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Thermal energy suppresses mutational defects in DNA unwinding at a yeast replication origin

(DNA replication/autonomously replicating sequences/*Saccharomyces cerevisiae*/supercoiled DNA/DNA unwinding element)

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ABSTRACT **Yeast replication origins contain a DNA sequence element whose biological activity correlates with hypersensitivity to single-strand-specific nucleases in negatively supercoiled plasmids. By using two-dimensional gel electrophoresis of plasmid topoisomers, we demonstrate that thermodynamically stable origin unwinding accounts for the nuclease hypersensitivity and, furthermore, that increased thermal energy facilitates stable origin unwinding *in vitro*. In living cells, increased thermal energy can suppress origin mutations that raise the free-energy cost for unwinding the nuclease-hypersensitive element. Specifically, mutational defects in autonomously replicating sequence (*ARS*)-mediated plasmid replication are less severe in cells grown at 30°C as compared to 23°C. Our findings indicate that the energetics of DNA unwinding at the nuclease-hypersensitive element are biologically important. We call the nuclease-hypersensitive sequence the DNA unwinding element (DUE) and propose that it serves as the entry site for yeast replication enzymes into the DNA helix.**

Origins of DNA replication, through a combination of genetic and physical mapping techniques, have been identified in the yeast *Saccharomyces cerevisiae* (1–4). The origins were originally defined as autonomously replicating sequences (*ARS*s). The DNA sequence requirements of *ARS* function have been studied through comparative and mutational sequence analysis (5–7). An 11-base-pair core consensus sequence [(A/T)TTTAT(A/G)TTT(A/T)] is required for origin function. The core consensus sequence is thought to be the recognition site for an initiator protein, since point mutations in the sequence lead to loss of *ARS* function (6). Additionally, a broad flanking sequence, 3′ to the core consensus, is also required for efficient replication (7). Sequence comparisons and mutational analyses have been less revealing about the role of the flanking sequence. Although high in A+T content in general, the flanking sequence exhibits little primary sequence homology from one *ARS* to another (5). Furthermore, the flanking sequence tolerates certain substitutions, insertions, and deletions within the required region (7, 8). A variety of roles have been proposed for the flanking DNA sequence (for review, see refs. 9 and 10); however, the actual role(s) of the *ARS* 3′ flanking sequence has not been firmly established.

Our laboratory has demonstrated that the 3′ flanking sequence of *ARS*s is hypersensitive to single-strand-specific endonucleases in negatively supercoiled DNA (11, 12). We presumed that the nuclease hypersensitivity reflects a low free-energy requirement for unwinding. We proposed that the ease of unwinding the origin is a determinant of replication initiation since the nuclease hypersensitivity of origin mutants correlates with autonomous replication (12).

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Here we directly demonstrate that the nuclease-hypersensitive element in the H4 *ARS* has a low free-energy requirement for DNA unwinding and, furthermore, that thermal energy facilitates origin unwinding *in vitro*. These findings, combined with the fact that the unwinding free energies of different nuclease-hypersensitive elements can be ranked by their position in the hierarchy of nuclease hypersensitivity (13), provided an experimental rationale for testing whether the energy cost for unwinding the *ARS* 3′ flanking sequence is important for biological function. We report that mutations in the nuclease-hypersensitive element that sufficiently increase the free-energy cost for origin unwinding lead to an enhanced dependence on elevated temperature for efficient plasmid replication. The data strongly support the hypothesis that the nuclease-hypersensitive element facilitates localized unwinding at the replication origin in yeast cells. We call the nuclease-hypersensitive sequence the DNA unwinding element (DUE) and propose that, like the DUE in the *Escherichia coli* chromosomal origin (14), it serves as the entry site for replication enzymes into the DNA helix.

MATERIALS AND METHODS

Enzymes. P1 nuclease, prepared according to Fujimoto *et al.* (15), was from Yamasa Shoyu (Choshi, Japan). T4 DNA ligase was from New England Biolabs and calf thymus DNA topoisomerase I was from Bethesda Research Laboratories.

DNA. All plasmids were grown in *E. coli* HB101 cells in Luria–Bertani broth. DNA was obtained from cells lysed by boiling in the presence of lysozyme (16). The extractable superhelical density of different plasmids prepared by this procedure is −0.067, as determined by agarose gel electrophoresis (17) in the presence of chloroquine. The DNA was purified by two rounds of equilibrium centrifugation in cesium chloride density gradients containing ethidium bromide (18).

