Ease of DNA unwinding is a conserved property of yeast replication origins

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Received October 16, 1992; Revised and Accepted December 18, 1992

ABSTRACT

Autonomously replicating sequence (ARS) elements function as plasmid replication origins. Our studies of the H4 ARS and ARS307 have established the requirement for a DNA unwinding element (DUE), a broad easily-unwound sequence 3' to the essential consensus that likely facilitates opening of the origin. In this report, we examine the intrinsic ease of unwinding a variety of ARS elements using (1) a single-strand-specific nuclease to probe for DNA unwinding in a negatively-supercoiled plasmid, and (2) a computer program that calculates DNA helical stability from the nucleotide sequence. ARS elements that are associated with replication origins on chromosome III are nuclease hypersensitive, and the helical stability minima correctly predict the location and hierarchy of the hypersensitive sites. All well-studied ARS elements in which the essential consensus sequence has been identified by mutational analysis contain a 100-bp region of low helical stability immediately 3' to the consensus, as do ARS elements created by mutation within the prokaryotic M13 vector. The level of helical stability is, in all cases, below that of ARS307 derivatives inactivated by mutations in the DUE. Our findings indicate that the ease of DNA unwinding at the broad region directly 3' to the ARS consensus is a conserved property of yeast replication origins.

INTRODUCTION

Sequences that allow extra-chromosomal maintenance of DNA in *Saccharomyces cerevisiae*, known as autonomously replicating sequences (ARS), have proven to be origins of plasmid replication (1, 2). ARS activity requires a short consensus sequence and a broad 3'-flanking region (reviewed in 3). The requirement for the 11-base pair (bp) consensus (WTTTATRTTTW; W = A or T, R = A or G [4]) in allowing autonomous replication of plasmids is firmly established. All ARS elements contain an essential sequence with a high degree of match to the 11-bp consensus—most often at eleven or ten positions (3, 5, 6), and, in one case, at nine positions (7). The consensus sequence is thus a general and essential component of yeast ARS elements.

The sequence requirements of the other component, the broad region 3' to the consensus T-rich strand, have been more difficult to discern. Deletion analyses have shown that efficient replication requires about 100 base pairs 3' to the consensus; however, the span of this required region is not easily defined since the contiguous vector sequence can modulate ARS activity (reviewed in 3). The primary sequence of the required region varies among ARS elements. Linker substitution analyses of the H4 ARS (8) and ARS1 (9) have identified no short sequence element that is essential for ARS function aside from the consensus and a few adjoining nucleotide residues; however, G+C-rich linker substitutions at certain positions reduce ARS1 efficiency (9).

The broad 3'-flanking region of the H4 ARS, ARS307 (formerly C2G1), and the 2 μ ARS is hypersensitive to single-strand-specific nucleases in negatively-supercoiled DNA (10–12), reflecting a low free energy cost for DNA unwinding (13, 14). Mutations in the broad 3'-flanking region of the H4 ARS that impair unwinding also reduce replication activity, and such mutations can be suppressed by cell growth at elevated temperature (10, 14). In addition, computer-assisted DNA helical stability analysis of ARS307 mutations reveals a quantitative association between ease of unwinding the 3'-flanking region and ARS efficiency (11). The required easily-unwound sequence is a novel genetic element and is termed a DNA unwinding element (DUE). We have proposed that the yeast DUE, like the DUE at the *Escherichia coli* origin (15, 16), facilitates origin unwinding after the initiator protein binds the consensus sequence, and serves as the entry site for the enzymes that start DNA replication (10, 14).

It remains to be determined whether the presence of a DUE is a general property of yeast ARS elements. In this paper, we examine the single-strand-specific nuclease hypersensitivity of several ARS elements derived from chromosome III (17) which have been shown to colocalize with chromosomal replication origins (18, 19). We also use a recently-developed computer program (11) to determine the DNA helical stability of these and a variety of other ARS elements, including ARS elements derived from prokaryotic DNA (20) and all yeast ARS elements whose essential consensus sequence has been identified by mutational analysis. We find that the ease of DNA unwinding at the broad 3'-flanking region is a property of all ARS elements examined, suggesting that a DUE is a conserved component of yeast replication origins.

