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The inefficient replication origin from yeast ribosomal DNA is naturally impaired in the ARS consensus sequence and in DNA unwinding

Charles A. Miller, Robert M. Umek and David Kowalski*

Cancer Genetics Department, Roswell Park Cancer Institute, Buffalo, NY 14263, USA

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ABSTRACT

Ribosomal DNA (rDNA) replication origins of *Saccharomyces cerevisiae* are known to function inefficiently, both in the context of the tandem rDNA repeats in the chromosome and as single copy autonomously replicating sequences (ARSs) in plasmids. Here we examined components of the rDNA *ARS* that might contribute to inefficient extrachromosomal replication. Like the efficient H4 *ARS*, the rDNA *ARS* requires a match to the 11 bp ARS consensus sequence (ACS) and a broad non-conserved region that may contain multiple elements, including a DNA unwinding element (DUE). Using a single-strand-specific nuclease hypersensitivity assay and by determining the superhelical density required for stable DNA unwinding, we found that the DNA of the rDNA *ARS* is not as easily unwound as the H4 *ARS*. Unwinding of the rDNA *ARS* required additional energy, similar to the unwinding of mutations in the H4 *ARS* that stabilize the double helix in the DUE region and impair replication. *In vivo* extrachromosomal replication of the rDNA *ARS* was cold sensitive, like H4 *ARS* mutants that require additional energy to unwind the DUE region but unlike the easily unwound, wild-type H4 *ARS*. Impairment of replication function at reduced temperature suggests that the elevated energy requirement for DNA unwinding inherent in the wild-type rDNA *ARS* contributes to inefficient replication function. We also examined the essential ACS match in the rDNA *ARS*, which is known to be imperfect at one position. A point mutation in the essential ACS that corrects the imperfect match increased the efficiency of extrachromosomal replication. Our results reveal that the essential ACS element and DNA unwinding in the rDNA *ARS* are naturally impaired, suggesting that inefficient function of the rDNA replication origin has a biological purpose.

INTRODUCTION

Duplication of the DNA in eukaryotic chromosomes initiates at multiple sites called replication origins. The nature of replication origins, while well defined in bacteria and in certain eukaryotic viruses, remains to be elucidated in most eukaryotic species (reviewed in 1). Among eukaryotes, the replicator, the collection of *cis*-acting components that activate a replication origin, is best defined in the yeast *Saccharomyces cerevisiae*. Yeast chromosomal replicators can be isolated as autonomously replicating sequences (ARSs) which function as plasmid replication origins. However, for unknown reasons, different replication origins can vary widely in efficiency and not every ARS within a chromosome is used in each S phase of the cell cycle (2,3).

A specific match to the 11 bp ARS consensus sequence (ACS), WTTTAYRTTTW, is an essential component of plasmid ARS elements and chromosomal replicators (4–7). An origin recognition complex (ORC), consisting of six different proteins, interacts with the ACS element and functions in initiation of DNA replication (reviewed in 8). In addition to the ACS, the flanking DNA, or B domain, contains multiple short sequence elements that stimulate ARS function (7,9–12). Of four B elements, B1–B4, two are known to interact with specific proteins. Element B1 contributes to ORC binding (13,14). Element B3 binds a known transcription factor (ABF1) which, depending on the ARS, functions either near the ACS or at a distance (7,15).

The essential B domain of ARS elements spans a broad, non-conserved region 3' to the T-rich strand of the required ACS match. The DNA in that broad region is easily unwound (16–21) and contains a DNA unwinding element (DUE). A DUE is a *cis*-acting sequence whose intrinsic helical instability facilitates replication origin activity (22). A DUE-containing region has been roughly localized to the B domain by deletion mutations within several ARS elements (see below). Helical stability analysis of high resolution linker substitution mutations in *ARS307* suggests that DUE function resides primarily in the B2 element (23). The B2 element is functionally conserved between *ARS307* and *ARS1* (9) and the *ARS1* B2 element can be functionally substituted by a broad, easily unwound sequence (23). DUEs are functionally important in replicators from other species, including *Escherichia coli oriC*, SV40 *ori* and bacteriophage T4 *ori* (22,24,25). In these replicators, the DUE corresponds to the DNA sequence which first unwinds during initiation of replication (25–28). By analogy with the

*To whom correspondence should be addressed. Tel: +1 716 845 4462; Fax: +1 716 845 5906; Email: kowalski@sc3101.med.buffalo.edu
Present address:
Charles A. Miller, Department of Environmental Health Sciences, Tulane University, New Orleans, LA 70112, USA

DUE in prokaryotic and viral replicators, the DUE that flanks the yeast ACS element is proposed to facilitate localized DNA unwinding during initiation, providing the entry point into the double helix for proteins involved in the subsequent steps of replication. Consistent with this proposal, the initiation of DNA synthesis in the yeast *ARS1* replicator starts in a region near element B2 (29).

A number of factors can determine the intrinsic ability of DNA to locally unwind and thus could influence DUE function. Previous studies from this laboratory using negatively supercoiled DNA have shown that temperature, superhelical stress and DNA sequence are determinants of the unwinding ability of DNA under specific ionic conditions *in vitro* (16,30,31). *In vivo*, sequence mutations and temperature variations have been exploited to examine the role of DNA unwinding ability in ARS-mediated plasmid replication in yeast. Deletion or substitution mutations in the DUE region that replace an easily unwound sequence with one that is more difficult to unwind reduce activity of a variety of ARS elements (6,16,19,31,32). Conversely, dissimilar sequences that are easily unwound can functionally substitute for the DUE region (6,19), suggesting that the ease of DNA unwinding is a determinant of replication origin function. The energetics of unwinding the replication origin are biologically relevant since ARS mutations with an elevated energy cost for unwinding the DUE region can be suppressed *in vivo* by increasing the temperature of cell growth (31).

rDNA replication origins in the tandem rDNA repeats of yeast chromosome XII function inefficiently (33–35). Only a small fraction of the potential rDNA origins in the 100–200 tandem repeats appear to be used to initiate DNA synthesis in yeast and the same is true for the tandem rDNA repeats of other organisms (36–38). A single copy rDNA *ARS* also functions inefficiently in extrachromosomal replication (32,39). Extrachromosomal rDNA replication is biologically important since rDNA circles containing the ARS element arise spontaneously via recombination in the tandem repeats (40) and the accumulation of rDNA circles in dividing yeast promotes cellular aging (41).

A *cis*-acting component closely associated with the rDNA *ARS* may contribute to the inefficiency of extrachromosomal replication. Different sized rDNA *ARS* fragments ranging from 2.7 kb down to a 107 bp minimal ARS function with similar inefficiency (32), minimizing the possibility that negative regulatory elements outside the minimal ARS are responsible for inefficient replication. Our previous characterization of the *cis*-acting components of a minimal rDNA sequence with full ARS activity has identified the essential ACS match and a broad, easily unwound region containing a specific sequence element and a DUE. However, the basis for inefficient extrachromosomal replication is not known.

