosystems synthesizer. Standard 50-μl reactions for PCR utilizing *Taq* polymerase were performed as described in the protocol from Perkin-Elmer Cetus. The PCR consisted of an initial denaturation for 45 s at 95°C followed by 35 cycles of annealing for 15 s at 35°C, extension for 45 s at 74°C, and denaturation for 15 s at 95°C. The process was carried out in a Perkin-Elmer model 9600 thermocycler. The initial primer used, YrAP1, contained a synthetic *Bam*HI site at its 5′ end and was used to generate the 107-bp parental fragment, r8. The sequence of YrAP1 is GAAATGGATC CTTTTGATTG TT. The primers YrAP3 (GAAATGGATC CTTTTGATTG TTTATGTAAT GTGTGATGAT), YrAP5 (GAAATGGATC CTTTTGATTG TTTATGTTTT GTGTGATGAT TTTACATAAT TGCAT AGTA), and YrAP4 (CCAGAATTCT ATGTAATCTT AC TCCTATGT CT) were used to create the mutations of the first, second, and third consensus sequences, respectively, producing r10, r11, and r12. The YrAP4 and YrAP5 primers were used in combination to produce the mutant r14 fragment. In addition to mutagenesis of the third consensus sequence, r12 and r14 have an additional change of sequence beyond the third consensus element due to the creation of an *Eco*RI restriction site by PCR with the YrAP4 primer.

Mitotic stability. Yeast cells that were transformed by rDNA *ARS* derivatives of the centromere-containing pVHA vector were grown under selection for the plasmid in synthetic minimal medium supplemented with the required auxotrophic factors except for uracil (40). Three or more colonies transformed by each derivative were tested for mitotic stability. Following overnight growth in liquid medium to saturation under selection for the plasmid, the cells were diluted and plated on selective (synthetic minimal medium without uracil) and nonselective (YPD) media (40). The number of colonies that formed on the selective plate was divided by the number of colonies on the nonselective plate to obtain the proportion of cells that contained the plasmid.

Helical-stability analysis. The Thermodyn program was used to calculate helical stability, the free energy requirement for DNA strand separation (34). Thermodynamic properties of nearest-neighbor dinucleotides (4) form the basis for this approach. Two types of analyses were used for different purposes: (i) a sliding-window analysis of the r1 fragment and (ii) a single-window analysis of wild-type and mutated *ARS*s.

The Thermodyn program performs a sliding-window analysis by calculating the free energy (ΔG) value for a given window of sequence and then moving a fixed number of base pairs (step) and repeating the calculation for the next window position. The free energy value obtained for each window is tabulated and assigned to the nucleotide position at the center of that window. The data obtained from the program are plotted by using the Slidewrite Plus program (Advanced Graphics Software, Inc.). From the approximate breadth (100 bp) of the rDNA *ARS* region that was nicked by mung bean nuclease in vitro, a 100-bp window size with a step of 1 bp was selected for the computer analysis. The region of the rDNA sequence analyzed in Fig. 3 includes the r1 fragment plus 50 bp of additional rDNA sequence to each side of the r1 fragment. The additional rDNA sequences were needed to obtain free energy values for the ends of the r1 fragment. The sliding-window analysis was used in the preliminary analysis of the r1 fragment from the rDNA to locate regions of helical instability that represent potential DUEs.

A single-window analysis was used compare the unwinding abilities of wild-type and mutated rDNA *ARS* sequences 3′ to the essential rDNA *ARS* consensus. The single-window analysis required the determination of the essential consensus sequence in order to position the window at a fixed site. A fixed window of 100 bp positioned immediately 3′ to the T-rich strand of the consensus was used for comparison of helical stability in the 3′-flanking region (35). This type of analysis yields a single free energy value for the 100-bp region of each derivative tested. The Thermodyn computer program is written for IBM-compatible computers and can be requested by sending an Internet address to KOWALSKI@VAX2.MED.BUFFALO.EDU.

RESULTS

The rDNA *ARS* colocalizes with a broad easily unwound sequence. Figure 1 shows a map of the *S. cerevisiae* rDNA repeats and illustrates the locations of transcripts in relation to the chromosomal replication origins (circles labeled ORI). The rDNA *ARS* is present in the 2.7-kb *Hin*dIII fragment (r2.7) derived from chromosome XII. Also shown is the location of the 769-bp restriction fragment, r1, that served as the starting point of this analysis. r1 contains 12 9- or 10-bp matches to the 11-bp *ARS* consensus sequence (Fig. 1, boxes). Unlike most *ARS* elements previously characterized, the rDNA *ARS* contains no perfect matches to the *ARS* consensus sequence. On the basis of our model for the minimal essential components of yeast replication origins (34, 35, 48, 49), we reasoned that consensus matches in which the T-rich strand is flanked 3′ by a broad easily unwound sequence represented the likely candidates for the essential consensus in the rDNA *ARS*. Previous results from this laboratory have demonstrated the propensity of yeast *ARS* elements to adopt an unwound structure that is thermodynamically stable in negatively supercoiled plasmids at 37°C, low ionic strength, and neutral pH (49). The stably unwound region displays hypersensitivity to cutting by single-strand-specific nucleases, such as mung bean nuclease or P1 nuclease. Mapping of the mung bean nuclease-hypersensitive region at nucleotide resolution within the r1 restriction fragment (Fig. 2) identified an easily unwound sequence that contained three 10-of-11-bp consensus matches. A region of

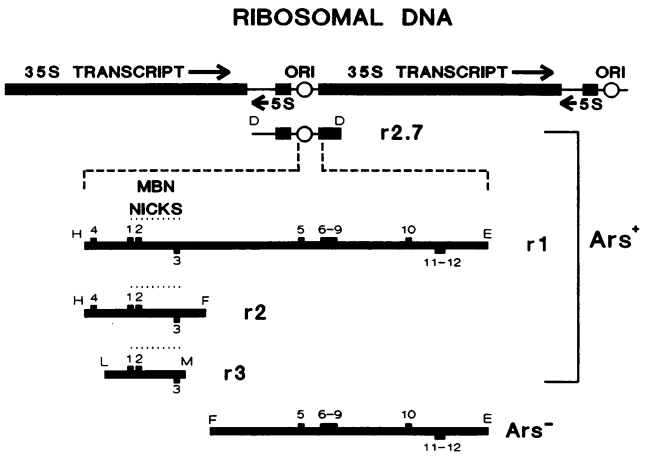

FIG. 1. Map of the repetitive rDNA of *S. cerevisiae*. The DNA sequences coding for the 35S Pol I and the 5S Pol III transcripts (heavy lines) are shown along with the nontranscribed spacer regions (thin lines). Circles represent the region associated with chromosomal origin activity (ORI). The 2.7-kb fragment, r2.7, was derived from a *Hin*dIII digest of genomic DNA. A 769-bp *Hae*III-*Eco*RI fragment, named r1, was derived from a nontranscribed spacer region and a small portion of the DNA encoding the 35S transcript. r1 is the parental *ARS* for those derivatives shown below. *ARS* activity is localized to the r2 (*Hae*III-*Fok*I, 227-bp) and r3 (*Mnl*I-*Mae*III, 139-bp) subfragments (Ars^+). The larger *Fok*I-*Eco*RI subfragment lacks *ARS* activity (Ars^-). The small numbered boxes above and below the restriction fragments mark the positions and strands (above for the top strand, below for the bottom strand) of the near matches (9- and 10-of-11-bp matches) to the T-rich strand of the *ARS* consensus. The dotted line above fragments with *ARS* activity shows the easily unwound region that is nicked by mung bean nuclease (MBN) (see Fig. 2). Restriction enzyme cutting sites: D, *Hin*dIII; E, *Eco*RI; H, *Hae*III; F, *Fok*I; M, *Mae*III; L, *Mnl*I.