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MATERIALS AND METHODS

Enzymes

P1 nuclease, prepared according to Fujimoto *et al.* (21), was from Yamasa Shoyu Co. Ltd., Choshi, Japan. Other enzymes were as follows: restriction endonucleases (New England Biolabs); calf intestine alkaline phosphatase (Boehringer Mannheim); polynucleotide kinase (United States Biochemical Corporation).

DNA

Chromosome III *ARS*-containing plasmids (17) derived from YIp5 were a gift from Carol Newlon (UMDNJ—New Jersey Medical School). *Escherichia coli* HB101 containing an *ARS* plasmid was grown in Luria-Bertani broth. Cells were lysed by boiling in the presence of lysozyme (22) and plasmid DNA was purified by two rounds of equilibrium gradient centrifugation in CsCl (23). The superhelical density of different plasmids prepared by this method is -0.065 ± 0.002 as determined by two-dimensional agarose gel electrophoresis of plasmid topoisomers (13).

Single-strand-specific nuclease reactions

Supercoiled plasmids (1.5 μ g) were pre-incubated at 37°C in 19 μ l of 10 mM Tris—HCl, pH 7.5/1 mM Na₂EDTA. P1 nuclease (0.5 ng in 1 μ l; 600 units/mg) was added and the reaction proceeded for 30 minutes. Reactions were quenched (24), and the DNA was purified by organic extraction and ethanol precipitation. Under the conditions used, the single-strand-specific nuclease introduces a single nick per molecule, since the initial nick converts the supercoiled plasmid to a nuclease-resistant relaxed form (25).

Global mapping of P1 nuclease nicks

P1 nuclease hypersensitive sites were determined by direct mapping of the specific nicks (10, 13, 16). The nicked circular DNA was cleaved at a unique restriction endonuclease site, dephosphorylated, and 5' end-labeled with ³²P. The DNA was irreversibly denatured with glyoxal (26) and separated by electrophoresis in an agarose gel. Similarly-denatured restriction fragments of known length served as molecular weight markers. The sites of P1 nuclease recognition were established by determining the distance from the labeled restriction endonuclease site to the nick for the DNA in a given band as described (10).

Helical stability analyses

A computer program called Thermodyn was used to calculate the free energy required for DNA strand separation under the specific conditions used in the nuclease hypersensitivity assay (11). Helical stability plots of the chromosome III *ARS* elements were generated as follows. The free-energy difference (between the duplex and single-stranded states), calculated based on nearest-neighbor dinucleotide composition (27), is determined for the first of a series of overlapping segments, or 'windows.' The program then slides along the sequence a discrete number of base pairs (steps) and resumes helical stability calculations on a new window. The program output is a tabulation of free-energy values for each window versus the nucleotide position at the center of that window. The output was converted to graphic form using the Slidewrite Plus computer graphics program (Advanced Graphics Software, Inc.). The window size used for all helical stability analyses was 100 base pairs.

The helical stability of the 3'-flanking region of the other *ARS* elements was determined for the single window whose first nucleotide residue is immediately 3' to the last base pair of the consensus sequence and which extends 100 bp 3' to the T-rich strand. In contrast to our earlier analysis of *ARS307* deletion derivatives (11), no variation in window position was permitted for the values reported.

The Thermodyn program used in this study runs on IBM-compatible personal computers. It is available via the Internet, and can be requested by sending your Internet address to CAMKOWAL@ubvms.cc.buffalo.edu.

