The DNA unwinding element in a yeast replication origin functions independently of easily unwound sequences present elsewhere on a plasmid

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ABSTRACT

We have previously identified a DNA unwinding element (DUE) in autonomously replicating sequences (ARSs) and demonstrated a correlation between single-strand-specific nuclease hypersensitivity of the DUE and ARS-mediated plasmid replication in yeast. The DUE in the H4 ARS is the most easily unwound sequence in a supercoiled DNA molecule, in the context of the Ylp5 plasmid. To determine whether sequences which are more readily unwound than the ARS can influence replication activity, we have inserted such sequences, called 'torsional sinks', into the plasmids at a site distal to the ARS. We show that the torsional sink sequences effect reduction or elimination of the nuclease hypersensitivity of a variety of H4 ARS derivatives. However, we detect no difference in the *in vivo* replication activity of an individual ARS plasmid with or without a torsional sink. Thus, the function of the DUE in a yeast replication origin is unaffected by easily unwound sequences present elsewhere on the same plasmid.

INTRODUCTION

Autonomously replicating sequences (ARSs) function as replication origins on plasmids in the yeast *Saccharomyces cerevisiae* (1,2) and are composed of two main components: a consensus sequence [(A/T)TTTAT(A/G)TTT(T/A)] (3,4), presumed to bind an initiator protein, and a broad, non-conserved 3'-flanking sequence (4-6), proposed to serve a variety of roles (reviewed in 7). Our laboratory has demonstrated that the 3'-flanking sequence of ARSs is hypersensitive to single-strand-specific endonucleases in negatively supercoiled DNA (8,9). The nuclease hypersensitivity of H4 ARS derivatives with mutations in the 3'-flank correlates with ARS-mediated plasmid replication *in vivo* (9). Thermodynamically-stable DNA unwinding accounts for the nuclease hypersensitivity of both a broad A+T-rich sequence in the H4 ARS 3'-flank (10) and a pBR322 sequence that can functionally substitute for the ARS 3'-flank (9,11).

Our single-strand nuclease hypersensitivity assay identifies the plasmid DNA sequence with the lowest free energy cost for

unwinding (11). Support for the hypothesis that the ease of unwinding the ARS 3'-flank is of biological importance is derived from the finding that increased thermal energy in living cells can suppress mutations that raise the free energy cost for ARS unwinding (10). We call the easily unwound sequence a DNA unwinding element (DUE) and have proposed that the DUE serves as the entry site for replication enzymes into the DNA helix.

It is unclear whether the presence of easily unwound sequences elsewhere on a closed-circular plasmid influences yeast ARS function. Replication-negative H4 ARS derivatives with mutations in the DUE unwind at a specific site in the vector instead of at the ARS (9). Plasmid DNA sequences compete to unwind in our nuclease hypersensitivity assay (11). It is not known whether easily unwound sequences on the same plasmid compete to unwind with the DUE inside yeast cells and affect ARS function. The purpose of this study is to determine whether distal sequences which are more readily unwound than the DUE influence ARS function.

MATERIALS AND METHODS

Enzymes

P1 nuclease prepared according to Fujimoto et al. (12) was from Yamasa Shoyu Co. Ltd., Choshi, Japan. Enzymes from commercial suppliers were as follows: restriction enzymes, T4 DNA ligase and E. coli DNA polymerase I, Klenow fragment (New England Biolabs), calf intestinal phosphatase (Boehringer Mannheim), T4 polynucleotide kinase (P-L Biochemicals).

DNA

All plasmids were grown in *Escherichia coli* HB101 cells in L-broth (13). DNA was obtained from cells lysed by boiling in the presence of lysozyme (14). The superhelical densities of different plasmids prepared by this procedure are similar as determined by agarose gel electrophoresis (15) in the presence of chloroquine (11). To prepare large quantities of plasmid DNA for nuclease studies, the DNA was purified by two rounds of equilibrium centrifugation in cesium chloride density gradients containing ethidium bromide (13). The location of P1 nuclease

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Figure 1. A map of the torsional-sink plasmids. The plasmids are derivatives of YIp5 (29). The solid lines are pBR322 sequences, the hatched box is the *URA3* gene and the open box is the *ARS* insert. The *ARS* inserts are sequence derivatives of the H4 *ARS* which substitute the small, unique EcoRI-HindIII or EcoRI-BamHI fragment of YIp5 (6). The torsional-sink sequences were cloned as blunt-ended fragments into the unique PvuII site on the plasmids (see Materials and Methods). The position of the unique NruI site in the plasmids used to map the nuclease-hypersensitive sites is indicated. The position of the nuclease hypersensitive site in YIp5 [pBR322 site 1, defined in (11)] is labeled the 'vector site'.

hypersensitive sites were consistent among different preparations of the same plasmid.