In the present work, we have examined components of the rDNA *ARS* that might contribute to inefficient extrachromosomal replication. To better understand the structural properties and function of the DUE region, we have characterized the intrinsic unwinding ability of the rDNA *ARS* as well as the temperature dependence of extrachromosomal replication. We find that the inefficient rDNA *ARS* requires more energy to unwind than the H4 *ARS*, an efficient replication origin. This finding appears to be biologically relevant since extrachromosomal replication mediated by the rDNA *ARS in vivo* is cold sensitive and similar cold-sensitive replication is seen with replication-defective H4 *ARS* mutants that require additional energy to unwind the DUE region. To test the biological significance of the imperfection in the essential ACS match that we had previously identified (32), we show that a mutation that corrects the imperfection *improved* the efficiency of extrachromosomal replication. Our results indicate that the essential ACS element and DNA unwinding in the rDNA *ARS* are naturally impaired, suggesting that inefficient function of the rDNA replication origin has a biological purpose. Possible biological purposes for natural impairments in the rDNA replicator are discussed.

MATERIALS AND METHODS

Plasmids and DNA

Plasmids were propagated in either DH5α or HB101 strains of *E.coli* as described previously (19). The pWE vector is a derivative of pBT3A (22). pBT3A is a pBR322 derivative that lacks nuclease hypersensitive sequences detected in pBR322 (30). pBT3A also lacks a portion of the ampicillin resistance gene (*Amp*) and the pBR322 segment from *Bsp*MII to *Tth*III-1. The pWE vector contains the pUC12 polylinker inserted between the *Pst*I and *Eco*RI sites of pBT3A (D.A.Natale and D.Kowalski, unpublished data). YIp5 is a pBR322-derived vector that contains the *URA3* gene as a selectable marker and has been described previously (42). pVHA (43) and YRp14CEN4 (44) are both centromeric plasmids that contain the *URA3* gene as a selectable marker. Both plasmids also contain the *SUP11* gene. The smaller derivative of *SUP11* present in pVHA does not contain the weak *SUP11*-associated *ARS3* (43). YIp5, YRp14CEN4 and pVHA were used in various experiments to determine ARS function at different temperatures in yeast. The pWE and YIp5 vectors were used to assess supercoil-induced DNA unwinding *in vitro*. The sequences of H4 *ARS* (H4, L35 and L96) and rDNA *ARS* (r1, r2 and r3) derivatives used in these experiments have been previously described (32,45). Plasmids used in P1 nuclease hypersensitivity assays or in two-dimensional gel analysis of topoisomers were isolated from *E.coli* by the boiling lysis method (46). This method of plasmid isolation consistently yields plasmids with a median superhelical density of –0.065 ± 0.002 (17,30). Chromosomal and plasmid DNA for Southern blot analysis were isolated from yeast by treating the cells with lyticase (Sigma Chemical Co.) followed by lysis in 0.5% SDS at 65°C for 30 min and processing as described previously (47). The DNA was extracted with phenol:chloroform:isoamyl alcohol (35:34:1 v/v) and precipitated with ethanol and resuspended in a solution of 10 mM Tris–HCl, pH 8, prior to restriction digestion.

Mapping P1 nuclease hypersensitive sites in rDNA *ARS* plasmids

The P1 nuclease is a single-strand-specific nuclease that was used to identify easily unwound regions in supercoiled DNA, as previously described (31). Under conditions of 37°C in 10 mM Tris–HCl (pH 7.5) and 1 mM Na_2EDTA, negative supercoiling induces stable unwinding at the most easily unwound site in a plasmid and this site is hypersensitive to nicking by a single-strand-specific nuclease (31). Treatment of YIp5 and pWE derivatives containing the rDNA *ARS* (r1 derivative) with P1 nuclease (2.4 ng in 2 µl, 600 U/mg) for

30 min resulted in complete conversion of the supercoiled DNA to a nicked circular form. The frequency of the nicking event is once per molecule because immediately after nicking the superhelical tension is lost and the locally unwound region renatures. After nicking, the plasmids were cut at a unique restriction site, end-labeled with ^{32}P, irreversibly denatured with glyoxal and separated in an agarose gel as described previously (19). The gel was dried and subjected to autoradiography. The P1 nuclease hypersensitive sites were mapped relative to known positions of restriction sites in the plasmids by determining the sizes of the denatured DNA products.

Two-dimensional gel electrophoresis of plasmid topoisomers

The level of negative supercoiling required to stably unwind the rDNA *ARS* plasmid was assessed by two-dimensional gel electrophoresis of plasmid topoisomers (30). For this experiment, the rDNA *ARS* (r1 derivative) was subcloned between the *Pst*I and *Eco*RI sites of the pWE vector. DNA topoisomers were generated by limited topoisomerase I relaxation of the negatively supercoiled plasmid isolated from *E.coli* (31). The first dimension of gel electrophoresis was conducted in 1% agarose at 37°C in 10 mM Tris–HCl (pH 7.5) and 1 mM Na_2EDTA. The lane from the first dimension gel was excised and imbedded in a molten agarose solution to form the second gel. The second dimension gel electrophoresis was conducted in 1% agarose at ambient temperature in a Tris–phosphate buffer (36 mM Tris, 30 mM NaH_2PO_4, pH 7.8) containing 5 μg/ml chloroquine. After electrophoresis, the gel was soaked in water to remove the chloroquine and then stained with ethidium bromide (1 μg/ml) to visualize the DNA topoisomers using UV transillumination.

Cells and culture conditions

The YPH3 strain of yeast (*ade 2-101*/*ade 2-101*, *ura3-52*/*ura3-52*, *lys2-801*/*lys2-801*) was used in all the experiments described in this report. Yeast were transformed with plasmids by a modification of the lithium acetate method (48). Aliquots of transformed cells were spread on synthetic dextrose minimal medium (SD) plates (47) supplemented with ammonium sulfate (5 g/l), adenine sulfate (20 mg/l) and lysine HCl (30 mg/l) and cultured at either 25 or 34°C for several days to assess the effect of temperature on colony growth rate. For analysis of plasmid stability by the colony color assay, cells containing plasmids were initially grown in liquid medium under selection for the plasmid (medium without uracil). The cells were diluted and plated on SD color indicator plates containing 4 mg/l adenine sulfate, supplemented with ammonium sulfate and lysine HCl (see above) and containing uracil (20 mg/l). The plates were immediately incubated at specific temperatures for 18 h and then switched to 30°C for 4 days of additional growth. For the experiments shown in Figure 3, colonies were scored for half-sectoring events as described previously (44) with pink-red half-sectored colonies being indicative of a plasmid loss event during the initial division of a cell. Mitotic stability (the fraction of plasmid-containing cells in the population) of the r8 and r15 derivatives was determined by plating yeast on non-selective and selective (without uracil) media prior to and after five generations of growth in non-selective liquid culture medium. The mitotic stability values were then used to calculate the plasmid loss rate per generation as described (6). For analysis of plasmid copy number by Southern blotting, primary transformants were grown to saturation under conditions selecting for plasmid retention (no uracil present) at 30°C in liquid SD supplemented with ammonium sulfate, adenine sulfate and lysine HCl. The cells were then diluted and grown in a non-selective medium, YPD (47), at specified temperatures for 25 generations.