Preparation of Plasmid Topoisomers and Two-Dimensional Gel Electrophoresis. Plasmid topoisomers were prepared using a modification of the protocol presented by Wang (19). Supercoiled pAB9 (8) was incubated with P1 nuclease and quantitatively converted to nicked-circular DNA (20). The nicks were then covalently closed using T4 DNA ligase in the presence of various concentrations of ethidium bromide. Twelve separate ligation reactions (containing ethidium bromide concentrations from 1.2 to 7.8 μg/ml) were pooled to generate the topoisomer population shown in Fig. 1*A*. Alternatively, the entire spectrum of topoisomers was generated by limited topoisomerase I relaxation of supercoiled molecules (21) extracted from *E. coli*. Gel electrophoresis of

Abbreviations: DUE, DNA unwinding element; *ARS*, autonomously replicating sequence.

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plasmid topoisomers was performed in submerged 1% agarose gels. Topoisomers were separated in the first dimension at 23°C or 37°C in the same solution conditions used for the P1 nuclease reaction (12). A 1-cm-wide strip containing the topoisomers separated in the first dimension was fused to a second-dimension gel. The second-dimension gel was equilibrated to 36 mM Tris/30 mM NaH_2PO_4/1 mM Na_2EDTA, pH 7.8, containing chloroquine (5 μg/ml). After second-dimension electrophoresis, the gels were soaked in distilled water to remove the chloroquine, then soaked in distilled water containing ethidium bromide (1 μg/ml), and photographed under UV illumination.

Yeast Transformations. The *S. cerevisiae* strain used to test plasmids for autonomous replication, YPH3 (*ura3-52, lys-801, ade2-101*), was obtained from Philip Hieter (Johns Hopkins University). The test plasmids were transfected into the recipient cells using the lithium acetate procedure originally described by Ito *et al.* (22) and modified by Boguslawski (23). Aliquots of the transformed cells were plated at 23°C or 30°C onto synthetic minimal medium containing adenine sulfate (20 μg/ml) and lysine hydrochloride (30 μg/ml). The transformation frequency was ≈3000 transformants per μg of plasmid DNA. The selective liquid medium used for cell doubling time experiments was the same as that used in the selective plates.

RESULTS AND DISCUSSION

Thermodynamically Stable DNA Unwinding at a Yeast Replication Origin. We have reported (11, 12) a single-strand-specific nuclease-hypersensitive site in the broad flanking sequence of yeast replication origins. Nuclease hypersensitivity of a pBR322 sequence has been shown to be the consequence of thermodynamically stable DNA unwinding, as opposed to transient unwinding ("breathing") (13). To test for stable unwinding of a yeast replication origin, we performed two-dimensional gel electrophoresis of plasmid topoisomers (24, 25) containing the H4 *ARS* (8). Plasmid topoisomers were electrophoresed in the first dimension in the same solution conditions in which the hypersensitive site is detected (12). Topoisomers differ in their linking number and, therefore, in their number of superhelical turns. In general, the greater the number of negative superhelical turns in the molecule, the greater its mobility in the resolving portion of the first-dimension gel. However, plasmid topoisomers that possess a stably unwound sequence migrate more slowly than expected on the basis of their linking number, since each helical turn unwound removes one superhelical turn. The second-dimension electrophoresis, perpendicular to the first, is conducted in a buffered solution containing chloroquine. Chloroquine unwinds the DNA by intercalation, reversing any stably unwound regions present and resulting in the separation of the topoisomers on the basis of linking number alone. Fig. 1*A* shows a two-dimensional gel analysis of the plasmid pAB9. The pAB9 plasmid has a 374-base-pair *Sau*3AI fragment containing the H4 *ARS* cloned into the *Bam*HI site of pUC8 (8). The positions of migration of certain topoisomers as well as the nicked circular (N) form are indicated. The majority of topoisomers are negatively supercoiled during the first-dimension electrophoresis. Selected negatively supercoiled topoisomers are labeled (topoisomers 7, 10, 11, and 15).