Positional specificity of P1 nuclease nicks using the glyoxal modification protocol

P1 nuclease is a single-strand-specific endonuclease that acts on negatively supercoiled DNA at neutral pH (16). Supercoiled plasmids were treated with P1 nuclease as previously described (17). The nicked-circular molecules generated were then linearized at a unique restriction endonuclease site (see Figure legends). The 5' ends of the site of linearization were then dephosphorylated using calf intestinal phosphatase and ^{32}P labeled using T4 polynucleotide kinase and $\gamma\text{-}^{32}\text{P}\text{-ATP}$ (13) (the nick does not label in this protocol). The nicked strands were separated from the intact strands by denaturation in glyoxal (18) and electrophoresis through 1.2% agarose gels (20 cm) at 30 volts for 18 hours. Radiolabeled restriction fragments of known length were also prepared and electrophoresed to serve as mobility markers. The agarose gels were dried onto Whatman 3MM paper using a slab gel drier and exposed to Kodak XAR film. The mobilities of restriction fragments of known length were used to calculate the number of base pairs in the experimental bands. The number of bases in DNA fragments was calculated using the CURVEFIT program (from M. Komaromy and H. Govan) with a digitizing tablet (Houston High-Pad) interfaced to an Apple IIe computer (19). The accuracy of this method for DNA fragments between 500 and 5500 bases was better than ± 50 bases.

Construction of the torsional-sink plasmids

A previously-characterized, nuclease-hypersensitive sequence from the transcription termination region of the yeast *LEU2* gene (8) was cloned into the PvuII sites of the parent plasmids 74/8, L110 and rL79 (6) to generate TS1, TS3 and TS2 respectively. The 226bp sequence is a HaeIII-AccI restriction fragment corresponding to positions +971 to +1197 of the *LEU2* gene (20). The AccI end was made flush by the addition of nucleotides to the 3' end with the large fragment of DNA polymerase (13). The sequence was then ligated into the PvuII site of recipient

plasmids using T4 DNA ligase in conditions previously described (9). The TS8 and TS9 plasmids were generated by cloning a 291bp BamHI-EcoRI fragment of the plasmid R9 into the PvuII sites of plasmids rL35 and rL79 (6), respectively. The ends of the 291bp restriction fragment were made flush and the fragment ligated as described above.

S. cerevisiae transformations

The yeast strain YPH3 (*ura3-52*, *lys2-801*, *ade2-101*), obtained from Philip Hieter, Johns Hopkins University, was used to test plasmids for autonomous replication. The plasmids were transfected into the recipient cells using the lithium acetate procedure originally described by Ito et al. (21) and modified by Boguslawski (22). Aliquots of the transformed cells were plated at 30°C onto synthetic minimal medium containing 20 $\mu\text{g/ml}$ adenine sulfate and 30 $\mu\text{g/ml}$ lysine-HCl (23). The transformation frequency was approximately 3000 transformants per μg of plasmid DNA.

RESULTS

Experimental Rationale

We inserted additional, easily unwound sequences, called 'torsional sinks', into plasmids at sites distal to the *ARS*. A general diagram of the plasmids is presented in Figure 1. A variety of H4 *ARS* derivatives cloned in YIp5 served as parent *ARS* plasmids into which a torsional sink was inserted. The parent *ARS* plasmids lacking a torsional sink exhibit a correlation between reductions in replication activity and reductions in nuclease hypersensitivity of the origin (9). Our aims were to test whether unwinding at the torsional sink could reduce nuclease hypersensitivity at the parent *ARS* on the plasmid and to determine whether the presence of the torsional sink influences *ARS* function.

We chose two DNA sequences, previously characterized as nuclease-hypersensitive sites in negatively supercoiled DNA (8,9), to serve as torsional sinks. One is a 226bp sequence from the transcription termination region of the yeast *LEU2* gene (8). The 226bp sequence has a free energy cost for unwinding similar to the pBR322 hypersensitive site (8,11), which is also present in YIp5 (9). The other sequence is a derivative of the H4 *ARS*, called R9, which is inactive as a replication origin due to a deletion in the *ARS* consensus sequence (6). The R9 sequence retains the H4 *ARS* DUE (9,10) and unwinds in preference to the pBR322 hypersensitive site (unpublished results).