Analysis of plasmid content by Southern blotting

DNA isolated from yeast was digested with *Eco*RI (New England Biolabs) and separated in a 0.8% agarose gel at 6 V/cm for 3 h in a Tris–phosphate buffer solution (TPE buffer). The DNA in the gel was partially depurinated and denatured (49) and was transferred to a Gene Screen II membrane (DuPont) using a Posiblot pressure blotting apparatus (Stratagene) according to the manufacturer's protocol. After transfer, the DNA was crosslinked to the membrane by exposure to 120 J m^{-2} UV light (254 nm) in a Stratalinker apparatus (Stratagene). The membrane was prehybridized with sheared salmon DNA, then treated with denatured radioactive DNA probe in a solution containing 10% dextran sulfate, 0.5% SDS, and 6× concentrated SSPE, allowed to react at 65°C overnight and extensively washed to remove remaining probe that did not bind (49). A PhosphorImager (Molecular Dynamics) was used to detect the radioactive signal from the probe that specifically bound to DNA on the membrane. ImageQuant computer software (Molecular Dynamics) was used to measure the relative amount of probe hybridized to chromosomal and plasmid sequences.

DNA probes for Southern analysis were isolated from restriction digests separated in low melting point agarose gels and further purified by electrophoresis through a 5% acrylamide gel. The probe fragments were excised from the gel and labeled with ^{32}P using the random oligonucleotide priming method (Prime-It II kit; Stratagene). The probes used were either an *Acc*I fragment from the *URA3* gene or a *Bgl*II–*Tth*III-1 fragment present within the CEN4 insert in pVHA and YRp14CEN4.

Site-directed mutagenesis of the rDNA ACS

The rDNA *ARS* was subjected to site-directed mutagenesis using a previously described PCR-based approach (1). Primer YrAP2 (5'-GAAATGGATCCTTTTGATTTTTTATGTTTT) and an external vector primer were used to amplify and mutate the r8 rDNA *ARS* derivative using the plasmid pWEr8 as a template. The resulting PCR product was digested with *Eco*RI and *Bam*HI and subcloned into the corresponding sites in pVHA. Sequencing of the r15 derivative showed that only the desired G→T point mutation at the first position of the rDNA ACS was present. This mutation changes the natural 10-of-11 bp consensus match rDNA *ARS* to a perfect 11-of-11 bp match.

RESULTS

The rDNA *ARS* is not as easily unwound as other ARS elements

All ARS elements we have previously examined are easily unwound by the energy of negative supercoiling in plasmid DNA (16–19). The most easily unwound site in a plasmid is recognized by a single-strand-specific nuclease under specific conditions in which negative supercoiling promotes localized and stable DNA unwinding (30). In the specific conditions of

Table 1. Rank of nuclease hypersensitive sites in ARS and vector sequences predicts the rank of superhelical density required to stably unwind DNA

Nuclease hypersensitive site[a]	Rank[b]	Superhelical density for stable unwinding[c]	References[d]
H4 *ARS* in YIp5	1	–0.038	19,31
YIp5 (pBR322) vector	2	–0.046	19,30
rDNA *ARS* in pWE	3	–0.051	This study
pWE vector	4	≤–0.057	22

[a]Sites hypersensitive to a single-strand-specific nuclease were determined in negatively supercoiled plasmids with a median superhelical density of –0.065.

[b]The rank of nuclease hypersensitivity in different plasmids is defined by the site that is exclusively cleaved in a supercoiled plasmid containing sequences of lower rank. For example, cleavage at the H4 *ARS* subcloned in YIp5 is dominant over that at the YIp5 (or pBR322) vector site, located 3' to the *Amp* gene. Cleavage of the latter site in the YIp5 vector is dominant over cleavage in the rDNA *ARS* subcloned in YIp5 (this study), while cleavage in the rDNA *ARS* subcloned in pWE is dominant over cleavage in the pWE vector (this study).

[c]Superhelical density for stable DNA unwinding was determined by two-dimensional gel electrophoretic analysis of plasmid topoisomers as described in the text.

[d]Data for nuclease hypersensitivity and two-dimensional gel electrophoresis are presented in detail in the references cited. The superhelical density for the pWE vector is a minimal estimate obtained from the pWE construct GC91 in which nuclease hypersensitivity occurs exclusively in the vector.

this assay, hypersensitivity of the ARS to nicking by a single-strand-specific nuclease suggests that the ARS has a lower energy requirement for DNA unwinding as compared to other sequences present in the test plasmid, YIp5. Two different single-strand-specific nucleases, P1 nuclease and mung bean nuclease, recognize the same sites in negatively supercoiled DNA (50) and we have used these enzymes interchangeably. Here we used P1 nuclease to probe for localized unwinding at the rDNA *ARS* in YIp5 (Fig. 1A). After nicking by P1 nuclease, the DNA was linearized at a unique restriction site and irreversibly denatured. Gel electrophoresis resolves a pair of bands of similar intensity for each nuclease hypersensitive site and the sum of the DNA lengths for each pair equals that of the full-length plasmid (19). Two band pairs, one major and one minor, are seen below the band containing the full-length plasmid, revealing two P1 nuclease hypersensitive sites (Fig. 1B, lane YIp5 + rDNA *ARS*). The major and minor P1 nuclease hypersensitive sites map in the vector, not in the rDNA *ARS* insert (Fig. 1A). The major site occurs 3' to the pBR322 *Amp* gene and the minor site occurs in the yeast *URA3* gene. The same hypersensitive sites are detected in the vector alone (Fig. 1B, YIp5). Thus, unlike other ARS elements we have previously examined, the rDNA *ARS* is not hypersensitive to single-strand-specific nuclease within the context of the YIp5 vector.