Fig. 1*A* shows that, in the first dimension (top to bottom), all topoisomers of negative superhelicity beyond topoisomer 10 migrate slower than expected for their linking number. The retarded mobility of these topoisomers is indicative of stable DNA unwinding. Stable DNA unwinding is detected in the pAB9 plasmid in the same solution conditions and temperature in which the H4 *ARS* flanking sequence is hypersensitive to single-strand-specific nuclease (12).

To firmly establish the relationship between stable unwinding of the plasmid and nuclease hypersensitivity of the yeast replication origin, pAB9 topoisomers were incubated with the single-strand-specific nuclease P1 prior to two-dimensional gel electrophoresis. The results are shown in Fig. 1*B*. The stably unwound topoisomers are preferentially cleaved and are converted to the nicked circular form (compare to Fig. 1*A*). Mapping of the P1 nuclease cleavages (data not shown) reveals that the yeast origin sequence is hypersensitive in the stably unwound topoisomers. Therefore, thermodynamically stable DNA unwinding accounts for the nuclease hypersensitivity of the yeast replication origin. We conclude that the nuclease-hypersensitive element has a low free-energy requirement for localized DNA unwinding relative to other sequences on the plasmid.

Thermal Energy Facilitates Stable Origin Unwinding. A+T-rich sequences that are nuclease hypersensitive on supercoiled plasmids at 37°C do not display this property at lower temperature (26). We wished to determine whether stable unwinding at the A+T-rich flanking sequence in a yeast replication origin is also temperature dependent. Topoisomers of pAB9 were subjected to two-dimensional gel electrophoresis as above except that the first-dimension electrophoresis was carried out at 23°C instead of 37°C. The results are shown in Fig. 1*C*. Each topoisomer migrates faster than topoisomers of fewer supercoils in the first dimension (top to bottom). Thus, stable DNA unwinding is not detectable in the pAB9 plasmid at 23°C in the range of superhelicity examined. Combined with the results presented in Fig. 1 *A* and *B*, the data demonstrate that elevated temperature facilitates localized DNA unwinding at the yeast replication origin *in vitro*.

Thermal Energy Can Suppress Origin Mutations That Increase the Free-Energy Cost for Unwinding the Nuclease-Hypersensitive Element. Our findings that the nuclease-hypersensitive element in the H4 *ARS* has a low free-energy cost for DNA unwinding and that increased thermal energy facilitates origin unwinding *in vitro* provided an experimental rationale for testing whether the energy required for unwinding the hypersensitive element is a determinant of replication efficiency *in vivo*. Thermal energy affects DNA unwinding *in vivo* in a general way and cell growth temperature is not normally thought to limit origin unwinding. However, if the energy cost for unwinding the nuclease-hypersensitive element is important for biological function, a mutation in the element that sufficiently increases the free-energy cost for unwinding is expected to enhance the temperature dependence of origin unwinding and *ARS* plasmid replication in living cells. In such a mutant origin, the replication defect is expected to be more severe at reduced temperature.

Fig. 2 (*Upper*) shows a schematic diagram of mutant H4 *ARS* derivatives containing progressive deletions in the nuclease-hypersensitive element. The demonstration that thermodynamically stable DNA unwinding accounts for the nuclease hypersensitivity of the *ARS* enables us to rank the free-energy requirement for unwinding mutant derivatives based on their position in the hierarchy of nuclease hypersensitivity. For stably unwound DNA sequences detected under our specific conditions, the hierarchy of nuclease hypersensitivity is such that the higher ranking sites reflect a lower free-energy cost for DNA unwinding (13). Based on their relative nuclease hypersensitivities in the same vector DNA context (12), the mutant H4 *ARS* derivatives increase in the free-energy cost for origin unwinding (i.e., become more difficult to unwind) in the following order: rL35 < rL96 < rL55.