Nuclease hypersensitivity and replication activity of the torsional sink plasmids

Three plasmids were constructed with the 226bp torsional sink cloned into the PvuII site of parent *ARS* plasmids. A schematic diagram of the H4 *ARS* derivatives in the parent and torsional sink plasmids is shown in Figure 2A. A linker-scanning derivative of the H4 *ARS* (74/8) is the parent of the TS1 plasmid, while H4 *ARS* derivatives resulting from deletions in the 3'-flank are the parents of the TS3 (deletion L110) and TS2 (deletion rL79) plasmids. All three *ARS* parent plasmids transform a *ura3* strain to uracil prototrophy at high frequency (6), indicative of autonomous replication. However, they differ in their replication efficiencies (6) and ease of origin unwinding (9). The replication efficiencies of the parent plasmids (74/8 > L110 > rL79) correlate with their ease of origin unwinding (9).

Nuclease-hypersensitive sites on the torsional-sink plasmids were mapped as previously described (9). Briefly, supercoiled DNA is incubated with P1 nuclease to generate one nick per

Figure 2. Nuclease-hypersensitive sites and *ARS* activity of plasmids containing the 226 base pair torsional-sink sequence. **A)** Schematic diagrams of the H4 *ARS* derivatives present in the parent and 226bp sink plasmids. The 74/8, L110 and rL79 derivatives retain the *ARS* consensus sequence (open box) but differ in the amount of the 3'-flanking sequence (terminal nucleotide position indicated by numbers above the line). The filled box shown for the 74/8 derivative indicates a 10bp linker-scanning substitution. **B)** P1 nuclease hypersensitive sites in torsional-sink plasmids TS1, TS2 and TS3. The plasmids contain the 226bp *LEU2* sequence cloned into the *PvuII* site (see Figure 1). Negatively supercoiled TS1, TS2 and TS3 plasmids were nicked with P1 nuclease (17), linearized and 5'-³²P labeled at the unique *NruI* site. The products were denatured in the presence of glyoxal (18) and electrophoresed through a 1.2% agarose gel. The identity of each plasmid construct is indicated above the lane. Each nuclease hypersensitive site leads to formation of a specific band pair. The arrows denote the band pairs in the TS1 lane. The bold filled arrows denote the positions of migration of the products of P1 cleavage in the *ARS*. The hollow arrows indicate the products of cleavage in the torsional-sink sequence, while the thin arrows denote the vector-site cleavage products. The slowest-migrating species seen at the top of the lane is the unit length strand (*i.e.*, the strand which is not nicked by P1 nuclease). The order of the bands in the TS3 and TS2 lanes is the same as the TS1 lane, while the absolute sizes vary due to the specific H4 *ARS* derivative and its position in each plasmid. The positions of migration of single-stranded DNA markers of known sizes (in bases) are indicated to the right of the TS2 lane. **C)** *ARS* activity of the TS1, TS3 and TS2 plasmids and their parent plasmids lacking torsional sinks. The torsional-sink plasmids and their parent plasmids were transfected into a *ura3* yeast strain which requires the persistence of the plasmid borne *URA3* gene for growth in selective media. After transformation, an aliquot of the cells was plated on selective media, incubated three days at 30°C and photographed. The plates are displayed with the torsional-sink containing transformants at the top (row a) above their respective parent-plasmid-containing transformants (row b). Approximately 3000 transformants per microgram of DNA was obtained for each plasmid.

molecule. The nicked DNA is then subjected to digestion by a restriction endonuclease with a unique recognition site in the molecule. The procedure generates one intact, unit-length strand and two sub-unit length strands from the P1 nuclease nicked strand. The strands are radiolabeled at the 5' ends generated by the restriction endonuclease, permanently denatured with glyoxal and separated by agarose gel electrophoresis. The sizes of the sub-unit length bands, in bases, maps the distance from the restriction endonuclease recognition site to the single-strand-specific nuclease hypersensitive site.

The locations of the nuclease hypersensitive sites on the torsional sink plasmids were unambiguously assigned by independent mapping experiments from two different restriction sites. An autoradiogram of the P1 nuclease cleavage products resulting from plasmids linearized at the *NruI* site (see map in Figure 1) is shown in Figure 2B. The arrows to the left of the autoradiogram indicate pairs of cleavage products from specific

nuclease-hypersensitive sites in the TS1 plasmid. The bold, filled arrows indicate the position of migration of cleavage products from the *ARS* sequence in TS1. The intensities of the bands, determined by densitometric analysis, reveal that the *ARS* is the predominant site of nuclease-detectable unwinding in the TS1 plasmid (82% of the cleavage products) as previously shown for the parent plasmid 74/8 (90% of the cleavage products) (9). Thus, the *ARS* contains the most easily unwound sequence in both the parent plasmid 74/8 and the TS1 plasmid with the 226bp torsional sink. Notice that the 226bp torsional-sink sequence (open arrow) is cleaved at a similar, albeit slightly lower, frequency to the vector site (thin arrow) in TS1. In fact, the torsional-sink site and the vector site band intensities are similar to each other in all three constructs analyzed in Figure 2B (central four bands in each lane). The results are consistent with our previous observation that the 226bp sequence and the pBR322 vector site have similar unwinding energy requirements (*i.e.* each account