Earlier we showed that single-strand-specific nuclease hypersensitivity of negatively supercoiled DNA in our assay condition is a consequence of thermodynamically stable, as opposed to transient, DNA unwinding (22,30,31). Thus, the nuclease hypersensitive site identifies the DNA sequence with the lowest free energy requirement for stable unwinding in the supercoiled molecule. Removal of the most easily unwound site permits supercoil-induced stable unwinding at a less easily unwound site remaining in the DNA molecule, rendering that site hypersensitive to the single-strand-specific nuclease (30). We tested for nuclease hypersensitivity at the rDNA *ARS* in a different vector, pWE (see map in Fig. 2A), which lacks the nuclease hypersensitive sequences present in YIp5. Previous nucleotide level mapping of single-strand-specific nuclease cleavages showed that the rDNA *ARS* is cleaved in the context of the pWE vector, but provided no information on whether the ARS or the vector contained the major nuclease hypersensitive site (32). After P1 nuclease nicking, linearization at a unique restriction site and denaturation, a single pair of bands is seen below the band containing the full-length plasmid (Fig. 2B), indicative of only one nuclease hypersensitive site in the entire plasmid. The site that is hypersensitive to the single-strand-specific nuclease maps to the rDNA *ARS* (Fig. 2A). This observation suggests that the rDNA *ARS* is more easily unwound than the sequences present in the pWE vector.

The hierarchy of nuclease hypersensitivity at different sites in supercoiled DNA reflects a hierarchy in the energy requirement for unwinding those sites (30). Table 1 shows that the rDNA *ARS* (in the context of pWE) ranks third in the hierarchy of nuclease hypersensitive sites present in the H4 *ARS* (in the context of YIp5), the YIp5 vector (major site, 3' to the pBR322 *Amp* gene) and the pWE vector. This ranking suggests that the rDNA *ARS* has a higher energy requirement for unwinding than the YIp5 vector sequences and the H4 *ARS* cloned in YIp5. The ranking also suggests that the rDNA *ARS* has a higher energy requirement for unwinding than other ARS elements (16,17) which, like the H4 *ARS*, are nuclease hypersensitive in the YIp5 vector.

Stable DNA unwinding at the nuclease hypersensitive site requires a certain threshold of superhelical density (22,30,31). The free energy change for stable DNA unwinding is directly related to the superhelical density (51,52). The superhelical density can be determined after identifying the topoisomer with the minimal number of negative supercoils required for stable DNA unwinding, i.e. the transition topoisomer. Stable DNA unwinding is detected after two-dimensional gel electrophoresis of a topoisomer population. The first dimension electrophoresis is performed in the same specific conditions of

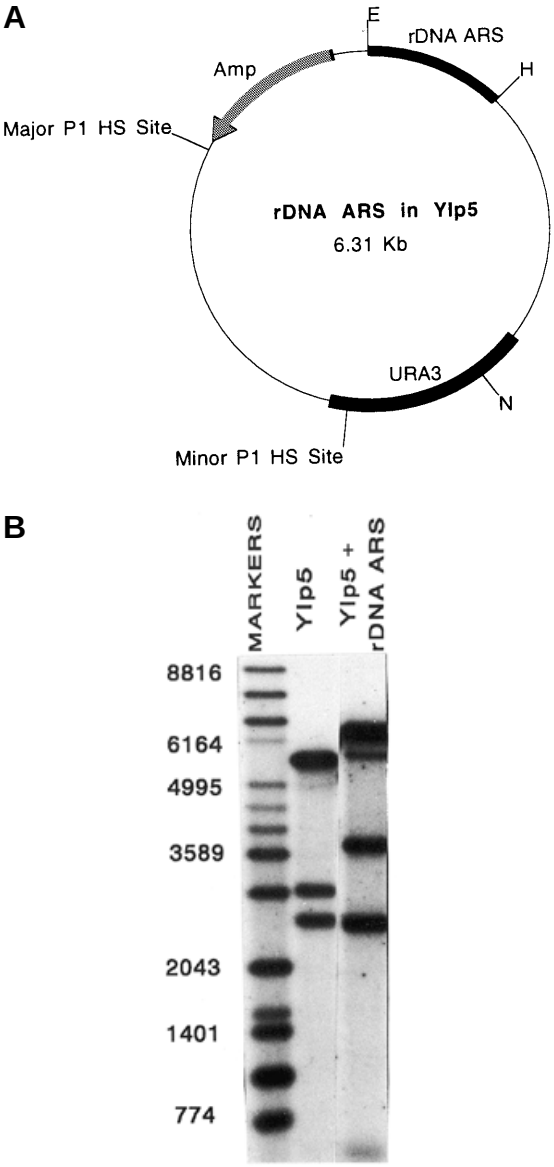

Figure 1. The rDNA *ARS* is not hypersensitive to P1 nuclease in a supercoiled YIp5 vector. (**A**) Diagram of the YIp5 vector containing the rDNA *ARS* insert (r1) at the *Eco*RI (E) and *Hin*dIII (H) restriction sites. The positions of the *Nco*I (N) restriction site and the major and minor P1 hypersensitive sites are shown. The locations of the ampicillin resistance gene (*Amp*) and the *URA3* insert are also indicated. (**B**) Localization of P1 nuclease hypersensitive (HS) sites in negatively supercoiled plasmids: YIp5 and the YIp5 vector containing the rDNA *ARS* insert. The lane 'markers' contains glyoxal-denatured DNA size markers. The lanes labeled YIp5 and YIp5 + rDNA *ARS* indicate the supercoiled plasmids tested. After nicking with P1 nuclease, DNA was linearized and ^{32}P-labeled at the *Nco*I site. DNA was irreversibly denatured, separated by gel electrophoresis and visualized by autoradiography. The slowest moving bands at the top of each lane correspond to the full-length DNA strands complementary to the strand nicked by P1 nuclease. Faster moving bands result from site-specific P1 nicking. A major and a minor band pair, the sizes of which sum to the full-length DNA, is seen for each P1 nuclease hypersensitive site (the minor band pair for YIp5 is difficult to see but is clearly seen in YIp5 + rDNA *ARS*). The sizes of the DNA products in this and other experiments localize the P1 nuclease nicks 3' to the *Amp* gene (major HS site) and within the *URA3* insert (minor HS site). The location of the major HS site corresponds to that seen previously at the DNA sequence level (30). The rDNA *ARS* is not detectably nicked.

