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Functional analysis of a replication origin from *Saccharomyces cerevisiae*: identification of a new replication enhancer

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ABSTRACT

Yeast replication origins have a modular arrangement of essential DNA sequences containing the ARS consensus sequence (ACS) flanked by auxiliary DNA elements which stimulate origin function. One of the auxiliary elements identified at several origins is a DNA replication enhancer that binds the Abf1p protein. We have isolated an ARS sequence from *Saccharomyces cerevisiae* based on its ability to bind Abf1p. Here we present a detailed molecular dissection of this ARS, designated ARS*1501*, and we demonstrate that it functions as a genomic replication origin on chromosome *XV*. Mutagenesis of the Abf1p DNA-binding sites revealed that these sequences did not contribute significantly to ARS function. Instead, a new DNA element important for replication, designated REN1501, has been located 5′ to the T-rich strand of the ACS. We show that REN1501 functions in either orientation and at variable distances from the ACS, defining this element as a DNA replication enhancer. Most significantly, point mutations within this element decreased the stability of plasmids bearing ARS*1501*, suggesting that REN1501 binds a protein important for replication initiation. Only three elements found at origins are known to specifically bind proteins. These include the ARS essential sequences and the Abf1p and Rap1p DNA-binding sites. We show that the function of REN1501 at the origin cannot be replaced by a Rap1p DNA-binding site or a site that binds the transcriptional factor Gal4p and can only be partially substituted for by an Abf1p recognition sequence. This implies that the role of the REN1501 element at the ARS*1501* origin is specific, and suggest that the frequency of origin firing in eukaryotic cells may be regulated by origin-specific enhancers.

INTRODUCTION

Replication of eukaryotic chromosomal DNA proceeds by numerous initiations along a chromosome, generating multiple replicons. Initiation at replicons of *Saccharomyces cerevisiae* occurs at specific sites corresponding to the autonomously replicating sequences (ARSs). Hence studies on the organization of functional domains at ARSs are crucial for understanding the mechanism and regulation of initiation of DNA replication. ARSs were first isolated as DNA fragments that confer on plasmids bearing them the ability to replicate autonomously in yeast (1). Some ARSs were shown to function as origins of replication *in vivo* in plasmids as well as in their native chromosomal context (2–7).

Recent molecular dissection of several ARSs has shown that origins are composed of multiple functional domains (6,9–11). These origins appear to consist of an essential core sequence containing an 11 bp [(A/T)TTTA(T/C)(A/G)TTT(A/T)] ARS consensus sequence and auxiliary elements flanking the core (12–13). In ARS*121* these elements, which can be connected by random DNA without apparent loss of origin activity, interact to elicit maximal origin function (6).

One of the DNA elements flanking the essential core sequence in several ARSs is the binding site for the multifunctional Abf1p (14–19). Previous studies have shown that this site functions as an enhancer of DNA replication in an orientation and distance independent manner (20). Another element (DUE/B2/ATR) shared by all ARSs is located 3′ to the T-rich strand of the ACS (13). In ARS*121* the correct positioning of this (A+T)-rich element relative to the essential sequences is critical for origin activity (6).

The modular arrangement of domains important for origin function raised the possibility that multiple protein factors interact with these elements. Recently, proteins (ORC and CBF) interacting specifically with the essential sequences of several ARSs have been identified (21–22). Other proteins such as MCMs have been implicated in the initiation of replication but their precise target at the origin has not yet been reported (23–24). Proteins interacting with the auxiliary elements present in some ARSs have also been identified and characterized. These include the multifunctional Abf1p and Rap1p proteins (14–19). Based on the genetic analysis of their cognate DNA-binding sites, these proteins fulfill multiple cellular functions including regulation of transcription and DNA replication.

The mechanism of DNA replication initiation in yeast is unknown. It is also unknown how the yeast enhancers of replication work. However, the recent *in vitro* reconstitution of a multiprotein

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complex at ARS*121*, which required the Abf1p, suggests a direct involvement of the enhancer in initiation. In this system Abf1p in concert with OBF2 (HDF), the Ku homolog in *S.cerevisiae*, stabilized the specific interaction of the ORC-related CBF with the essential sequences of ARS*121* (25).