Figure 3. Nuclease-hypersensitive sites and ARS activity of plasmids containing the 291 base pair torsional-sink sequence. **A)** Schematic diagrams of the H4 ARS derivatives in the parent and 291bp sink plasmids. The rL35 and rL79 derivatives retain the ARS consensus sequence (open box) but differ in the amount of the 3'-flanking sequence (terminal nucleotide position indicated by numbers above the line). **B)** P1 nuclease-hypersensitive sites in the TS8 and TS9 plasmids and their parent plasmids lacking torsional sinks. The nuclease cleavage products were generated and separated as described in Figure 2B. The identity of each plasmid construct is indicated above the lanes. The bold, filled arrows and the thin arrows refer to the ARS and vector site cleavage products respectively in the rL35 and rL79 lanes, while the open arrows refer to the positions of the torsional-sink site cleavage products in the TS8 and TS9 lanes. Lane M contains single-stranded DNA markers, of which some of the sizes (in bases) are indicated on the right. **C)** ARS activity of the TS8 and TS9 plasmids and their parent plasmids lacking torsional sinks. The plasmids were transfected, cells grown and plates photographed as in Figure 2C. The torsional-sink-containing transformants are displayed in the top row (a) above their respective parent-plasmid-containing transformants (b).

for similar proportions of the nuclease cleavage products when no other nuclease-hypersensitive sites are present) (8). In contrast to the effects of the torsional sink in the TS1 construct, the torsional sink in the TS3 and TS2 plasmids *does* result in significant reductions in the relative nuclease hypersensitivity of the ARS. In the TS3 plasmid, all three hypersensitive sites are cleaved at near equal frequency, since the intensities of the bands are nearly equal. The L110 ARS accounts for 37% of the nuclease cleavage products in TS3 (Figure 2B), while the same ARS accounts for 70% of the products in the parent plasmid (9). In the TS2 plasmid, the rL79 ARS accounts for only 17% of the nuclease cleavage products, while 55% of the nuclease cleavages occur in the same ARS in the parent plasmid (17). We conclude that the 226bp torsional sink significantly competes with the ARS for unwinding within the TS3 and TS2 plasmids.

To compare the replication competence of the torsional-sink plasmids with their parents, the plasmids were transfected into a *ura3* strain of yeast which requires the plasmid-borne *URA3* marker for growth in selective medium. The transfected cells were then plated on medium lacking uracil. A photograph of the plates three days after transfection is shown in Figure 2C. Transformation of the *ura3* strain to uracil prototrophy at high frequency is indicative of autonomous plasmid replication. All of the parent plasmids (row b) yield transformants at high frequency consistent with previous results (6). In addition, all of the torsional-sink plasmids transform the *ura3* strain at high frequency (row a). Thus, the torsional-sink plasmids are replication competent in the high frequency transformation assay despite significant competition for unwinding between the ARS and the 226bp sink in the TS2 and TS3 plasmids *in vitro*.

Additional information on plasmid replication efficiency can be gained from the colony sizes on the plates presented in Figure 2C. Colony size on selective medium correlates with replication efficiency of ARS plasmids harboring a selectable marker (5,10,24,25). In row b of Figure 2C, colony size decreases from left to right reflecting decreasing replication efficiency (74/8 >

L110 > rL79). The torsional-sink-containing transformants show a similar decrease in colony size from left to right in row a of Figure 2C. In fact, the colony size of each of the torsional-sink-containing transformants appears to be most similar to transformants harboring the respective parent plasmid. Thus, no detectable reductions in replication efficiency accompany the reductions in ARS unwinding observed between the parent and respective torsional-sink plasmids.

The 226bp torsional-sink sequence effects only modest reductions in unwinding of ARS derivatives which require the least free energy for unwinding (and are the most efficient replication origins). In an attempt to further reduce the nuclease hypersensitivity of more readily unwound ARS derivatives, the 291bp R9 sequence (described above) which requires less free energy for unwinding than the 226bp sequence, was used to construct torsional-sink plasmids. The 291bp sequence was cloned into the PvuII sites of the parent ARS plasmid rL35 to generate TS8 and into plasmid rL79 to generate TS9 (Figure 3A). The rL35 ARS replicates efficiently (6) and unwinds in preference to the YIp5 vector site (17). The rL79 ARS unwinds at near equal frequency to the vector site. The rL79 plasmid is the parent ARS plasmid described above for TS2.