Plasmids containing the mutant *ARS* derivatives were transfected into a yeast strain that requires the plasmid-borne *URA3* gene for growth in selective medium. Aliquots of the transformations were plated at 30°C and 23°C. At 6 days after transfection, all of the origin derivatives tested yielded transformants at high frequency (data not shown), indicative of autonomous replication at both 30°C and 23°C. At shorter

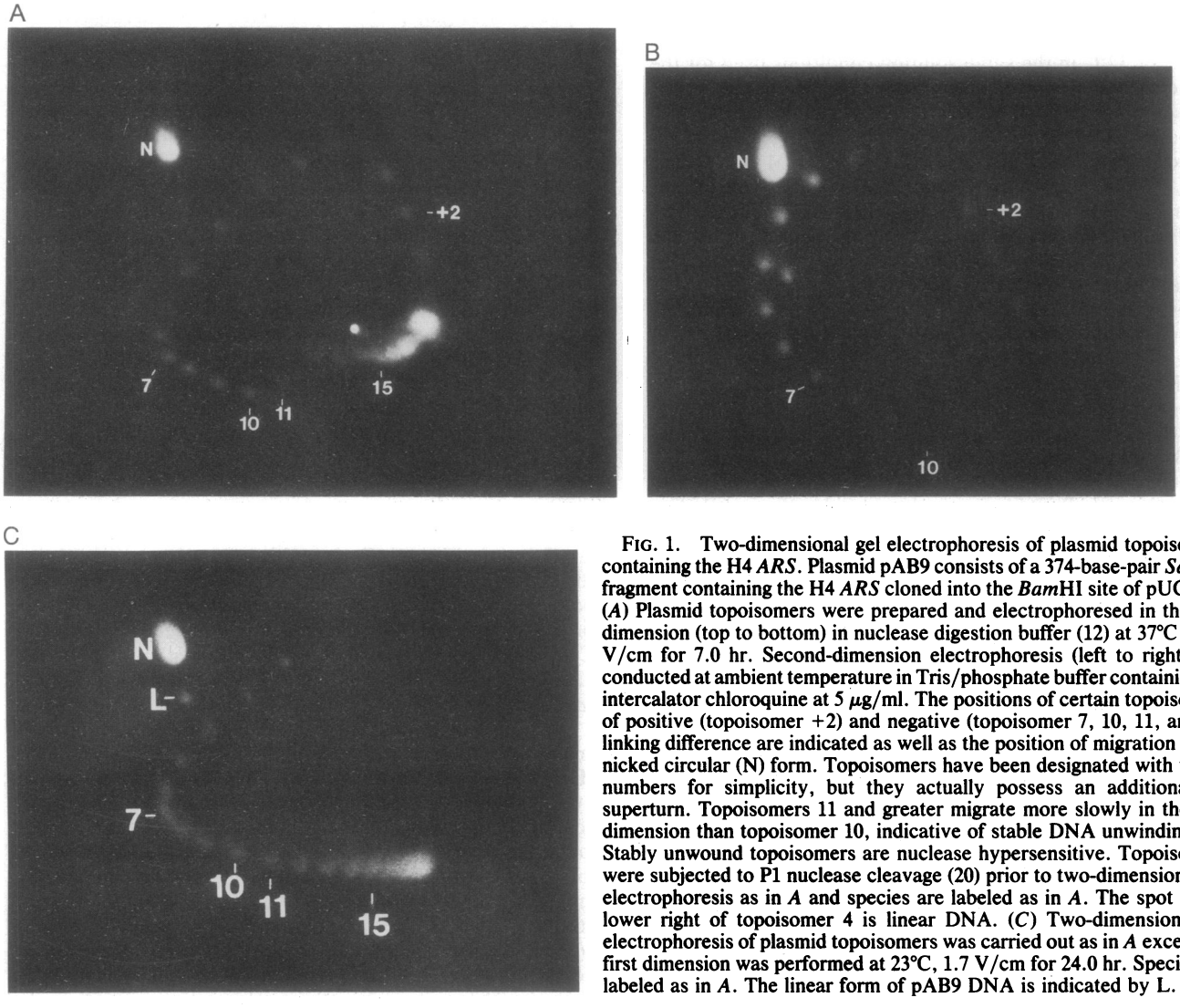

FIG. 1. Two-dimensional gel electrophoresis of plasmid topoisomers containing the H4 *ARS*. Plasmid pAB9 consists of a 374-base-pair *Sau*3AI fragment containing the H4 *ARS* cloned into the *Bam*HI site of pUC8 (8). (*A*) Plasmid topoisomers were prepared and electrophoresed in the first dimension (top to bottom) in nuclease digestion buffer (12) at 37°C at 4.8 V/cm for 7.0 hr. Second-dimension electrophoresis (left to right) was conducted at ambient temperature in Tris/phosphate buffer containing the intercalator chloroquine at 5 μg/ml. The positions of certain topoisomers of positive (topoisomer +2) and negative (topoisomer 7, 10, 11, and 15) linking difference are indicated as well as the position of migration of the nicked circular (N) form. Topoisomers have been designated with whole numbers for simplicity, but they actually possess an additional 0.5 superturn. Topoisomers 11 and greater migrate more slowly in the first dimension than topoisomer 10, indicative of stable DNA unwinding. (*B*) Stably unwound topoisomers are nuclease hypersensitive. Topoisomers were subjected to P1 nuclease cleavage (20) prior to two-dimensional gel electrophoresis as in *A* and species are labeled as in *A*. The spot to the lower right of topoisomer 4 is linear DNA. (*C*) Two-dimensional gel electrophoresis of plasmid topoisomers was carried out as in *A* except the first dimension was performed at 23°C, 1.7 V/cm for 24.0 hr. Species are labeled as in *A*. The linear form of pAB9 DNA is indicated by L.

times after transfection, examination of the plates revealed differences in the average colony size of transformants containing the different *ARS* deletion derivatives. The 3-day transformants are displayed in the plates shown in Fig. 2 (*Lower*). Comparison of the colonies containing the individual *ARS* mutants at 30°C and at 23°C reveals a temperature dependence of colony size for certain *ARS* mutants. That is, the size of the rL96 (B) and rL55 (C) colonies is greatly reduced at 23°C relative to 30°C, in marked contrast to the size of the rL35 (A) colonies, which is only slightly reduced. Colony size on selective medium reflects replication efficiency for *ARS* plasmids bearing a selectable marker in *S. cerevisiae* (7). The results tentatively identify rL96 and rL55 as *ARS* mutants with a temperature dependence for plasmid replication that is enhanced relative to that for rL35.