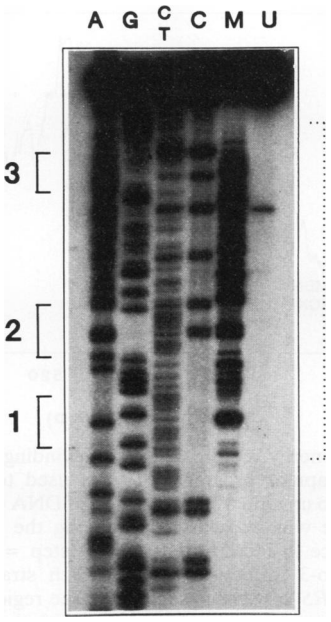

FIG. 2. Sequence level mapping of the mung bean nuclease-hypersensitive region within the rDNA *ARS*. The supercoiled pWEr1 plasmid was reacted with mung bean nuclease to nick the DNA at the easily unwound region. The plasmid was cut at the *Bam*HI sites in the polylinker and in the tetracycline resistance gene. The DNA was dephosphorylated with bacterial alkaline phosphatase and labeled with ^{32}P by using polynucleotide kinase and [γ-^{32}P]ATP. After digestion with *Fok*I, the *Bam*HI-*Fok*I *ARS* fragment containing a single 5′ end label was isolated from an 8% polyacrylamide gel. The sample treated with mung bean nuclease (lane M) and the sample derived from untreated DNA (lane U) were denatured by heating and resolved in an 8% sequencing gel. Reaction products generated by the Maxam-Gilbert method (lanes labeled A, G, $\frac{C}{T}$, and C) were separated in the adjacent lanes. The dotted line indicates the region of DNA (approximately 100 bp) that is hypersensitive to mung bean nuclease. The hypersensitive region includes three 10-of-11-bp matches to the *ARS* consensus sequence that are labeled 1 to 3.

approximately 100 bp that extends from consensus 1 through consensus 3 was specifically nicked by mung bean nuclease.

The site that is hypersensitive to a single-strand-specific nuclease identifies the site in the plasmid DNA sequence with the lowest helical stability (28). Thus, the broad DNA region recognized by mung bean nuclease in the rDNA *ARS* derivative r1 has a low helical stability. DNA helical stability is the free energy difference (ΔG) between native and melted DNA. Helical stability can be reliably calculated for a particular sequence by using experimentally determined thermodynamic properties of nearest-neighbor dinucleotides (4). Rapid assessment of the helical stabilities of all sequences within a given DNA segment is possible by using a recently developed computer program. The Thermodyn program performs a sliding-window analysis and determines the helical stability under the same conditions used to detect the nuclease-hypersensitive site (34). We analyzed the helical stability of the rDNA *ARS* derivative r1 by using a window of 100 bp, corresponding to the approximate breadth of the nuclease-hypersensitive region. As seen in Fig. 3, the region with the lowest helical stability colocalizes with the DNA sequence that is specifically nicked (dotted line) by mung bean nuclease. Thus, both the nuclease hypersensitivity assay and the thermodynamic calculation of helical stability identify the same easily unwound DNA region in the rDNA *ARS*-containing fragment.

Only a small subset of the *ARS* consensus matches present in the r1 fragment colocalizes with the region identified by the nuclease hypersensitivity assay and by the helical-stability analysis (Fig. 2 and 3). A large portion of the nuclease-hypersensitive region (Fig. 2, dotted line) is positioned 3′ to the T-rich strand of each of the three 10-bp matches to the *ARS* consensus sequence. We considered that any of these consensus matches could be essential, since, like the essential consensus sequences in other *ARS* elements examined (34, 35), all three had easily unwound sequences in the 3′ consensus-flanking DNA. Restriction digestions were used to reduce the size of the 769-bp fragment to identify subfragments with *ARS* activity. As suggested from the colocalization of the broad easily unwound region with consensus matches 1, 2, and 3, the smaller *Hae*III-*Fok*I restriction fragment (Fig. 1, r2) and the internal *Mnl*I-*Mae*III fragment (r3) contain the *ARS* activity, while the larger *Fok*I-*Eco*RI fragment has no *ARS* activity (Ars^-).

Deletions in the easily unwound region and in *ARS* consensus matches. We introduced deletions in the *ARS*-containing fragment r3 by taking advantage of the fact that in a negatively supercoiled plasmid, the broad easily unwound region is a specific target for nicking by mung bean nuclease. Linearization and deletion at the nicked site were accomplished by the combined 3′-to-5′ exonuclease and single-

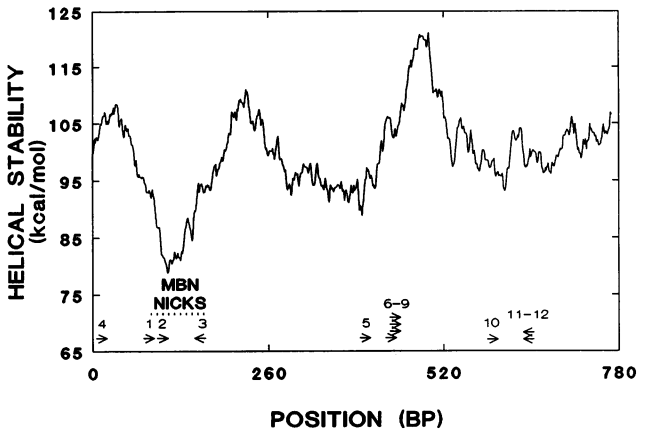

FIG. 3. Free energy requirement for unwinding the rDNA *ARS* sequence. A computer program (34) was used to determine the energy required to unwind a 100-bp region of DNA (window) (1 kcal = 4.184 kJ). The window was moved along the length of the r1 fragment sequence in increments of 1 bp (step = 1). The arrows indicate the 5'-to-3' direction of the T-rich strand of the near matches to the *ARS* consensus sequence. The region of low helical stability (low free energy values) coincides with the dotted line that indicates the region nicked by mung bean nuclease (MBN). A broad region of easily unwound DNA is located 3' to each of the *ARS* consensus matches numbered 1, 2, and 3.