RESULTS

Chromosome III *ARS* elements are hypersensitive to single-strand-specific nuclease

The H4 and 2 μ *ARS* elements are hypersensitive to single-strand-specific nucleases in negatively-supercoiled DNA (10, 12). *ARS307*, recently demonstrated to colocalize with a chromosome III replication origin (19), is also nuclease hypersensitive on a supercoiled plasmid (11). We tested additional *ARS* elements from chromosome III (17), including two (*ARS305* and *ARS309*) that colocalize with chromosomal replication origins (18, 19), to determine if nuclease hypersensitivity is a general property of *ARS* elements. Supercoiled plasmids containing individual *ARS* elements from chromosome III were singly nicked with P1 nuclease and then linearized at a single restriction endonuclease site. The vector used, YIp5, was processed in a similar manner for comparison. The products were ³²P end-labeled, denatured, and separated by agarose gel electrophoresis (Figure 1). The position of the products of nuclease cleavage at the previously characterized vector site (13) is indicated by the arrow to the left of the autoradiogram. The vector site is present in each of the *ARS* plasmids and thus serves as a reference. Products migrating faster than the reference band are the result of nuclease cleavage at the *ARS* insert (lanes 2–5). The intensity of the faster-migrating band relative to the intensity of the reference band in the same lane reflects the frequency of cleavage in the *ARS* insert relative to the cleavage at the reference site. In all the *ARS* plasmids examined, the *ARS* sequence is cleaved more frequently than the reference vector site. Nuclease hypersensitivity, in our assay, is a characteristic of sequences that have a low free energy cost for DNA unwinding (13). Thus, ease of DNA unwinding is a property that is shared among the H4, 2 μ , and four chromosome III-derived *ARS* elements.

Helical stability analysis of the DNA sequence predicts the nuclease hypersensitive sites in the chromosome III *ARS*-containing plasmids

Computer analysis of DNA helical stability has been used as an alternative approach to detect easily unwound sequences (11). DNA helical stability is the free energy difference (ΔG) between the duplex and single-stranded states, and is governed by the base sequence. The free energy required for strand separation can be reliably calculated from the base sequence using experimentally-determined thermodynamic parameters for the component nearest-neighbor dinucleotides (27). This important finding has been exploited in a recently-developed computer program (11). The program can take into account the conditions used for the single-strand-specific nuclease reactions, and the data can be used

Figure 1. Nuclease hypersensitivity of chromosome III *ARS* elements. Supercoiled plasmids containing *ARS* elements from chromosome III were nicked with P1 nuclease, linearized at a restriction endonuclease site, 32 P end-labeled and denatured prior to agarose gel electrophoresis. The arrow to the left of the autoradiogram indicates the position of the band resulting from specific nicks at the reference vector site. The plasmids used are as follows: Lane 1, YIp5; Lane 2, pA6C (*ARS305*); Lane 3, pJ11D1 (*ARS309*); Lane 4, pC2G1 (*ARS307*); Lane 5, pH9G (*ARS310*). After P1 nuclease treatment, plasmids were linearized with either *Nco*I (lanes 1–4) or *Sal*I (lane 5). Lane M is a marker lane with sizes of selected bands (in nucleotides) indicated to the left.

to obtain a helical stability profile of a plasmid of interest. We have previously shown that the helical stability minima correctly predict both the location and hierarchy of the sites that are hypersensitive to single-strand-specific nuclease in an *ARS307*-containing plasmid (11). We generated helical stability profiles for the other chromosome III *ARS*-containing plasmids examined above to compare the sites of nuclease hypersensitivity to the helical stability minima. The free energy required to strand-separate a given 100-bp DNA sequence (window) was graphed versus the nucleotide position at the center of that sequence (window position) (Figure 2). For each plasmid, the minimum helical stability (dashed line) occurs within the *ARS* insert (bar), and colocalizes with the position of the major nuclease-hypersensitive site (heavy arrow) determined from the data in Figure 1. The region with the next-lowest helical stability colocalizes with the minor nuclease-hypersensitive site in the vector (light arrow). Thus, the computer program has correctly predicted both the sites and hierarchy of nuclease hypersensitivity for each of the *ARS*-containing plasmids.