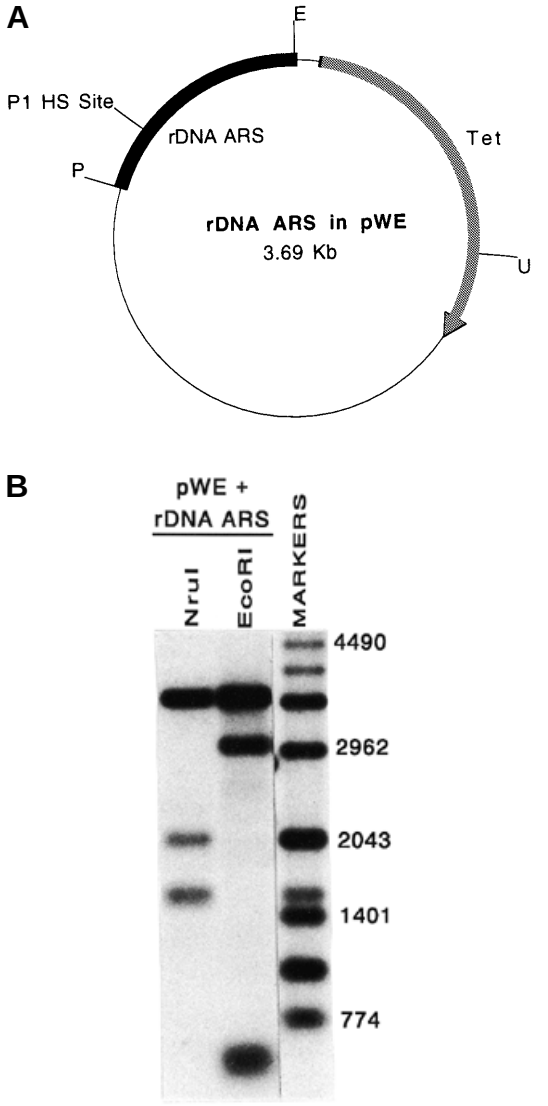

Figure 2. The rDNA *ARS* is hypersensitive to P1 nuclease in a supercoiled plasmid that lacks the easily unwound sites of YIp5. (**A**) Map of the pWE plasmid containing the rDNA *ARS* insert at the *Eco*RI (E) and *Pst*I (P) sites. The positions of the *Nru*I (U) site and the region hypersensitive (HS) to nicking by P1 nuclease are shown. Tet indicates the open reading frame for the tetracycline resistance gene. (**B**) Mapping nicks at P1 nuclease hypersensitive sites in a negatively supercoiled pWE vector containing the rDNA *ARS*. DNA was treated with P1 nuclease, linearized with either *Nru*I or *Eco*RI and the products labeled, separated and visualized as described in Figure 1. Below the full-length DNA band at the top, one band pair is seen, indicating a single P1 nuclease hypersensitive site. The sizes of the DNA products localize the P1 nuclease hypersensitive site to the rDNA *ARS*. High resolution analysis at the DNA sequence level has previously shown that the cleavages occur in the DUE and ACS of the rDNA *ARS* (32).

temperature and ionic strength required to detect the specific nuclease hypersensitive site. Previously we demonstrated that the stably unwound DNA topoisomers, detectable as a retardation of the transition topoisomer as well as more negatively supercoiled topoisomers in the first dimension electrophoresis, are also the nuclease hypersensitive topoisomers (30,31). For

the rDNA *ARS* in pWE, the linking number difference of the transition topoisomer in two independent experiments was –18. The superhelical density of the transition topoisomer is –0.051 (–18/3691 bp/10.5 bp/turn). As shown in Table 1, the superhelical density for stable unwinding at the rDNA *ARS* is more negative than that for the YIp5 (pBR322) vector site and the H4 *ARS*. The hierarchy of the superhelical density required for stable unwinding is consistent with the independently determined rank of nuclease hypersensitivity (Table 1). Our findings indicate that the rDNA *ARS* requires more negative superhelical stress, i.e. more energy, to stably unwind than does the H4 *ARS* or other ARS elements we previously found to be nuclease hypersensitive in the YIp5 vector (16,17).

The rDNA *ARS*, like replication-defective H4 *ARS* mutations that are difficult to unwind, mediates cold-sensitive extrachromosomal replication

The energetics of replication origin unwinding have important implications concerning the initiation of replication *in vivo*. A large increase in the energy cost for unwinding caused by mutations in the DUE region of a number of ARS elements severely diminishes replication origin function (6,16,23,32). Smaller increases in the energy cost for unwinding the DUE region are accompanied by a partial loss of function and by a strong temperature dependence of ARS function under selective conditions. Elevated temperature suppressed the mutational defects in DNA unwinding at the H4 *ARS* since the defect was less severe in cells grown at 30°C compared to those at 23°C (31). Thus, if the elevated energy cost for unwinding the wild-type rDNA *ARS* is relevant to the function of the ARS as a replication origin *in vivo*, we expect that the rDNA *ARS* would display a cold-sensitive replication function.

The previously published experiments performed on H4 *ARS* mutations were carried out under selection for *URA3* gene expression in the YIp5 vector (32). We could not rule out the possibility that the H4 *ARS* mutations confer a temperature dependence on the expression of the *URA3* gene by some unknown mechanism. Additionally, certain *cis*-acting elements associated with ARSs can contribute a segregation function to plasmids in the absence of a centromere (53,54). Given that the YIp5 vector lacks a centromere to provide efficient segregation function, we could not rule out the possibility that the H4 *ARS* mutations provide a temperature-dependent segregation function.

To rule out these possibilities, we analyzed ARS activity in the absence of selection for *URA3* gene expression using a vector that contains a centromere (CEN) element. A colony color assay developed by Heiter *et al*. (44) permits quantitative assessment of ARS replication efficiency in a single cell division. The vector also contains a suppressor tRNA gene (*SUP11*) that suppresses the homozygous *ade2-101* mutation present in the diploid genome of the YPH3 yeast strain. The color of colonies that results from the degree of suppression of the *ade2-101* mutation by *SUP11* allows for the inference of plasmid copy number. Red colonies contain no or low percentages of plasmid, pink colonies contain one plasmid per cell and white colonies are composed of cells that contain two or more copies of the *SUP11* plasmid. A pink-red half-sectored colony ultimately results when, just after plating, a cell containing one copy of the plasmid (a 'pink' cell) fails to replicate the plasmid before the first cell division (a 1:0 segregation event). The cell containing the plasmid gives rise to the pink half of the colony and the cell with no plasmid forms the red half of the colony. The lower the proportion of pink-red half-sectored colonies in the population, the lower the frequency of plasmid loss due to replication failure and the higher is the plasmid replication efficiency.