Figure 3B shows an autoradiogram of the cleavage products of the TS8 and TS9 torsional-sink plasmids harboring the 291bp sequence as well as the cleavage products of the parent plasmids. The bold, filled arrows indicate the positions of the ARS cleavage products and the thin arrows point to the vector site cleavage products in the parent plasmids (lanes rL35 and rL79). The open arrows denote the position of torsional-sink sequence cleavage products in the TS8 and TS9 lanes. Densitometric analysis of the rL35 lane reveals that the ARS accounts for 80% of the nuclease cleavage products, in agreement with previous results (17). However, in the TS8 plasmid which contains the rL35 ARS, the predominant site of plasmid unwinding is the torsional sink. Similarly, in the TS9 construct, the torsional-sink sequence unwinds in such preference that unwinding at the rL79 ARS is

barely detected. Instead, nuclease-detectable unwinding in the TS9 plasmid occurs almost exclusively at the torsional sink. Thus, the insertion of the 291bp torsional-sink sequence into the parent plasmids leads to near elimination of *ARS* unwinding in the TS8 and TS9 plasmids. The replication competency of the TS8 and TS9 plasmids were compared with that of the respective parent plasmids as described above. Figure 3C shows a photograph of transformants obtained after transfection of the plasmids into a *ura3* strain. All plasmids generate uracil prototrophs at high frequency. Furthermore, the colony sizes of the torsional-sink-containing transformants are similar to the colony sizes of the transformants harboring the respective parent plasmids (compare plates within a column). Thus, the replication efficiencies of the TS8 and TS9 plasmids are not detectably reduced relative to those of their respective parents. The plate of the rL79 parent (Figure 3C, row b, column TS9) is the same plate presented in the photograph in Figure 2C (row b, column TS2). We note that the colony sizes of the transformants containing the rL79, TS2 and TS9 plasmids appear equal, while the occurrence of unwinding at the *ARS* within the plasmids varies greatly.

DISCUSSION

Earlier we showed that the DUE in the H4 *ARS* is the most easily unwound sequence in a supercoiled plasmid and that mutations that decrease the occurrence of unwinding at the DUE are accompanied by decreased plasmid replication activity (9,10). The results presented above demonstrate that it is possible to decrease the occurrence of DNA unwinding at the *ARS* in the plasmid by inserting 'sinks' for DNA torsional stress at a distal site. However, we detect no difference in *ARS*-mediated replication of plasmids with or without torsional sinks. These findings are consistent using a variety of H4 *ARS* derivatives that differ in replication efficiency and the ease of unwinding the DUE. Our findings are also consistent for two different genetic elements acting as the torsional sink: the *LEU2* gene terminator region and an inactive H4 *ARS* derivative which lacks the consensus sequence. We conclude that the DUE in a yeast replication origin functions independently of easily unwound sequences present elsewhere on the same plasmid.

Our findings suggest that in yeast cells, in contrast to our *in vitro* assay, the *ARS* does not compete to unwind with other DNA sequences present on a closed-circular plasmid. The mechanism of unwinding yeast replication origins *in vivo* is not known; however, the functional requirement for both the 11bp consensus sequence (3,4) and an easily unwound sequence (9,10) suggests a role for a specific DNA binding protein in addition to the DUE. Initiation of DNA unwinding at bacterial replication origins (26–28) and at a mammalian viral (29) replication origin requires binding of a specific initiator protein. We postulate that at yeast origins, as is the case for *E. coli oriC* (30), only a particular easily unwound sequence, the DUE, which is properly positioned in relation to an initiator protein binding site, is a substrate for the unwinding activity of the replication initiation complex.

The ease of unwinding sequences elsewhere on an *ARS* plasmid, while unimportant to *ARS* function *in vivo*, governs the ability to detect a DUE using our nuclease hypersensitivity assay. Kipling and Kearsley (31) observed that certain *ARS* plasmids are hypersensitive in the vector but not at the *ARS*. As shown here, insertion of a sequence more readily unwound than the resident *ARS* in a plasmid can eliminate nuclease hypersensitivity at the *ARS*. Thus, as suggested by Kipling and Kearsley (31) and

demonstrated here, easily unwound sequences elsewhere in the plasmid can mask the nuclease hypersensitivity at the *ARS*.

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