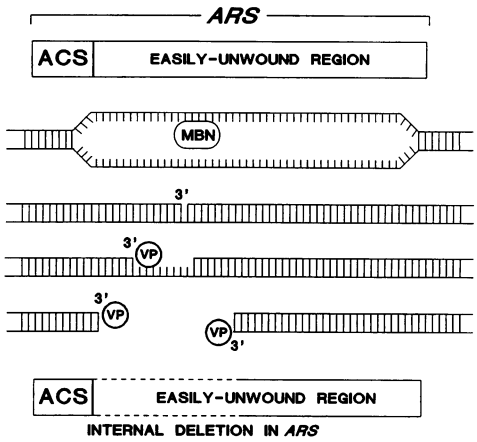

FIG. 4. A method of *ARS* mutagenesis based on localized DNA unwinding. A negatively supercoiled plasmid (pWEr3) unwinds at the rDNA *ARS* insert at neutral pH and low ionic strength at 37°C to relieve superhelical stress. Mung bean nuclease (MBN) introduces a single nick in the unwound region of each molecule (29). Linearization opposite the nicked site and deletion of DNA are achieved by limited digestion with snake venom phosphodiesterase (VP) (38). The venom phosphodiesterase first attacks the nicked plasmid with a 3'-to-5' exonuclease activity. The single-stranded gap that forms is attached by the single-strand-specific endonuclease activity of the venom phosphodiesterase, forming a new 3' OH end. Subsequent 3'-to-5' exonuclease activity and single-strand-specific endonuclease activity of venom phosphodiesterase generates bidirectional deletions. The linear DNA is then circularized by joining the deletion endpoints by using ligase to create an internal deletion in the *ARS*. ACS represents the essential 11-bp *ARS* consensus sequence. The easily unwound region corresponds primarily to the broad region (ca. 100 bp) directly 3' to the T-rich strand of the ACS, but it also can include the ACS.

strand-specific endonuclease activities of snake venom phosphodiesterase, as outlined in Fig. 4. Although the intent was to create internal deletions, occasionally the exonuclease activity of the venom phosphodiesterase created the equivalent of external deletions because of extensive digestion. Additional external deletions near consensus 3 were made by using BAL 31 exonuclease because the mutagenesis by mung bean nuclease and venom phosphodiesterase described above did not yield any small deletions in that region.

Figure 5 shows a portion of the *ARS*-containing r3 derivative as well as the sequences of the deletion derivatives and their *ARS* phenotypes. Internal deletions that removed consensus 1 and/or 2 abolished the transforming activity of the *ARS*. Internal deletions between consensus sequences 2 and 3 are tolerated; however, yeast cells transformed with one such derivative, r311, grew slowly under conditions that select for plasmid retention.

External 3' deletions into consensus 3 that reduce its similarity to the *ARS* consensus do not affect transforming activity (r6.5, r6). Deletions that proceeded past consensus 3 displayed no transforming activity in YIp5 (r346 and r7). An external deletion from the 5' side shows that the sequence up to 9 bp 5' to consensus 1 is dispensable (r8). However, 5' deletions that proceed through consensus 1 abolish *ARS* activity (r365 and r366).

Double point mutations in matches to the *ARS* consensus sequence. Deletion mutagenesis did not resolve the identity of the essential consensus sequence. To identify the essential consensus sequence, we introduced point mutations by utilizing a PCR-based approach. A primer that overlaps consensus 1 and an external vector primer were used to create r8, the parental *ARS* derivative used to construct the consensus mutations described below (the full sequence of r8 is shown in Fig. 5). Cells were transformed at high frequency with plasmids containing the 107-bp r8 derivative and grew at the same rate as transformants containing larger rDNA *ARS* derivatives. Double point mutations consisting of a change from TT to AA at the 9th and 10th positions of consensus sequences 1, 2, and 3 were created by various combinations of PCRs (Table 1). The derivative r10 contains the mutated consensus sequence GTTTATGTAAT. The double point mutation of consensus sequence 1 eliminated the ability of the rDNA *ARS* fragment to transform cells when tested in the YIp5 vector. In contrast, mutations in consensus sequences 2 and 3 were tolerated; however, a reduction in colony growth rate was observed for cells transformed by the r11 and r14 derivatives. The data indicate that consensus 1, GTTTATGTTTT, is essential for the function of the rDNA *ARS* and that consensus sequences 2 and 3 are nonessential.

Mitotic stability of rDNA *ARS* deletion derivatives. To obtain quantitative data regarding their replicative functions, a subset of rDNA *ARS* derivatives was subcloned into the centromere-containing pVHA vector, transformed into the YPH3 strain of *S. cerevisiae*, and tested for mitotic stability under selection for the plasmid. The mitotic stability corresponds to the percentage of cells in the population that contain the plasmid. The higher the mitotic stability is, the greater is the replication efficiency mediated by the *ARS* element in the plasmid. The mitotic stabilities of the 2.7-kb r2.7 derivative (41% ± 17%) and the 139-bp r3 derivative (54% ± 9%) are similar given the error associated with the assay. Thus, sequences required for full *ARS* efficiency are contained within the 139-bp r3 derivative.

Internal deletions in the r3 derivative that remove se-

INTERNAL DELETIONS OF THE r3 FRAGMENT

DERIVATIVE	CONSENSUS 1	CONSENSUS 2	CONSENSUS 3	Ars
r3	TGATTTTGAT TGTTTATGTT TTGTGTGATG	ATTTTACATT TTTGCATAGT ATTAGGTAGT CAGATGAAAG ATGAATAGAC ATAGGAGTAA	GAAAACATAG AATAGTTACg	+
r399	TGATTTTG-- ---------- ----GTGATG	ATTTTACATT TTTGCATAGT ATTAGGTAGT CAGATGAAAG ATGAATAGAC ATAGGAGTAA	GAAAACATAG AATAGTTACg	-
r344	TGATTTTGAT TGT------- ----------	---------- ---------- ATTAGGTAGT CAGATGAAAG ATGAATAGAC ATAGGAGTAA	GAAAACATAG AATAGTTACg	-
r386	TGATTTTGAT TGTTTATGTT TTGTGTG---	---------- ---------- ---------T CAGATGAAAG ATGAATAGAC ATAGGAGTAA	GAAAACATAG AATAGTTACg	-
r3131	TGATTTTGAT TGTTTATGTT TTGTGTGA--	---------- ---------- ----GGTAGT CAGATGAAAG ATGAATAGAC ATAGGAGTAA	GAAAACATAG AATAGTTACg	-
r397	TGATTTTGAT TGTTTATGTT TTGTGTGATG	ATTTTACAT- ---------- ---------- CAGATGAAAG ATGAATAGAC ATAGGAGTAA	GAAAACATAG AATAGTTACg	-
r389	TGATTTTGAT TGTTTATGTT TTGTGTGATG	ATTTTACATT TTTGCAT--- ---------- --GATGAAAG ATGAATAGAC ATAGGAGTAA	GAAAACATAG AATAGTTACg	+
r311	TGATTTTGAT TGTTTATGTT TTGTGTGATG	ATTTTACATT TTTGCATAGT ATTA------ ---------- ---------- ---------A	GAAAACATAG AATAGTTACg	+