ARS305, *ARS309*, and *ARS310* do not contain a perfect match to the *ARS* consensus sequence (17). Instead, they contain multiple copies of different sequences that, at best, match the consensus in 10 of 11 positions. These *ARS* elements are uncharacterized with respect to the identity of the essential consensus sequence. Most of the consensus match sequences within the *ARS* insert (shown as dots in Figure 2) occur in the vicinity of the nuclease hypersensitive site and helical stability minimum. However, the consensus sequence essential for *ARS* function need not be within the plasmid sequence with the lowest helical stability (28). Instead, as our studies on the H4 *ARS* and *ARS307* have demonstrated, a broad region 3' to the essential consensus, where the DUE is located, must have a sufficiently low helical stability

Figure 2. Helical stability (ΔG) of chromosome III *ARS*-containing plasmids. The free energy (kcal/mol) required to strand-separate a 100-bp window of DNA sequence is graphed versus the central position of the window within the *ARS* plasmid. A two base pair increment between window positions (step=2) was used. The dashed line indicates the lowest level of helical stability for the plasmid. The location of the major and minor P1 nuclease-hypersensitive sites are indicated by the heavy and light arrows, respectively. The *ARS* insert is indicated by the bar, and *ARS* consensus matches (≥ 10 of 11 bp) within the insert are indicated by dots.

for the *ARS* to function (10, 11). Therefore, we determined the free energy required to unwind the 100-bp region 3' to every consensus match in the three uncharacterized chromosome III *ARS* elements from the data in Figure 2. The free energy values range from 78.8 to 87.0 kcal/mol. These values fall within the range of those found for well-studied *ARS* elements (see below). To assess how the free energy values for the chromosome III *ARS* elements relate to those for replication-defective *ARS*

Figure 3. Helical stability (ΔG) of the 3'-flanking region of well-studied ARS elements. The free energy (kcal/mol) required to strand-separate the 100-bp sequence directly 3' to the consensus is shown for ARS elements whose essential consensus has been defined by mutational studies (4–9, 33). The free energy required to unwind certain ARS307 3'-flanking region mutations is shown as a reference. The values given for the ARS307 mutations are the helical stabilities of external deletion derivatives that bring the same vector sequence into the 3'-flanking region. The +/– represents the $\Delta 133$ derivative (29), which has the largest deletion that is still able to promote high frequency transformation. The – represents the $\Delta 155$ derivative (29), which is the derivative with the smallest deletion that is unable to promote high frequency transformation.

Figure 4. Helical stability (ΔG) of novel M13 ARS elements. The free energy (kcal/mol) required to strand-separate the 100-bp sequence directly 3' to the newly-created ARS consensus match is shown for mutations within the M13 vector sequence that gave rise to ARS+ plasmids. The dashed lines labeled – and +/- indicate the free energy required to unwind the ARS307 mutations described in Figure 3.

elements with mutations in the DUE, we compared them to the free energy required to unwind the 100-bp region 3' to the essential consensus for mutant ARS307 derivatives. ARS activity is severely impaired in a mutant in which the free energy required to unwind the 3'-flanking region is 114.0 kcal/mol, and is

abolished in another mutant in which the free energy is 122.3 kcal/mol (see legend to Figure 3). The helical stability of the 100-bp region 3' to each consensus match in the uncharacterized chromosome III ARS elements is well below the helical stabilities for replication-defective ARS derivatives with mutations in the 3'-flanking region.

The broad 3'-flanking region of well-studied ARS elements has a low helical stability

It is not known which of the consensus matches is essential for the activity of the uncharacterized chromosome III ARS elements, and it can not be predicted (6). We wished to determine if a low helical stability is a general property of the broad 3'-flanking region of well-studied ARS elements whose essential consensus sequence has been identified by mutational analysis. The helical stability of the 100-bp 3'-flanking region of all ARS elements that fit this criterion is shown in Figure 3. The free energy required to unwind the replication-defective ARS307 derivatives containing mutations in the 3'-flanking region (discussed above) is shown as a reference. The helical stability values of the 3'-flanking region of well-studied ARS elements range from 71.2 to 97.9 kcal/mol. Even the highest value, exhibited by the HO ARS, falls below the values for inactivation or severe impairment seen for the mutant ARS307 derivatives. Thus, all ARS elements examined contain a broad easily-unwound sequence 3' to the essential consensus.