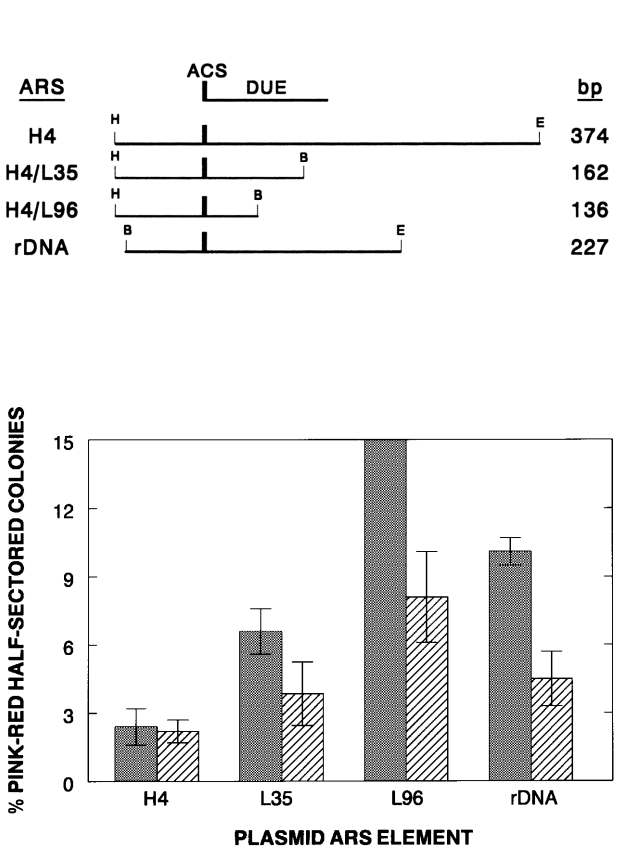

Figure 3. Cell growth temperature affects plasmid replication mediated by the wild-type rDNA *ARS* or by H4 *ARS* derivatives with DUE mutations. (**A**) The schematic diagram illustrates DNA fragments containing the wild-type H4 *ARS*, mutant H4 *ARS* derivatives (deletions L35 and L96) and the wild-type rDNA *ARS* (r2) that were inserted into YRp14CEN4. The DNA sequences of the ARS derivatives were previously described (32,45). The sizes of the DNA fragments in base pairs (bp) are shown on the right. The diagram above illustrates the location of the T-rich strand of the essential ACS element (box) in each DNA fragment and the 100 bp 3'-flanking region (line) that contains the DUE in the wild-type rDNA *ARS*. Restriction sites used for cloning are indicated by: E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII. (**B**) The colony color half-sectoring assay (44) was used to determine the frequency of replication failure events for the ARS elements indicated on the lower axis. The cells containing the respective plasmids were plated on color indicator medium at 20 (solid bars) or 30°C (cross-hatched bars) to determine the effect of temperature on formation of pink-red half-sectored colonies. The higher the percentage of pink-red half-sectored colonies, the higher the frequency of replication failure mediated by the ARS element in the plasmid. The pink-red half-sectoring value for the L96 plasmid at 20°C is a minimal estimate. The actual value is likely even greater but could not be measured since exclusively red colonies were formed, reflecting an extremely high loss rate for the plasmid at 20°C.

As shown in Figure 3B, the plasmid containing the wild-type H4 *ARS* replicates efficiently at both 20 and 30°C, as indicated by the low proportion of pink-red half-sectored colonies (~2% or only two 1:0 segregation events per 100 colonies). The results show that replication mediated by the H4 *ARS* is not affected by temperature. At the culture temperature of 30°C the rDNA *ARS* plasmid shows a half-sectoring frequency of 5.5%, while at the lower temperature of 20°C the frequency of half-sectoring events is 10.5%, an ~2-fold increase. The results show that the rDNA *ARS* is less efficient than the H4 *ARS* in mediating plasmid replication at either temperature. Importantly, the results indicate that plasmid replication mediated by the H4 *ARS* is temperature independent, while that mediated by the rDNA *ARS* is cold sensitive.

H4 *ARS* mutations L35 and L96 (45) were also examined in this experiment. These mutations have deletions in the DUE region that are substituted by vector sequences (Fig. 3A). The vector sequences increase the energy requirement for DNA unwinding relative to the wild-type ARS and the L96 mutation is more difficult to unwind than L35 (19). The frequency of plasmid loss, as indicated by the percentage of pink-red half-sectored colonies in Figure 3B, reflects the relative difficulty in unwinding the derivatives: the L96 plasmid is lost at a greater frequency than is the L35 plasmid at the same temperature. At 20°C the percentage of half-sectoring for colonies containing L35 and L96 is increased relative to that seen for the same derivative at 30°C, indicating more inefficient plasmid replication at the lower temperature. The results show that the H4 *ARS* mutations exhibit a cold-sensitive replication phenotype, as was seen for the wild-type rDNA *ARS*. The greater the degree of difficulty in unwinding the DUE region (L96 > L35), the more severe is the cold-sensitive replication phenotype. Overall, our results indicate that the wild-type rDNA *ARS* shares structural and functional properties with replication-defective H4 *ARS* mutations in the DUE region: both require an elevated energy to both unwind and mediate cold-sensitive extrachromosomal replication.

Growth temperature severely affects the content of rDNA *ARS* and the mutant H4 *ARS* plasmids in dividing cells

An independent assay that reflects extrachromosomal replication efficiency mediated by ARS elements is the residual content of a centromere-containing plasmid after multiple cell divisions in the absence of selection for the plasmid. Cell cultures, initially grown at 30°C under selection for the plasmid (medium lacking uracil), were released from selection by subsequent growth in non-selective (YPD) medium for the same number of generations at 23 or 34°C. A Southern blot of *Eco*RI-digested plasmid and genomic DNA was probed with an *Acc*I fragment of the *URA3* gene common to both the plasmid and the chromosome. The ratio of the specific hybridization signals obtained from the common plasmid and chromosomal DNA sequences reflects the residual content of the various ARS plasmids after multiple cell divisions. The quantitative results for each plasmid ARS element compared at the two growth temperatures are shown in Figure 4. The effect of growth temperature on the content of the wild-type rDNA *ARS* plasmid is severe compared to that seen for the wild-type H4 *ARS* plasmid. The effect seen for the wild-type rDNA *ARS* plasmid resembles that seen for each plasmid containing an H4 *ARS* mutation with a deletion in the DUE region (L35 and L96). The wild-type rDNA *ARS* shows a 5-fold change in plasmid content with temperature and

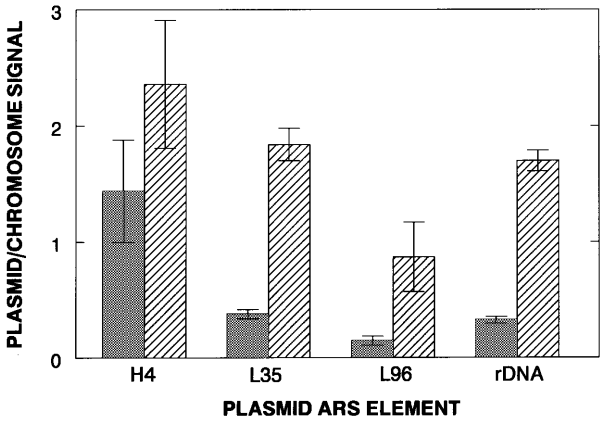

Figure 4. Effects of growth temperature on the content of different ARS plasmids in dividing cells. The plasmids used in this experiment contained one of the following ARS inserts: wild-type H4 *ARS*, a mutant H4 *ARS* derivative (L35 or L96) with a deletion in the DUE region or the wild-type rDNA *ARS*. The ARS inserts were described in Figure 3A. The plasmid content of the cells grown at 34 (cross-hatched bars) and 23°C (solid bars) is reflected by the average plasmid:chromosome ratio. Total DNA was isolated from yeast, cleaved with *Eco*RI and analyzed by Southern blotting to determine the plasmid:chromosome ratio in cells grown at 34 and 23°C for the same number of generations in non-selective medium. The ratio of plasmid *URA3*:chromosome *ura3-52* hybridization signal was quantified using a phosphorimager and determined for DNA from cultures of three independent colonies at each growth temperature.