EXTERNAL DELETIONS OF THE r3 FRAGMENT

DERIVATIVE	CONSENSUS 1	CONSENSUS 2	CONSENSUS 3	Ars
r313	TGATTTTGAT TGTTTAgggc gagctcgaat	tcttgaagac gaaagggcct cgtgatacgc ctatttttat aggttaatgt catgataata	atggtttctt agacgtcagg	-
r379	TGATTTTGAT TGTTTATGTT TTGTGTGAgg	gcgagctcga attcttgaag acgaaagggc ctcgtgatac gcctatttt ataggttaat	gtcatgataa taatggtttc	-
r314	TGATTTTGAT TGTTTATGTT TTGTGTGATG	ATTTTACATT TTTGCAgctc gaattcttga agacgaaagg gcctcgtgat acgcctattt	ttataggtta atgtcatgat	-
r346	TGATTTTGAT TGTTTATGTT TTGTGTGATG	ATTTTACATT TTTGCATAGT ATgggcgagc tcgaattctt gaagacgaaa gggcctcgtg	atacgcctat ttttataggt	-
r7	TGATTTTGAT TGTTTATGTT TTGTGTGATG	ATTTTACATT TTTGCATAGT ATTAGGTAGT CAGATGAAAG ATGAATAGAC ATAGGAGgaa	ttcttgaaga cgaaagggcc	-
r6.5	TGATTTTGAT TGTTTATGTT TTGTGTGATG	ATTTTACATT TTTGCATAGT ATTAGGTAGT CAGATGAAAG ATGAATAGAC ATAGGAGTAA	GAAAACAcga attcttgaag	+
r6	TGATTTTGAT TGTTTATGTT TTGTGTGATG	ATTTTACATT TTTGCATAGT ATTAGGTAGT CAGATGAAAG ATGAATAGAC ATAGGAGTAA	GAAAACATAc gaattcttga	+
r8	ccATTTTGAT TGTTTATGTT TTGTGTGATG	ATTTTACATT TTTGCATAGT ATTAGGTAGT CAGATGAAAG ATGAATAGAC ATAGGAGTAA	GAAAACATAG AATAGTTACg	+
r365	cgatgcgtcc ggcgtagagg aatccccagt	ATTTTACATT TTTGCATAGT ATTAGGTAGT CAGATGAAAG ATGAATAGAC ATAGGAGTAA	GAAAACATAG AATAGTTACg	-
r366	ggcgccggtg atgccggcca cgatgcgtcc	ggcgtagagg aatccccagt ggTAGGTAGT CAGATGAAAG ATGAATAGAC ATAGGAGTAA	GAAAACATAG AATAGTTACg	-

FIG. 5. Relationship between mutations in the rDNA *ARS* and transforming ability. The various mutations made in the r3 fragment were subcloned into the YIp5 vector and transformed into *S. cerevisiae* under conditions that select for the expression of the plasmid-borne *URA3* gene. The left 30 bases (AGTGGACAGA GGAAAAGGTG CGGAAATGGC) of the r3 fragment and the internal and external derivatives (except r8, r365, and r366) have been omitted from this figure. Internal deletion mutations are shown by dashes, and the vector sequences that replace the external deletions are in lowercase. The Ars column indicates high-frequency transformation of the plasmids. +, plasmid that transformed cells at high frequency (>1,000 colonies per μg of plasmid DNA); −, plasmid that was unable to transform cells with high frequency (<10 colonies per μg of plasmid DNA).

quences between essential consensus 1 and nonessential consensus 3 (r386, r3131, r397, r389, and r311) negatively affect mitotic stability (Table 2). The deletion of sequences from the 3′ side of the T-rich strand of the essential consensus 1 affects mitotic stability when the deletion endpoint proceeds through consensus 3, as seen in the examples of the 3′ external deletions r346 and r7. Although Ars$^-$ in YIp5, the r7 derivative was stabilized sufficiently in the centromere-containing pVHA plasmid such that weak *ARS* function is detectable. The reduction of nonessential consensus 3 from a 10-of-11-bp match to a 9-of-11-bp match in conjunction with an alteration of the adjacent 3′ sequence (r6) does not cause a significant change in mitotic stability.

The ease of DNA unwinding is a determinant of rDNA *ARS* function. Sequences required for rDNA *ARS* function (Fig. 5; Tables 1 and 2) colocalize with a broad region of easily unwound DNA (Fig. 1 to 3). In the H4 *ARS* and *ARS307*, a broad easily unwound sequence 3′ to the essential consensus is required for *ARS* function (34, 48). The *cis*-acting sequence whose helical instability is required for replication origin function is termed a DUE (27). DUEs are identified by the effects of mutations on helical instability as determined by (i) hypersensitivity to single-strand-specific nucleases (27, 48), (ii) thermodynamically stable DNA unwinding in two-dimensional gel electrophoresis of plasmid topoisomers (27), and (iii) the thermodynamic properties of the nearest-neigh-

TABLE 1. Identification of the essential consensus sequence in the rDNA *ARS*[a]

Derivative	Consensus 1	Consensus 2	Consensus 3	Ars
r8	GTTTATGTTTT	TTTTACATTTT	TTCTATGTTTT	+
r10	--------AA-	----------	----------	−
r11	----------	--------AA-	----------	+
r12	----------	----------	--------AA-	+
r14	----------	--------AA-	--------AA-	+

[a] The parental rDNA *ARS* fragment used for the generation of point mutations was the 107-bp r8 derivative. Double point mutations (TT to AA) were introduced at the 9th and 10th positions of each 11-bp consensus sequence. Plasmids containing these rDNA *ARS* derivatives were scored for high-frequency transformation (Ars$^+$ or Ars$^-$) as for Fig. 5. The essential consensus sequence, GTTTATGTTTT, that is inactivated by the double point mutation shown for derivative r10 corresponds to positions 1855 to 1865 in the published sequence of this region (43). Derivatives with mutations in the third consensus (r12 and r14) also include a deletion of sequences 3′ to the third consensus.

TABLE 2. Mitotic stability of rDNA *ARS* plasmids[a]

Derivative	Mitotic stability (%)
r3	54 ± 9
r6	49 ± 7
r389	38 ± 5
r311	19 ± 9
r7	8 ± 6
r346	<1
r386	<1
r3131	<1
r397	<1

[a] Mitotic stability (percentage of plasmid-containing cells; mean ± standard deviation) was determined under conditions that selected for retention of the *URA3* gene carried on the centromere-containing pVHA vector. Derivatives whose mitotic stability is given as <1% were Ars$^-$ in the high-frequency transformation assay.