The broad 3'-flanking region of yeast ARS elements created in prokaryotic DNA has a low helical stability

The above findings imply that any DNA, from any source, that has ARS activity in *S. cerevisiae* would contain an ARS consensus element and a broad region of reduced helical stability positioned immediately 3' to the consensus T-rich strand. Kipling and Kearsy described the isolation of novel ARS elements generated by mutations within a vector derived from the DNA of the prokaryotic phage M13 (20). Sequencing of the M13 ARS elements revealed that close matches to the 11-bp *S. cerevisiae* ARS consensus were generated, and the authors attributed the ARS activity to the creation of these consensus matches. An examination of the unwinding ability of these sequences, using the nuclease hypersensitivity assay developed in our lab, failed to detect a hypersensitive site at the new ARS sequences (20). However, as the authors noted, and as Umek and Kowalski have demonstrated (28), easily unwound sequences at ARS elements can be masked from detection by a more easily unwound sequence within the same plasmid molecule. To avoid this complication, we examined the helical stability of the novel M13 ARS sequences by computer analysis. The free energy required to unwind the 100-bp region 3' to the newly-created consensus matches is shown in Figure 4. The dashed lines represent the helical stability of the mutant ARS307 derivatives used as a reference above. The data reveal that the free energy requirement of the broad 3'-flanking region of each M13 ARS element is below that required to inactivate or severely impair ARS307 by mutations in the DUE. Thus, the novel ARS elements were created in regions of the M13 vector which exhibit a low helical stability.

DISCUSSION

We have previously shown that the H4 ARS, ARS307, and the 2μ ARS are hypersensitive to single-strand-specific nuclease in negatively supercoiled DNA, and that the site of nuclease

hypersensitivity is a broad region 3' to the essential consensus sequence (10–12). Here we demonstrate that the chromosome III *ARS* elements *ARS305* (A6C), *ARS309* (J11D1), and *ARS310* (H9G) are also nuclease hypersensitive. This indicates that the *ARS* elements have a low free energy requirement for DNA unwinding, since our nuclease hypersensitivity assay identifies the plasmid sequence with the lowest free energy cost for unwinding (13). The low free energy required for unwinding nuclease-hypersensitive sites was previously inferred from the stable DNA unwinding observed after two-dimensional gel electrophoresis of plasmid topoisomers (13, 14, 16). Here we further demonstrate the low free energy requirement of the chromosome III *ARS* elements by analysis of DNA helical stability using a recently-developed computer program (11). Analysis of three plasmid DNA sequences containing different chromosome III *ARS* elements shows that the helical stability minimum for a 100-bp window occurs within the *ARS* insert and colocalizes with the nuclease-hypersensitive site. The findings presented here extend our previous findings that yeast *ARS* elements contain a broad easily-unwound region, and illustrate the predictive power of the helical stability analysis using our computer program.

We examined the helical stability of all *ARS* elements whose essential consensus sequence has been defined by mutation, including *ARS1*, *ARS121*, the HMRE, HO, H4, and 2μ *ARS* elements, and *ARS307* (Figure 3). Our results show that the helical stability of the 100-bp region spanning the required sequence 3' to the essential consensus is low in comparison to the helical stability of replication-defective *ARS307* derivatives with mutations in the 3'-flanking region. In *ARS305*, *ARS309*, and *ARS310*, where the essential consensus is not known, the helical stability values of the 100-bp region 3' to each consensus match are within the range of those of the well-studied *ARS* elements and are low in comparison to those of the replication-defective *ARS307* mutants. Thus, all *ARS* elements examined have a low helical stability in the 100-bp sequence 3' to a consensus match. Our findings indicate that a broad easily-unwound sequence located 3' to the consensus T-rich strand is a conserved component of yeast *ARS* elements.