Although an Abf1p DNA-binding site was identified at several origins, including ARS*120*, ARS*121*, ARS*1* and *HMRE* (14–19), not all studied ARSs were found to contain a site that binds Abf1p. To extend our functional analysis on the role of Abf1p in replication, we searched for additional origins containing a Abf1p DNA-binding site. This involved screening a yeast genomic library for DNA fragments that bind Abf1p and confer on plasmids the ability to transform yeast at a high frequency. Several such clones have been isolated. Here we report a detailed molecular dissection of one of these clones, ARS*1501*, which we demonstrate is an origin of replication on chromosome *XV*. These studies have unexpectedly revealed the presence of a new DNA replication enhancer, REN1501, not observed in other characterized ARSs. Our results indicate that this enhancer is unique and cannot be functionally replaced by other known enhancers, suggesting that frequency of origin firing in eukaryotic cells may be regulated by origin-specific enhancers.

MATERIALS AND METHODS

Strains and growth conditions

The *Escherichia coli* strain used for transformation and plasmid propagation was HB101. *Saccharomyces cerevisiae* strain FY251 (*MAT***a**, *ura3-52*, *his3*Δ*200*, *trp1*Δ*63*, *leu2*Δ*1*) was used for all plasmid transformations and maintenance studies. The yeast strain was kindly provided by Fred Winston, Harvard. Bacteria and yeast were grown as before (20).

Isolation of ARS*1501*

A Sau3A1 yeast genomic library was prepared in pUC19 DNA essentially as described before (26). Plasmid DNA, bearing yeast genomic fragments, was incubated with Abf1p in a reaction mixture described previously (16). After incubating at room temperature, the reaction mixture was supplemented with anti Abf1p polyclonal antibodies. The entire mixture was then loaded on a protein A–Sepharose column. The column was washed with a buffer containing 10 mM Tris–HCl, pH 7.5, 5 mM EDTA and 0.2 M NaCl. DNA that bound to the protein A resin through its interaction with Abf1p was eluted by a wash with the Tris buffer containing 1 M NaCl. The eluted DNA was concentrated by ethanol and transformed into *E.coli* HB101. DNA was prepared from individual transformants and used in DNA competition experiments to test for Abf1p binding by the procedure described before (16). Using this approach, a number of plasmid clones containing yeast genomic DNA capable of binding Abf1p have been identified. To test their ability to transform yeast, the yeast inserts were excised from these clones by double digestion with *Eco*RI and *Hin*dIII and then subcloned into the yeast shuttle vector YIp5. Several clones were identified capable of both binding Abf1p and transforming yeast at a high frequency. ARS*1501* DNA interacted most tightly with Abf1p.

Construction of the FY251(a1) strain

The FY251(a1) strain was constructed by replacing the genomic ARS*1501* sequence with an ARS*1501* mutated in the ACS. This construction was carried out by transforming the FY251 strain with the YIp5AB1501 (a1) plasmid, mutated in the ACS, cut once with the *Mfe*I restriction enzyme. Nucleotides 474–479 of ARS*1501* comprise a *Mfe*I restriction site. Transformants were identified by growth on plates containing ura$^-$ synthetic medium. Integration of plasmids bearing the mutated ARS DNA was confirmed by Southern analysis. Since the integration of the cut DNA leads to a duplication of the ARS sequence, a 5′-fluoro-orotic acid selection (27) was applied to identify cells that lost one of the two copies of the ARS*1501* sequence. Cells containing the mutated ARS*1501* were again identified by Southern analysis.

Yeast transformation and mitotic stability analysis

The origin of replication assay used was high frequency transformation of *S.cerevisiae* and mitotic stability analysis of ARS*1501*-containing plasmids. These were performed essentially as described before (6). Cells harboring an ARS-containing plasmid were propagated in a non-selective medium. After 14 generations, the fraction of cells (expressed as percentage) retaining the plasmid was estimated by plating the cells on agar plates containing non-selective and selective synthetic medium. Each mitotic stability was determined for four to six independent clones. Mitotic stabilities were determined only for the ARSs cloned into the YCp5-3 vector, which contains the CEN3 centromeric sequence.