TABLE 3. Effects of double point mutations in the easily unwound region on mitotic stability and helical stability[a]

Derivative	Mitotic stability (%)	Helical stability (ΔG, kcal[b]/mol)
r8	70 ± 15	88.7
r11	19 ± 12	87.5
r12	50 ± 8	86.6
r14	15 ± 5	85.4

[a] The determinations of mitotic stability (percentage of plasmid-containing cells; mean ± standard deviation) and helical stability were the same as in Table 2 and Fig. 6. The helical stability was determined for the 100-bp sequence directly 3′ to the T-rich strand of essential consensus 1. The *ARS* consensus matches present in r8 and in the mutated derivatives are shown in Fig. 1.

[b] 1 kcal = 4.184 kJ.

bor dinucleotides that constitute the DNA region of interest (34). The latter approach is particularly advantageous because, unlike other methods, it is free from interference by more easily unwound sequences present elsewhere on a plasmid (35) and it allows quantitation of DNA helical stability. DNA helical stability determined by this approach accurately reflects the experimentally determined helical stability (4) and accurately predicts the site that is hypersensitive to single-strand-specific nuclease in supercoiled DNA (34, 35) (Fig. 3).

The r3 fragment and all of its derivatives shown in Fig. 5 that retained an intact essential consensus (consensus 1) and a common 5′-flanking sequence were analyzed for a correlation between helical stability and mitotic stability. All of the r3 derivatives shown were cloned at the same location and in the same orientation in order to normalize any effects of vector DNA context. In Fig. 6, the DNA helical stability of the 100-bp 3′-flanking region is plotted against the mitotic stability values obtained for r3 and its deletion derivatives. An inverse relationship between helical stability and mitotic stability is seen. The relationship exhibits a high degree of correlation (correlation coefficient, −0.91). The data show that a low helical stability (i.e., easily unwound DNA) favors a high mitotic stability (i.e., efficient plasmid replication). Compared with the r3 derivative (wild type), mutations that increase helical stability are less efficient at or incapable of stabilizing the plasmid through multiple cell divisions. Comparison of the data in Fig. 6 with the DNA sequences of the rDNA *ARS* derivatives (Fig. 5) shows that, collectively, the mutations analyzed span the broad region 3′ to the essential consensus (consensus 1) that is required for *ARS* function. Our findings suggest that the intrinsic ease of DNA unwinding in the broad 3′-flanking region of the rDNA *ARS* derivatives is a determinant of autonomous replication.

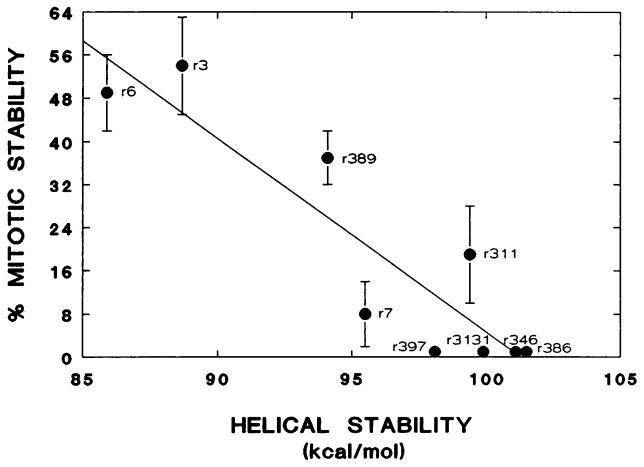

FIG. 6. Correlation between free energy required to unwind the DUE of the rDNA *ARS* and mitotic stability. The mitotic-stability values (mean and standard deviation) for deletion derivatives of r3 are plotted versus the helical stability (free energy in kilocalories per mole [1 kcal = 4.184 kJ]) required to unwind the 100 bp (fixed window) positioned 3′ to the end of the T-rich strand of the essential consensus sequence. The derivatives analyzed in the plot are listed in Table 2. The straight line represents the best fit to the data and was generated by the method of least squares. A correlation coefficient of −0.91 was obtained for this analysis.

Specific point mutations in the easily unwound region affect *ARS* activity without increasing helical stability. The r8 derivative (Fig. 5) and the double point mutations in the essential and nonessential consensus matches (Table 1) were assessed for the mitotic stability that they provide to the pVHA vector. r8 was derived by deleting 32 bp from the 5′ end of r3. r8 contains the essential consensus, the 3′ region of easily unwound DNA, and 9 bp 5′ to the essential consensus. Two nonessential matches to the *ARS* consensus are also present in the easily unwound region. The 107-bp r8 derivative is a minimal fragment that retains full *ARS* function in mitotic-stability assays (Table 3). The mitotic stability of r10 (double point mutation in essential consensus 1) could not be measured because it did not give rise to *URA3*$^+$ colonies. The double point mutation of consensus 2 (r11) and the combination of double point mutations at the nonessential consensus 2 and 3 sequences (r14) resulted in 3.7- and 4.7-fold reductions in mitotic stability, respectively (Table 3). The mitotic stability of the consensus 3 mutation alone (r12) was not significantly different from that of the parental r8 derivative. The results show that residues within nonessential consensus 2 are important for the replication efficiency of the rDNA *ARS*.

Consensus sequences 2 and 3 reside within the easily unwound region that flanks the essential consensus. To assess the effects of double point mutations on the ease of unwinding, the DNA helical stability for the 100-bp region 3′ to the essential consensus was determined for r8 and the mutated derivatives (Table 3). None of the double point mutations increase the helical stability above that of the parental r8 derivative. Nonetheless, derivatives r11 and r14

TABLE 4. *ARS* elements that require an imperfect match to the consensus[a]

ARS element	Essential consensus	Match
rDNA *ARS*	g T T T A T G T T T T	10
HO *ARS*	T T T a A T A T T T T	10
ARS121	T g T T t T G T T T A	9
ACS	A T T T A T G T T T A	11
	T A T	

[a] Mismatches to the essential 11-bp *ARS* consensus sequence (ACS) are indicated by lowercase letters. The essential consensus sequences of the HO *ARS* (24) and *ARS121* (52) have been reported by others.

exhibit reduced mitotic stability compared with the r8 derivative. Thus, point mutations in nonessential consensus 2 reduce the efficiency of the rDNA *ARS* without increasing DNA helical stability.

Sequence conservation of *ARS* components among the rDNA repeats. Identification of a minimal rDNA sequence required for efficient *ARS* function allowed us to address whether sequence polymorphisms in the chromosomal rDNA repeats might account for the low frequency of origin usage. Polymorphisms in the region associated with rDNA *ARS* function could explain why fewer than one-third of the *ARS*s present in rDNA repeats in the chromosome are actually used in a given S phase. To test this possibility, we isolated four independent rDNA clones from genomic DNA without selection for *ARS* activity and analyzed the sequence through the region that included the essential *ARS* consensus and the required 3′-flanking region. The DNA sequence of the four clones over this region is identical to that of the rDNA *ARS* clone (r1) which was used in the mutational analysis (see "Sequence analysis" in Materials and Methods). Like r1, the four independent rDNA clones possess *ARS* activity. All five rDNA clones are equal in their ability to transform *S. cerevisiae* and are similar in replication efficiency as assessed by the growth rate of the resulting colonies under selective conditions. We conclude that the low-frequency usage of origins present in the rDNA repeats is not due to polymorphisms within the sequences that we find to contribute to rDNA *ARS* function.