L35 and L96 show ~5- and 6-fold changes in plasmid content, respectively. In all three cases, a cold-sensitive phenotype is indicated by the severe reduction in plasmid content at the lower growth temperature as compared to the higher temperature. Analysis of the effect of growth temperature on plasmid content in a different vector, pVHA (43), confirmed the cold-sensitive phenotype for the rDNA *ARS* (data not shown). Thus, analysis of the residual plasmid content after multiple cell divisions shows that the cold-sensitive replication phenotype displayed by the wild-type rDNA *ARS* is similar to that seen for H4 *ARS* mutations in the DUE region with an elevated energy cost for DNA unwinding.

An imperfection in the essential ACS element contributes to inefficient function of the rDNA *ARS*

Analysis of the essential ACS element in the rDNA *ARS* has revealed an imperfect match to the canonical WTT-TAYRTTTW sequence (32). A mismatch occurs at the first position in which a G is present instead of an A or T. We reasoned that the mismatch in the essential ACS element may contribute to the inefficient replication property of the rDNA *ARS* and that correcting the mismatch to a perfect match might enhance replication efficiency.

The mismatched G at the first position of the rDNA *ARS* consensus was changed to a T using PCR-directed mutagenesis (Table 2). The parental r8 derivative and the 'corrected' G→T derivative, r15, were evaluated for replication efficiency in the context of the pVHA plasmid, which contains *CEN4* and *SUP11* sequences (43). A qualitative colony color assay which

is based on *SUP11* suppression of a chromosomal *ade2* mutation in the host yeast strain indicated that the r15 plasmid containing the mutated rDNA *ARS* was maintained more efficiently than the plasmid utilizing the r8 plasmid containing the wild-type rDNA *ARS* (data not shown). The results of a quantitative plasmid loss rate analysis are shown in Table 2. The r8 plasmid containing the wild-type rDNA *ARS* is lost almost twice as frequently as is the r15 plasmid containing the mutated rDNA *ARS*. Thus, the mutation in the rDNA *ARS* that corrected the G→T in the essential ACS resulted in more efficient replication. These results demonstrate that the natural imperfection in the essential ACS element of the rDNA *ARS* accounts in part for the inefficient extrachromosomal replication.

Table 2. A point mutation correcting the natural mismatch in the essential ACS element increases replication efficiency

rDNA ARS DERIVATIVE [1]	ACS ELEMENT	PLASMID LOSS RATE [2]
r8 (Wild Type)	GTTTATGTTTT	19.3% per generation
r15 (Mutation)	TTTTATGTTTT	11.8% per generation
	$^{T}_{A}TTTA^{T}_{C}{}^{G}_{A}TTT^{T}_{A}$	
	ARS Consensus Sequence	

[1]The wild-type rDNA ACS element (r8) was altered in the natural mismatch (G) using site-directed mutagenesis to create a perfect match (r15) to the ARS consensus sequence.

[2]The plasmid loss rate was determined for the wild-type and the mutated rDNA ARS derivatives in the context of pVHA, a CEN plasmid. Cells were allowed to grow for five generations in non-selective medium when determining plasmid loss rate essentially as previously described (6). The ~2× lower plasmid loss rate for the r15 ARS is indicative of improved initiation efficiency for the r15 derivative relative to the wild-type ARS in r8.

DISCUSSION

We have previously shown that the DNA of several yeast ARS elements is easily unwound *in vitro* and that the easily unwound sequence resides primarily in a functionally essential region 3' to the ACS element. Mutations in this region that reduce either nuclease-detectable unwinding or DNA helical instability are accompanied by a reduction in ARS activity in living cells, suggesting that the essential region contains a DUE (6,16,19,32). The functionally essential region 3' to the ACS element also contains two or three B elements (7,9,10,12). Analysis of high resolution mutations in *ARS307* suggests that, in addition to a known specific sequence requirement, the B2 element contains a DUE (23). The rDNA *ARS* contains a DUE and the nuclease hypersensitive region resides primarily 3' to the ACS (32), as in other ARS elements we have tested, however, as shown here, the rDNA *ARS* is not as easily unwound as other ARS elements. Unlike the H4 *ARS* (19) and chromosome III-derived *ARS305*, *ARS307*, *ARS309* and *ARS310* (16,17), the rDNA *ARS* is not hypersensitive to a single-strand-specific nuclease in a negatively supercoiled YIp5 vector. Instead, the major hypersensitive site occurs in the YIp5 vector at a site 3' to the pBR322 *Amp* gene (Fig. 1), an easily unwound site which we had previously identified (30). However, the rDNA *ARS* is the major nuclease hypersensitive site in the context of the pWE vector, which lacks the sequence 3' to the *Amp* gene and certain other easily unwound sequences present in YIp5 (Fig. 2). The results presented here, together with our previous analyses of the nuclease hypersensitivity of other ARS plasmids (16,17,19), show that hypersensitivity to the single-strand-specific nuclease decreases in the following order: H4 *ARS* and other ARSs analyzed > sequence 3' to the *Amp* gene > rDNA *ARS* > pWE vector. The hierarchy in hypersensitivity to a single-strand-specific nuclease in our specific assay conditions implies a hierarchy in energy cost for localized unwinding at the nuclease hypersensitive sites (30). The observed hierarchy in nuclease hypersensitivity suggested that the rDNA *ARS* requires extra energy to unwind compared to the H4 *ARS* and other ARSs tested and this was confirmed by determining the superhelical density required to induce the stably unwound structure at the same temperature and ionic condition required for nuclease hypersensitivity of the structure (Table 1). We conclude that the rDNA *ARS* requires more energy for localized DNA unwinding than does the H4 *ARS* or other ARS elements previously analyzed.