Plasmid construction and DNA manipulations

Plasmids pUC19, YIp5 and YCp5-3 (YIp5/CEN3) have been previously described (6). p19AB1501, YRpAB1501 and YCpAB1501 were ARS*1501* DNA (617 bp) cloned as a *Hin*dIII/*Eco*RI fragment into pUC19, YIp5 and YCp5-3, respectively. ARS*1501′* was created first in p19AB1501′ by deleting nt 1–117 from ARS*1501*. This was achieved by cutting the plasmid with *Hin*cII and *Sna*BI restriction enzymes. The plasmid backbone containing the remaining part of the ARS was then isolated and religated. YCpAB1501′ contains ARS*1501′* cloned into the YCp5-3 vector DNA.

Linker substitution and deletion mutagenesis

Linker substitution mutations and some deletions were introduced into ARS*1501* using PCR (polymerase chain reaction). All mutations were created in ARS*1501′*, which we refer to as wild type ARS, unless indicated otherwise. The DNA substrates for the PCR reaction were either p19AB1501′ or YCpAB1501′. Synthetic oligonucleotides containing either a *Pst*I, *Xho*I or *Sph*I restriction sequence were prepared. In the first PCR cycle two complementary oligonucleotides were used to prime DNA synthesis at a site of interest. This resulted in the production of two DNA fragments with an overlapping sequence, each containing the mutation. These fragments were then used in a second PCR cycle to regenerate a complete mutated ARS. Multiply mutated ARS sequence was generated by PCR as above except that ARS*1501′* DNA containing a mutation served as a template for further mutagenesis.

Deletions from the 5′ end of ARS*1501′* were formed by utilizing the *Pst*I linker substitution mutations. The plasmid bearing the mutated ARS DNA was cut with *Pst*I and *Hin*dIII and the resulting DNA fragment (minus the deletion) was moved into pUC19 cut with *Pst*I and *Hin*dIII or *Eco*RI. The cloned ARS was then moved into the YCp5-3 vector as an *Hin*dIII/*Eco*RI DNA fragment. Occasionally, deletions were also formed by first cutting

the plasmid bearing the mutated ARS with *Pst*I and *Hin*dIII and then religating the plasmid using *Pst*I/*Hin*dIII single-stranded linkers, essentially as described before (6). Deletion from the 3′ end were formed by utilizing the *Xho*I linker substitution mutations. The YCpAB1501′ plasmid bearing the mutated ARS was cut with the *Xho*I and *Hin*dIII restriction enzymes. The termini of the plasmid were then filled in and religated to yield a plasmid bearing ARS*1501′* with a deletion of interest.

Internal deletions within the ARS sequence were generated by the PCR procedure described above, except that the synthetic oligonucleotides used contained an internally deleted sequence. All mutations introduced *in vitro* were confirmed by DNA sequencing.

Analysis of replicating intermediates by two-dimensional electrophoresis in agarose gels

The Brewer and Fangman procedure (2) was used to analyse genomic replicating intermediates. The DNA was prepared and analysed essentially as described before (6).

RESULTS

Isolation of ARS*1501* and definition of the essential core sequence

ARS*1501* was isolated from a yeast genomic library on the basis of its ability to bind Abf1p. This screen, performed as described in Materials and Methods, yielded several plasmid clones that bound Abf1p and transformed yeast at a high frequency. ARS*1501* was selected for a detailed molecular analysis because it bound the Abf1p with high affinity.

As shown in Figure 1, the DNA sequence of the 617 bp ARS*1501* fragment revealed the presence of several 11 bp sequences that resemble the ARS consensus sequence (10/11 matches). As expected, we also found DNA sequences corresponding to the Abf1p recognition site, TCN$_7$ACG (19). These are located at nt 118–129 and 561–572. Both sites interact with Abf1p, although only the site at nt 561–572 bound Abf1p at a high affinity (unpublished observations).