DISCUSSION

We have identified a specific DNA sequence that is essential for the function of the rDNA *ARS*. Like other *S. cerevisiae ARS* elements that have been examined by mutational analysis, the rDNA *ARS* requires one match to the 11-bp *ARS* consensus sequence. However, unlike the essential consensus demonstrated in most *ARS* elements, the essential consensus in the rDNA *ARS* is an imperfect match. The requirement for a 10-of-11-bp match, GTTTATGTTTT, is demonstrated by the unique sensitivity of this match, and not other 10-of-11-bp matches, to point mutations (Table 1). Only two other *ARS* elements characterized to date have been shown to require imperfect matches to the *ARS* consensus sequence: the HO *ARS* (24) and *ARS121* (52). A comparison of the essential consensus sequence in each of these *ARS*s with that in the rDNA *ARS* is shown in Table 4. No two of the essential consensus sequences are the same. Positions 6 through 11 of all the essential sequences match the *ARS* consensus; however, mismatches to the consensus occur in positions 1, 2, 4, and 5.

The G residue at position 1 is the mismatched position in the essential consensus of the rDNA *ARS*. In the essential consensus of *ARS307* (ATTTATGTTTT), a mutation from A to G at position 1 creates a sequence that is identical to the essential consensus in the rDNA *ARS*. The G mutation in *ARS307* causes a reduction in replication efficiency (50). Thus, the G mismatch at the first position of the essential consensus in the rDNA *ARS* may provide an explanation for the inefficient *ARS* activity observed here and elsewhere (26). The G mismatch is present in five independent rDNA *ARS* clones that we sequenced, suggesting a low frequency or absence of polymorphisms at position 1 in the essential consensus among the *ARS*s present in the rDNA repeats in chromosome XII. It is possible that the G mismatch in the *ARS* consensus also weakens origin activity in a chromosome and accounts for the low frequency of origin usage observed at the rDNA locus (6, 30, 41, 53). The G mismatch may result in a relatively weak interaction with the initiation factors that recognize the *ARS* consensus sequence.

In addition to the essential consensus sequence, two consensus matches that are not essential are present in the wild-type rDNA *ARS* derivative r8 (Table 1). The three consensus matches are very similar in sequence, and each matches the *ARS* consensus at 10 of 11 positions (Table 1). Consensus 2 is a perfect match to the revised *ARS* consensus sequence proposed by Van Houten and Newlon, which includes a C at position 6 (50). For this reason, we initially considered consensus 2 to be the best candidate for an essential consensus. Another reason to favor consensus 2 is that the base variations present in the other rDNA consensus matches (consensus 1, G at position 1; consensus 3, C at position 3) (Table 1) cause an inhibition of *ARS* activity when introduced into the *ARS307* consensus. However, consensus 2 is clearly not essential, since the rDNA *ARS* retained transforming activity after the mutation of positions 9 and 10 of consensus 2 (Table 1). Essential consensus sequences in other *ARS* elements are absolutely conserved at positions 9 and 10 (50) (Table 4). In the rDNA *ARS*, only in consensus 1 do mutations at these positions lead to inactivation of the *ARS* (Table 1). A DNA sequence search for matches to the revised *ARS* consensus (50), which is present in the current release (6.0) of the transcription factor data base (18), finds nonessential consensus 2 and misses essential consensus 1 of the rDNA *ARS*. A more complete understanding of the DNA sequence requirements of the *ARS* consensus will require the identification of the essential consensus in additional *ARS* elements as well as point mutation analysis of more essential consensus sequences.

Our deletion analysis revealed that a minimal rDNA *ARS* with full *ARS* efficiency is contained in a 107-bp DNA fragment (r8 derivative). Sequences up to 9 bp 5′ of the essential consensus are dispensable for *ARS* activity (Fig. 5, r8) and for full *ARS* efficiency (Table 3). A broad region 3′ to the T-rich strand of the essential consensus is required for full *ARS* efficiency. The precise length of the required 3′-flanking region is difficult to assess, since, as shown for other *ARS* elements (1, 9, 24, 48), contiguous vector sequences can influence *ARS* function.

A broad, easily unwound region in the rDNA *ARS* was located by nucleotide level mapping of nicks introduced by mung bean nuclease. The same DNA region was identified by analysis of DNA helical stability based on known thermodynamic properties of the nearest-neighbor dinucleotides (4) and automated by a computer program (34). These findings, in conjunction with knowledge of the positions of the *ARS* consensus matches, directed us to the region of the rDNA fragment that is essential for *ARS* activity (Fig. 1 to

3). A role for the ease of DNA unwinding in rDNA *ARS* function is suggested by the inverse correlation of DNA helical stability with the mitotic stability of the deletion mutations in the broad region directly 3′ to the essential consensus sequence (Fig. 6). The series of deletion mutations that alter different portions of the broad 3′-flanking sequence has an effect of both raising the helical stability and reducing rDNA *ARS* efficiency. Mutated *ARS* derivatives with the highest helical stabilities are inactive (Fig. 6). These findings suggest that the ease of unwinding the broad 3′-flanking sequence is a determinant of rDNA *ARS* function. Analysis of a similar series of mutations has indicated a requirement for DNA helical instability in the 3′-flanking sequence of *ARS307* and the H4 *ARS* (34, 48). The DNA sequence whose helical instability is required for replication origin function is termed a DUE (27).

The essential consensus in the rDNA *ARS* is also easily unwound as indicated by its susceptibility to nicking by single-strand-specific nuclease in our assay (Fig. 2). The same observation has been made for other *ARS* elements (34, 47, 48). Also, thermodynamic analysis of the DNA sequence indicates that the essential consensus sequence of *ARS307*, as well as consensus near matches, exhibits helical instability (34). It is possible that the helical instability of the essential consensus contributes to its biological function; however, the essential consensus does not appear to be a part of the DUE. The DUE in *ARS* elements is defined by mutations that stabilize the DNA helix and reduce *ARS* activity (34, 48). In contrast to a DUE, the essential consensus can be completely inactivated by point mutations which do not stabilize the DNA helix. For example, the substitution of AA for TT in the essential consensus of the rDNA *ARS* (Table 1, consensus 1) destabilizes, rather than stabilizes, the DNA helix in the essential consensus ($\Delta G_{r10} - \Delta G_{r8} = -1.2$ kcal [ca. -5.0 kJ/mol). Nonetheless, the AA substitution inactivates *ARS* function. Thus, the essential consensus sequence in the rDNA *ARS* and other *ARS*s exhibits a response to mutations that is clearly distinct from that of a DUE.