The replication efficiency of *ARS* plasmids largely, if not completely, determines plasmid persistence in a dividing cell population. *ARS* plasmids lack a centromere element and are poorly segregated to daughter buds during cell division (27). Plasmids containing weaker *ARS* elements generate more plasmid-less cells in the dividing cell population (7). The death of plasmid-less cells in the selective growth medium results in an increased doubling time for the population.

To quantitate plasmid replication mediated by the *ARS* mutants as a function of temperature, single colonies from the 30°C plates shown in Fig. 2 were used to inoculate selective liquid medium and the percentage of plasmid-containing cells in the population and the population doubling time were determined for exponentially growing cultures. The results are shown in Table 1. Comparison of the plasmid replication properties of the individual *ARS* derivatives at 30°C and 23°C reveals a marked temperature dependence for the rL96 and rL55 mutants. Specifically, the population doubling time of cells containing the rL35 plasmid increases only 14% with a temperature decrease from 30°C to 23°C, while the doubling times of cells containing the rL96 and rL55 plasmids increase by 46% and 111%, respectively, with the same temperature variation. Additionally, the same temperature decrease leads to a reduction in the percentage of plasmid-containing cells in the rL96 and rL55 populations but not in the rL35 population. The results presented in Table 1 are consistent with the comparative colony growth rates observed in Fig. 2 in that the rL96 and rL55 plasmid-containing cells are more sensitive to temperature variation than the rL35 plasmid-containing cells. The preservation of these differences, despite inoculation of the 23°C liquid cultures with single colonies picked from the 30°C plates (see legend to Table 1), confirms that we have not selected for irreversible alterations in cell growth properties on the plates. The results indicate that the replication efficiency of the rL96 and rL55 *ARS* plasmids is more dependent on temperature than that of the rL35 *ARS* plasmid.

The free-energy cost for unwinding the nuclease-hypersensitive element in the origin mutants increases in the following order: rL35 < rL96 < rL55. This information plus the above results reveal that origin mutants with a greater free-energy cost for unwinding the nuclease-hypersensitive