The easily-unwound sequence required for replication origin function is termed a DNA unwinding element (DUE) (14, 16). We have proposed that an initiator protein(s) recognizes the essential consensus sequence and establishes a protein-DNA initiation complex that, together with the intrinsic helical instability of the DUE, brings about localized origin unwinding (10, 14). The helical instability of the DUE is crucial, since mutations that significantly increase helical stability, *e. g.* G+C-rich linker substitutions and certain deletions or insertions, can diminish or destroy *ARS* activity (10, 11, D.N. and D.K., unpublished data). The detection of an easily-unwound sequence 3' to the essential consensus sequence of a variety of *ARS* elements suggests that our model for the initiation of origin unwinding may be general for all yeast *ARS* elements.

Kipling and Kearsley (20) described novel *ARS* elements created by mutation within prokaryotic sequences of an M13 vector. Two of the three *ARS* elements described (A17 and B12) contained mutations that generated a consensus match in close proximity to pre-existing consensus match sequences. These data were interpreted to indicate that *ARS* activity can arise as a result of the duplication of near match sequences. However, both the newly-created near match in B12 (GTTAATATTTT) and the improved consensus match in A17 (TGTTATATTTT) are in

regions with a low free energy requirement for DNA unwinding (Figure 4). Therefore, the possibility exists that it is the creation of a consensus match in a sequence environment that favors unwinding, not the duplication *per se*, that accounts for the *ARS* activity. Consistent with this interpretation, a different M13 mutation, D10, was created next to a broad easily-unwound sequence (Figure 4) but not close to any other consensus match (20). Our analysis of the novel M13 *ARS* elements shows that they all contain a broad easily-unwound region 3' to a consensus match.

The findings presented here support our previous proposal that *ARS* elements are minimally composed of two essential components: a consensus element and a DNA unwinding element (10, 14). For a given *ARS* element, appropriate mutations in either component alone can abolish autonomous replication. Among *ARS* elements, variations can occur in both the specific sequence of the consensus (3) and the energy required for unwinding the DUE (Figure 3). Such variations contribute to differences in *ARS* efficiency (6, 11); however, since both components contribute to *ARS* function, it is not possible to rank the efficiencies of different *ARS* elements by considering either component alone. An example that illustrates this point is a comparison between the novel A17 and B12 *ARS* elements. An attempt to predict their relative replication efficiencies based on helical stability would lead to the conclusion that B12, which has the lower helical stability (Figure 4), would be more efficient than A17. A prediction based on the extent of match to the *ARS* consensus would lead to the opposite conclusion—that A17, with a 10/11 match, would be more efficient than B12, which has a 9/11 match. However, neither prediction is correct since each *ARS* element has an identical replication efficiency (20).

ARS efficiency can also be influenced by additional sequence elements, such as replication enhancers (7) or short 3' sequence elements (9), each of which can overlap the DUE. The occurrence or activity of such additional sequence elements can vary among *ARS* elements (30–32). Only an appreciation of the quantitative contributions of all of the components (consensus, DUE, and additional sequence elements) will account for differences in *ARS* efficiency and help resolve certain paradoxical findings on different *ARS* elements. For example, base variations in the consensus that are inactive in the context of *ARS307* can be active in the context of other *ARS* elements (6, 7). Also, the DUE in a highly efficient *ARS* element, such as *ARS1*, can have a higher free energy requirement than the DUE of a less efficient *ARS* element, such as *ARS307* (Figure 3). Thus, while our findings indicate that the determinants of autonomous replication—a consensus element and a 3' DUE—are few, clearly the determinants of *ARS* efficiency can be many.

ACKNOWLEDGEMENTS

We thank Carol Newlon for providing the chromosome III *ARS*-containing plasmids, and Joel Huberman, Charles Miller, and Christine Brun for critical reading of the manuscript. This research was supported by grants (GM30614 and GM44119) from the National Institutes of Health.

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