Unlike the more easily unwound H4 *ARS*, the rDNA *ARS* confers cold-sensitive extrachromosomal replication in yeast grown at different temperatures. The rDNA *ARS* plasmid is lost more frequently at the lower versus the higher temperature while the H4 *ARS* plasmid loss is not detectably affected in a single cell division (Fig. 3B). The cold-sensitive replication conferred by the rDNA *ARS* was expected only if the energetics of replication origin unwinding are limiting for its function *in vivo*. Previous analysis revealed that H4 *ARS* mutations which sufficiently increase the energy cost for unwinding the DUE region confer cold-sensitive phenotypes with respect to both colony growth and cell doubling under selection for the ARS plasmid (31). In the present study, the assays that were used provided a more direct measure of plasmid replication efficiency mediated by the ARS since selection for plasmid gene expression was not enforced and since the vectors contained a centromere to ensure efficient segregation. For both the wild-type rDNA *ARS* and for the mutant H4 *ARS* derivatives, the analyses show that plasmid replication efficiency is cold sensitive. A reduced replication efficiency at the lower versus higher temperature in dividing cells is reflected in both an increased frequency of plasmid loss in a single division (Fig. 3B) and an extremely reduced plasmid content after multiple divisions (Fig. 4). In these assays, the wild-type rDNA *ARS* functions like replication-defective H4 *ARS* derivatives containing mutations that increase the energy cost for unwinding the DUE region. Our results indicate that the wild-type rDNA *ARS* shares structural and functional properties with replication-defective H4 *ARS* mutations in the DUE region.

In vivo, site-specific DNA binding proteins are thought to be major effectors in initiating localized unwinding at replication origins. Proteins such as ORC that bind the ACS in yeast replicators have been isolated and ORC contributes to initiation *in vivo* (reviewed in 8). However, the complete initiator protein complex has yet to be positively identified and reconstituted *in vitro*. At the SV40 *ori* and at *E.coli oriC*, binding of the cognate initiator protein facilitates opening of the DNA helix at a specific site in the origin *in vitro* (26,27). At both *ori* regions, the specific site is intrinsically easy to unwind and replication-defective mutations which increase DNA helical stability, as well as replication-positive mutations which maintain a low helical

stability, indicate that the specific site contains a DUE (22,24). Furthermore, at both the SV40 *ori* and *E.coli oriC*, a threshold temperature exits below which the cognate initiator protein can still bind but cannot unwind the origin *in vitro* (26,55). Failure to unwind DNA at the origin results in a failure to initiate DNA replication. The properties of the rDNA *ARS* described here, elevated energy cost for DNA unwinding and cold-sensitive plasmid replication, are consistent with the possibility that the available thermal energy modulates the activity of the DUE *in vivo*. It is known that thermal energy can modulate local unwinding at a DUE region in naked supercoiled DNA (31). *In vivo*, the elevated energy cost for unwinding the rDNA *ARS* may render helix opening at the DUE more prone to fail at lower temperatures, despite the presence of the required initiator proteins and enzymes.

Our previous identification of the essential ACS element of the rDNA *ARS* and the sequencing of several independently isolated rDNA *ARS* clones indicated that the ACS element contains an imperfect match to the ARS consensus sequence (32). The particular mismatch, a G in place of A or T at position 1, reduced replication efficiency when introduced as a mutation to the ACS element in *ARS307* (43). This result prompted us to test the effect of correcting the natural ACS mismatch in the rDNA *ARS* to that of a perfect ACS. Replacing a G with a T at the first position of the ACS improved rDNA *ARS*-mediated plasmid stability as determined by a quantitative plasmid loss rate assay (Table 2). To our knowledge, this is the first report of a mutation in an essential element that *increases* the activity of a replication origin. Since the mutation is in the essential ACS element, the activity increase may result from improved recognition or activation by ORC or other initiation factors that interact directly or indirectly through that element. Identification of a functional impairment in the essential ACS element suggests that there is selective pressure to maintain inefficient function in the rDNA *ARS*.

While the replication efficiency of the rDNA *ARS* containing the ACS mutation was enhanced (i.e. plasmid loss was reduced), it still did not display the efficiency of the H4 *ARS* and other optimally functioning replication origins in yeast (plasmid loss = 1.0–5.0% per generation). The absence of a functional B1 element is an unlikely explanation for inefficient function since the rDNA *ARS* resembles the efficient *ARS1* in that both have a functionally important TT sequence at the same position 3' to the ACS, within the B1 element of *ARS1* (9,32). The rDNA *ARS* has no ABF1 binding site (B3 element), but such a site is not required for efficient function in other ARSs and in some contexts actually inhibits function (23). It is possible that there is a novel B element in the rDNA *ARS*. The only other elements known to be present in ARSs are B2 and B4 and these are associated with a DUE (23). The failure of the ACS mutation to result in fully efficient function of the rDNA *ARS* is consistent with our observations on the unusual helical stability of the rDNA *ARS* DUE and a role for DNA unwinding in the initiation of DNA replication.

Our findings presented here reveal that the inefficient rDNA *ARS* is naturally impaired in both the essential ACS element and in DNA unwinding. What are the biological consequences for the chromosome of having an inefficient rDNA replicator that is used at low frequency? In the case of the multiple, closely spaced replicators at the tandemly repeated rDNA locus, the absence of firing at many of the individual replicators appears to be normal and not detrimental (33–35). Even in an extreme case, if only one in 10 rDNA replicators fired, the resulting replication origins would still be sufficiently closely spaced to passively replicate all rDNA repeats containing replicators that did not fire, based on estimates of the length of DNA that can be duplicated by a single replication fork (3).

What is the biological purpose for inefficient functioning of the rDNA replicator? In the rDNA repeats in the chromosome, rDNA replicators are positioned between the transcriptionally active rDNA genes. Replication and transcription of rDNA genes occur concurrently throughout S phase (33,56). The inefficient function of the rDNA replicator may have evolved to restrict initiation of replication to a small fraction of the tandem rDNA repeats, minimizing interference between replication and transcription of the rDNA genes.

Another possible biological purpose for inefficient function of the rDNA *ARS* concerns extrachromosomal replication and cellular aging. Extrachromosomal rDNA circles (ERCs) containing the ARS element are spontaneously released from the tandem rDNA repeats in the chromosome by recombination (40). ERCs preferentially accumulate in mother cells when the mother and daughter cells divide and ERC accumulation is accompanied by progressive enlargement and fragmentation of the nucleolus, arrest of cell division and eventual cell death (41). Mutations in a nucleolar DNA helicase, *SGS1*, promote accumulation of ERCs and shorten the lifespan of yeast cells (57). Inefficient function of the rDNA replicator, on the other hand, would tend to minimize accumulation of ERCs. Thus, the natural impairments we identified in the ARS consensus and in DNA unwinding that contribute to inefficient extrachromosomal rDNA replication may have evolved as determinants of the normal lifespan of yeast cells. The low efficiency of the rDNA replication origin may be a compromise between a level of efficency high enough to completely duplicate the tandem rDNA repeats in the chromosome and a level of efficiency low enough to prevent rapid accumulation of ERCs.

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