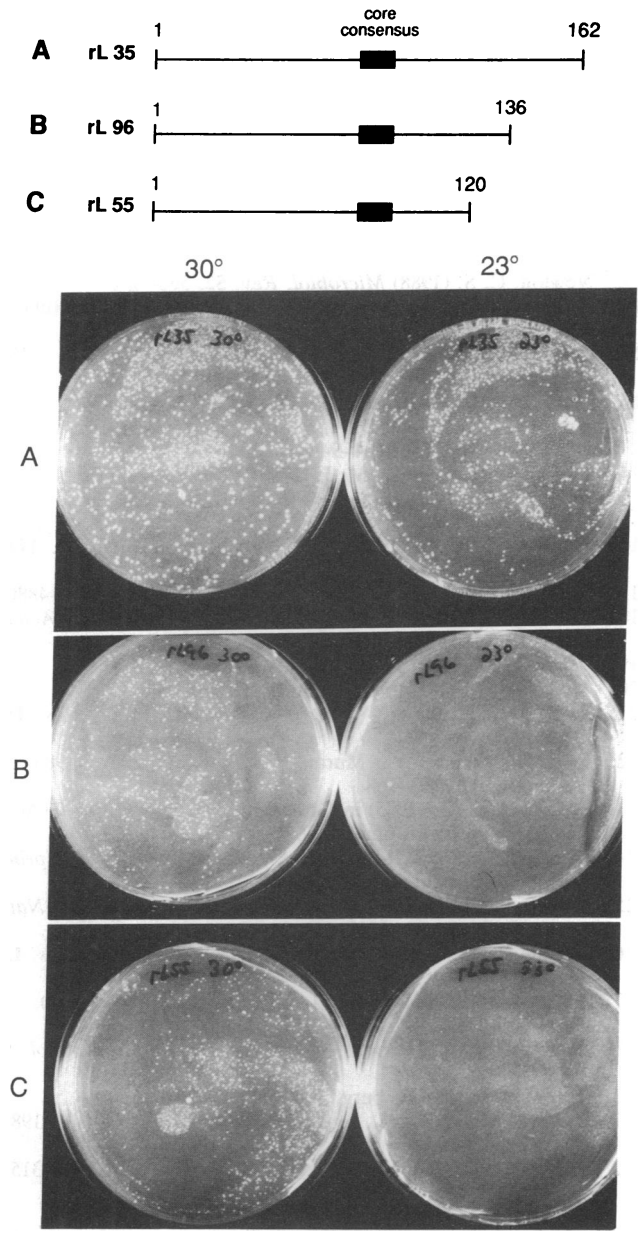

FIG. 2. Temperature-dependent plasmid replication for H4 *ARS* deletion derivatives *in vivo*. (*Upper*) Schematic diagrams of the H4 *ARS* derivatives studied. The rL35 (diagram *A*), rL96 (diagram *B*), and rL55 (diagram *C*) derivatives retain the core consensus sequence but differ in the amount of 3′ flanking sequence that comprises the nuclease-hypersensitive element (12). The numbers above the diagram for each derivative refer to the nucleotide positions retained (8). (*Lower*) The mutant derivatives of the H4 *ARS* shown above, present in place of the *Eco*RI–*Hin*dIII fragment on the YIp5 plasmid (8), were transfected into a *ura3* strain, YPH3, using lithium acetate (23), and aliquots were plated on selective medium at 30°C and 23°C. The plates were photographed 3 days after transfection. Plates A, B, and C correspond to diagrams A, B, and C, respectively (*Upper*).

element (rL96 and rL55) exhibit an enhanced temperature dependence of *ARS*-mediated plasmid replication. The mutational defect is less severe in cells grown at 30°C as compared to 23°C (Table 1). We conclude that increased thermal energy in the living cell can suppress mutational defects in unwinding the nuclease-hypersensitive element at the replication origin.

Biological Role for the Yeast Replication Origin Element with a Low Free-Energy Cost for DNA Unwinding. Our investigation into the basis of the nuclease hypersensitivity of the

Table 1. Plasmid replication efficiencies of H4 *ARS* derivatives as a function of cell growth temperature

ARS derivative	Doubling time, hr		% plasmid-containing cells	
	30°C	23°C	30°C	23°C
rL35	3.5	4.0	45	58
rL96	3.5	5.1	24	4.4
rL55	3.6	7.6	18	7.1

A single colony from the 30°C plates shown in Fig. 2 was used to inoculate selective liquid medium and grow saturated cultures. The time required to reach saturation varied with the *ARS* derivative (24 hr for rL35 or 48–72 hr for rL96 and rL55). The saturated cultures were diluted to 1×10^6 cells per ml. Aliquots of the diluted cultures were kept in a shaker incubator at 30°C or 23°C. Doubling time was monitored by counting cells in a hemocytometer from 1×10^6 through 1×10^7 cells per ml. At 5×10^6 cells per ml, 200–300 cells were removed from the 30°C and 23°C cultures and plated on selective and nonselective plates and these plates were then incubated at 30°C. The percentage of plasmid-containing cells is the number of colonies present on the selective plates divided by the number of colonies on the nonselective plates. In the absence of selection (medium supplemented with uracil), the doubling times of the nontransformed cells were 1.8 hr at 30°C and 2.2 hr at 23°C.