It is possible that certain mutations in the broad 3′-flanking region reduce *ARS* function by altering a *cis*-acting element that is distinct from, but overlaps, the DUE. If this is true, then we expect that the helical stability of such mutations would not correlate with *ARS* efficiency. The double point mutations r11 and r14 fall in this category. The mutations decrease *ARS* efficiency (Table 3) by changing a TT to AA in nonessential consensus 2 (Table 1); however, the helical stability of each mutation is not increased relative to that of the parental r8 derivative. In fact, each of the consensus 2 mutation derivatives has a slightly lower helical stability than the wild-type parent (r8). A role for the specific sequence of consensus near matches has been proposed (36). However, in the H4 *ARS*, the specific sequence of consensus near matches appears unimportant, since simultaneous point disruption of all nonessential consensus matches in the required 3′-flanking region has no effect on plasmid replication efficiency (20). These point mutations occur in the DUE of the H4 *ARS* (48, 49) and have an insignificant effect on DNA helical instability (34). Additional experiments on the rDNA *ARS* are necessary to define the extent of the element revealed by double point mutations in consensus 2.

Linker substitution studies on *ARS1* and *ARS121* indicate that short sequence elements 3′ to the essential consensus contribute to *ARS* function (31, 52). In *ARS1*, the best characterized of these elements is a consensus binding site for ABF1. There is no match to the ABF1 consensus (8, 12) in the minimal rDNA *ARS* (r8 derivative). The precise DNA sequence requirements of the other short sequence elements that are present in the required 3′-flanking sequences of *ARS1* and *ARS121* are not yet defined by point mutations. Thus, it is not yet possible to search for the presence of these elements in the sequence of the rDNA *ARS*. It is still unclear whether linker substitutions in *ARS1* and *ARS121* identify specific sequence elements, a part of a DUE, or both. The requirement for a DUE has not been examined in *ARS1* and *ARS121*, although the 100-bp 3′-flanking region in each has a low helical stability (35). The linkers used in the substitution studies on *ARS1* and *ARS121* are G+C rich and may reduce replication efficiency by increasing the helical stability of the easily unwound 3′-flanking sequence (33).

The DUE of the rDNA *ARS* is positioned 3′ to the T-rich strand of the essential consensus sequence, as is the case for other *ARS* elements in which a DUE has been implicated by mutational analysis (34, 48). The data presented here indicate that the rDNA *ARS* provides yet another example of the conservation of this genetic organization at a yeast replication origin. A general model proposed for yeast replication origins suggests that origin recognition by initiator proteins at the essential consensus sequence is followed by unwinding of the DNA helix 3′ to the *ARS* consensus within the DUE (48, 49). The initial unwinding of sequences 3′, but not 5′, to the T-rich strand of the consensus is consistent with the conserved orientation of the DUE relative to the asymmetric consensus sequence. Once unwound, the DUE is the proposed site for other replication proteins, such as a helicase(s), single-strand binding proteins, primase, and polymerases, to enter the origin and begin the process of DNA synthesis. The motif of a DUE flanking a binding site for the initiation complex is conserved among diverse bacterial species and may reflect a general theme for the organization of bidirectional replication origins (27).

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REFERENCES

1. **Bouton, A. H., and M. M. Smith.** 1986. Fine-structure analysis of the DNA sequence requirements for autonomous replication of *Saccharomyces cerevisiae* plasmids. Mol. Cell. Biol. **6**:2354–2363.
2. **Bramhill, D., and A. Kornberg.** 1988. Duplex opening by dnaA protein at novel sequences in initiation of replication at the origin of the *E. coli* chromosome. Cell **52**:743–755.
3. **Brand, A. H., G. Micklem, and K. Nasmyth.** 1987. A yeast silencer contains sequences that can promote autonomous plasmid replication and transcriptional activation. Cell **51**:709–719.
4. **Breslauer, K. J., R. Frank, H. Blocker, and L. A. Marky.** 1986. Predicting DNA duplex stability from the base sequence. Proc. Natl. Acad. Sci. USA **83**:3746–3750.
5. **Brewer, B. J., and W. L. Fangman.** 1987. The localization of replication origins on *ARS* plasmids in *S. cerevisiae*. Cell **51**:463–471.
6. **Brewer, B. J., and W. L. Fangman.** 1988. A replication fork barrier at the 3′ end of yeast ribosomal DNA genes. Cell **55**:637–643.
7. **Broach, J. R., Y.-Y. Li, J. Feldman, M. Jayaram, J. Abraham, K. A. Nasmyth, and J. B. Hicks.** 1983. Localization and sequence analysis of yeast origins of DNA replication. Cold Spring Harbor Symp. Quant. Biol. **47**:1165–1173.
8. **Buchman, A. R., W. J. Kimmerly, J. Rine, and R. D. Kornberg.** 1988. Two DNA-binding factors recognize specific sequences at

silencers, upstream activating sequences, autonomously replicating sequences, and telomeres in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8**:210–225.