H4 *ARS* has revealed that the hypersensitive element has a low free-energy cost for DNA unwinding relative to other sequences in the vector. The relative free-energy costs for unwinding different *ARS* mutants can be ranked by their relative nuclease hypersensitivities in the same vector DNA context. The free-energy cost for unwinding the hypersensitive sequence *in vitro* is indicative of the energy requirement for unwinding the same DNA sequence in living cells since free energy is a thermodynamic parameter and is independent of the unwinding mechanism (e.g., supercoiling, DNA unwinding enzymes, and DNA binding proteins). The energetics of DNA unwinding at the nuclease-hypersensitive element are important for biological function as evidenced by our discovery that increased thermal energy can suppress origin mutations that raise the free-energy cost for unwinding the element. Thus our findings strongly support the hypothesis (12) that the nuclease-hypersensitive element facilitates localized unwinding at the replication origin in living yeast cells.

We call the nuclease-hypersensitive sequence the DNA unwinding element (DUE). The DUE in the H4 *ARS* (12), the 2-μ plasmid origin (11), and other *ARS* elements tested (unpublished results) resides in a broad sequence 3′ to the thymidine-rich strand of the core consensus sequence, as illustrated in Fig. 3. We presume that an initiator protein specifies the replication origin by binding the core consensus sequence. This is consistent with the observation that *ARS* function requires a core consensus (protein recognition) sequence that is sensitive to single-base mutations (6). Our working model (10) is that, after the initiator protein binds, the DUE becomes locally unwound (either melted or partially unwound). Localized unwinding at the DUE *in vivo* is consistent with our observations that replication activity of *ARS* derivatives containing mutant DUEs can be modulated by both the free-energy cost for unwinding the DUE and cell growth temperature. We propose that unwinding the DNA helix at the DUE ultimately facilitates the entry of yeast replication enzymes, which are otherwise unable to access the individual strands of the DNA template.

An alternative to a role for the *ARS* 3′ flanking region in DNA unwinding has been suggested. Palzkill and Newlon (28) proposed that the 3′ flanking region functions by providing *ARS* consensus-related sequences that serve as additional binding sites for the putative initiator protein. Holmes and Smith (29) ruled out this hypothesis for the H4 *ARS* since replication function is fully retained after simultaneous dis-

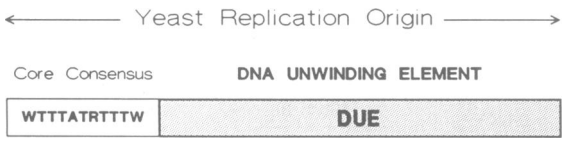

FIG. 3. Organization of DNA sequence elements in a *S. cerevisiae ARS* (see text). In the consensus sequence, W represents A or T and R represents A or G.

ruption of all consensus-related sequences in the required 3′ flanking region. The point mutations used for disruption were multiple A → T and T → A inversions that maintained the wild-type base composition of the 3′ flanking sequence. We have shown that the ease of unwinding the 3′ flanking DNA is not affected by multiple point mutations, even those that increase the G+C composition (12). Thus, the findings of Holmes and Smith (29) are consistent with our proposal that the 3′ flanking region functions in DNA unwinding (refs. 10 and 12; this report).

The organization of DNA sequence elements we have described for replication origins of *S. cerevisiae* (Fig. 3) is similar to the sequence organization of the *E. coli* chromosomal replication origin (*oriC*). We have found that *oriC* contains a DUE located adjacent to the initiator protein (DnaA) binding sites (14). The DUE maps to the same sequence that unwinds in a DnaA-protein- and temperature-dependent reaction to facilitate entry of the replication enzymes into the DNA (30). The DUE may be a general component of DNA replication origins in which localized DNA unwinding is induced by initiator protein binding (30–32).

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