9. **Celniker, S. E., K. Sweder, F. Srienc, J. E. Baily, and J. L. Campbell.** 1984. Deletion mutations affecting autonomously replicating sequence *ARS1* of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **4**:2455–2466.
10. **Deshpande, A. M., and C. S. Newlon.** 1992. The *ARS* consensus sequence is required for chromosomal origin function in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **12**:4305–4313.
11. **Diffley, J. F. X., and B. Stillman.** 1988. Purification of a yeast protein that binds to origins of DNA replication and a transcriptional silencer. Proc. Natl. Acad. Sci. USA **85**:2120–2124.
12. **Dorsman, J. C., A. Gozdzicka-Jozefiak, W. C. van Heeswijk, and L. A. Grivell.** 1991. Multi-functional DNA proteins in yeast: the factors GFI and GFII are identical to the *ARS*-binding factor ABF1 and the centromere-binding factor CPFI respectively. Yeast **7**:401–412.
13. **Dubey, D. D., L. R. Davis, S. A. Greenfeder, L. Y. Ong, J. Zhu, J. R. Broach, C. S. Newlon, and J. Huberman.** 1991. Evidence suggesting that the *ARS* elements associated with silencers of the yeast mating type locus HML do not function as chromosomal replication origins. Mol. Cell. Biol. **11**:5346–5355.
14. **Fangman, W. L., and B. J. Brewer.** 1991. Activation of replication origins within yeast chromosomes. Annu. Rev. Cell Biol. **7**:375–402.
15. **Ferguson, B. M., B. J. Brewer, A. E. Reynolds, and W. L. Fangman.** 1991. A yeast origin of replication is activated late in S phase. Cell **65**:507–515.
16. **Francesconi, S. C., and S. Eisenberg.** 1991. The multifunctional protein OBF1 is phosphorylated on serine and threonine residues in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **88**:4089–4093.
17. **Geitz, D., A. St. Jean, R. Woods, and R. H. Schiestl.** 1992. Improved method for high-efficiency transformation of intact yeast cells. Nucleic Acids Res. **20**:1425.
18. **Ghosh, D.** 1992. TFD: the transcription factor data base. Nucleic Acids Res. **205**:2091–2093.
19. **Heiter, P., C. Mann, M. Snyder, and R. W. Davis.** 1985. Mitotic stability of yeast chromosomes: a colony color assay that measures nondisjunction and chromosome loss. Cell **40**:381–392.
20. **Holmes, S. G., and M. M. Smith.** 1989. Interaction of the H4 autonomously replicating sequence core consensus sequence and its 3′-flanking domain. Mol. Cell. Biol. **9**:5464–5472.
21. **Hsiao, C.-L., and J. Carbon.** 1979. High-frequency transformation of yeast by plasmids containing the cloned yeast *Arg-4* gene. Proc. Natl. Acad. Sci. USA **76**:3829–3833.
22. **Huberman, J. A., L. D. Spotila, K. A. Nawotka, S. M. El-Assouli, and L. R. Davis.** 1987. The *in vivo* replication origin of the yeast two micron plasmid. Cell **51**:473–481.
23. **Huberman, J. A., J. Zhu, L. R. Davis, and C. S. Newlon.** 1988. Close association of a DNA replication origin and an *ARS* element on chromosome III of the yeast, *Saccharomyces cerevisiae*. Nucleic Acids Res. **16**:6373–6384.
24. **Kearsey, S.** 1984. Structural requirements for the function of a yeast chromosomal replicator. Cell **37**:299–307.
25. **Koshland, D., J. C. Kent, and L. H. Hartwell.** 1985. Genetic analysis of the mitotic transmission of minichromosomes. Cell **40**:393–403.
26. **Kouprina, N. Y., and V. L. Larionov.** 1983. The study of a rDNA replicator in *Saccharomyces*. Curr. Genet. **7**:433–438.
27. **Kowalski, D., and M. J. Eddy.** 1989. The DNA unwinding element: a novel, *cis*-acting component that facilitates opening of the *Escherichia coli* replication origin. EMBO J. **8**:4335–4344.
28. **Kowalski, D., D. A. Natale, and M. J. Eddy.** 1988. Stable DNA unwinding, not "breathing" accounts for single-strand-specific nuclease hypersensitivity of specific A+T-rich sequences. Proc. Natl. Acad. Sci. USA **85**:9464–9468.
29. **Kowalski, D., and J. P. Sanford.** 1982. Action of mung bean nuclease on supercoiled PM2 DNA. J. Biol. Chem. **257**:7820–7825.
30. **Linskens, M. H. K., and J. A. Huberman.** 1988. Organization of replication of ribosomal DNA in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8**:4927–4935.
31. **Marahrens, Y., and B. Stillman.** 1992. A yeast chromosomal origin of DNA replication defined by multiple functional elements. Science **255**:817–823.
32. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
33. **Natale, D. A., and D. Kowalski.** Unpublished data.
34. **Natale, D. A., A. E. Schubert, and D. Kowalski.** 1992. DNA helical stability accounts for mutational defects in a yeast replication origin. Proc. Natl. Acad. Sci. USA **89**:2654–2658.
35. **Natale, D. A., R. M. Umek, and D. Kowalski.** 1993. Ease of DNA unwinding is a conserved property of yeast replication origins. Nucleic Acids Res. **21**:555–560.
36. **Palzkill, T. G., and C. S. Newlon.** 1988. A yeast replication origin consists of multiple copies of a small conserved sequence. Cell **53**:441–450.
37. **Petes, T.** 1979. Yeast ribosomal DNA genes are located on chromosome XII. Proc. Natl. Acad. Sci. USA **76**:410–414.
38. **Pritchard, A. E., D. Kowalski, and M. Laskowski, Sr.** 1977. An endonuclease activity of venom phosphodiesterase specific for single-stranded and superhelical DNA. J. Biol. Chem. **252**:8652–8659.
39. **Rivier, D. H., and J. Rine.** 1992. An origin of replication and a transcriptional silencer require a common element. Science **256**:659–663.
40. **Rose, M. D., F. Winston, and P. Heiter.** 1990. Methods in yeast genetics: a laboratory course manual, p. 177–179. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
41. **Saffer, L. D., and O. L. Miller.** 1986. Electron microscopic study of *Saccharomyces cerevisiae* rDNA chromatin replication. Mol. Cell. Biol. **6**:1148–1157.
42. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
43. **Skryabin, K. G., M. A. Eldarov, V. L. Larionov, A. A. Bayev, J. Klootwijk, V. C. H. F. de Regt, G. M. Veldman, R. J. Planta, O. I. Georgiev, and A. A. Hajiolov.** 1984. Structure and function of the nontranscribed spacer regions of yeast rDNA. Nucleic Acids Res. **12**:2955–2968.
44. **Stinchcomb, D. T., K. Struhl, and R. W. Davis.** 1979. Isolation and characterization of a yeast chromosomal replicator. Nature (London) **282**:39–43.
45. **Struhl, K., D. T. Stinchcomb, S. Scherer, and R. W. Davis.** 1979. High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proc. Natl. Acad. Sci. USA **76**:1035–1039.
46. **Sweder, K. S., P. R. Rhode, and J. L. Campbell.** 1988. Purification and characterization of proteins that bind to yeast *ARS*s. J. Biol. Chem. **263**:17270–17277.
47. **Umek, R. M., and D. Kowalski.** 1987. Yeast regulatory sequences preferentially adopt a non-B conformation in supercoiled DNA. Nucleic Acids Res. **15**:4467–4480.
48. **Umek, R. M., and D. Kowalski.** 1988. The ease of DNA unwinding as a determinant of initiation at yeast replication origins. Cell **52**:559–567.
49. **Umek, R. M., and D. Kowalski.** 1990. Thermal energy suppresses mutational defects in DNA unwinding at a yeast replication origin. Proc. Natl. Acad. Sci. USA **87**:2486–2490.
50. **Van Houten, J. V., and C. S. Newlon.** 1990. Mutational analysis of the consensus sequence of a replication origin from yeast chromosome III. Mol. Cell. Biol. **10**:3917–3925.
51. **Walker, S. S., S. C. Francesconi, and S. Eisenberg.** 1990. A DNA replication enhancer in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **87**:4665–4669.
52. **Walker, S. S., A. K. Malik, and S. Eisenberg.** 1991. Analysis of the interactions of functional domains of a nuclear origin of replication from *Saccharomyces cerevisiae*. Nucleic Acids Res. **19**:6255–6262.
53. **Walmsley, R. M., L. H. Johnston, D. H. Williamson, and S. G. Oliver.** 1984. Replicon size of yeast ribosomal DNA. Mol. Gen. Genet. **195**:260–266.
54. **Warner, J.** 1989. Synthesis of ribosomes in *Saccharomyces cerevisiae*. Microbiol. Rev. **53**:256–271.