To assess the contribution of the identified DNA elements to origin function, it was first necessary to delineate the ARS essential sequences. The approach to this analysis was similar to the one we used for dissecting the functional domains of ARS*121* (6,20). First, to define the essential sequences it was necessary to find the functional ACS. Two observations pointed to the element at nt 326–336 (Fig. 1) as the functional homolog of the ACS. First, the construction of ARS*1501′* (Materials and Methods) which lacked an ACS-like sequence normally present at nt 66–76 functioned as an effective origin of replication. Second, a sequence comparison of the essential core of ARS*121* with the ARS*1501* DNA (Fig. 1) revealed >60% homology in the region containing nt 317–350. This region contained another ACS-like sequence.

To test the potential function of this ACS-like sequence, we introduced a series of *Pst*I linker substitution mutations in the region encompassing nt 312–396 as described in Figure 2. For convenience, we refer in this study to DNA sequences positioned 3′ and 5′ to the T-rich strand of the ACS as downstream and upstream sequences, respectively. This analysis demonstrated that an alteration of the ACS-like sequence (a1 mutation) resulted in total inactivation of the origin as judged by the inability of the mutated ARS to transform yeast at a high frequency. Inactivation of the ARS was also obtained by the d1 mutation positioned downstream and

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GCCCTGCTGA TTATGTTACG GATAACAAGA TAGAATATCC TTTGGAGCTC    50
                   10/11
GACAATTCTA CATAGTATTA TATTTTTCTC TTCTTCTTTT CTTTGTTCAT
                        ABF1(II)
TTCCATTGTC ATATTTACGT ATTTGCTGAT GATTGTTGGC ATTCCATAAT   150

ATAGATAACA TTATGATAAT AATTTCGTCT AACAATCTGT ATCATCTTAT

CAAACCATAC ATAGCAATAT ACTACTGATT TAAAACCAGC TAATACAAGA   250

TATTTGATCT CTATTTCAAA CAAAGGATTT TAAATGTTCT TCAATATGGT
                              10/11
CTTCTTTTTT CATTTATTTT TTTTGATTAA TATTTTGTTG AAAAGTAAAA   350

GTTTTGAGAC ATGCAACTTG TATTTCTCAG AAGTGAATAA AAATAAATAT

ATGAGATTTA AGATAGAAAA AACAAATCAT CTTTAAAAAC TTTCTTTCCT   450
             10/11
AGTATAGCCA CTTTTTATAG TTACAATTGT AAAGAACTGC CTATAAACAA

TAAGTCTGTC TGCCATATCT ACGTTGTTAT TTAATTTCAC TAAACAACCG   550
          ABF1(I)
CCCAGAAAAT CGTACAGTGT GATTTTCTTT CGTCATCTAA TATTTCCCAA

TGAGGTAAAA GGTAAAT                                       617
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Figure 1. The ARS*1501* DNA sequence. The ARS*1501* DNA was isolated as described in Materials and Methods. The DNA sequence was determined by the standard primer extension method. The 11 bp sequence that resembles the ARS consensus (10/11 matches) is underlined with a heavy line. DNA sequences corresponding to the Abf1p recognition sites are underlined with a thin line.

juxtaposed to the ACS (Fig. 2A). All other mutants described in Figure 2A could transform yeast. Nonetheless, to determine whether these mutants affected the efficiency of the origin, we performed mitotic stability analysis of plasmids bearing the mutated ARS DNA. This analysis showed that the d3 mutation positioned 12 nt downstream to the ACS significantly lowered plasmid mitotic stability. All other linker substitution mutations, spanning a region of 59 nt outside the ACS (Fig. 2C), had no major effect and did not reveal any other DNA element important for origin function.

To identify other functional regions in ARS*1501*, we performed deletion analysis. This analysis also delineated the size of the minimal sequence required for ARS activity. As shown in Figure 2B and D, the Δu1 and Δd4 deletions defined the upstream and downstream boundaries of the minimal sequence, respectively. The minimal sequence that can support origin function spans a region of ~29 nt starting with the ACS and including the sequence defined by the d3 mutation. This suggests a bipartite structure for the essential core sequence of ARS*1501* similar to the organization of the essential sequences of other ARSs (6,9,10,28).

ARS*1501* is an origin of replication on chromosome *XV*

To test whether ARS*1501* functions as a genomic origin, we first mapped the genomic location of ARS*1501* by probing a gel containing separated yeast chromosomes with ^{32}P-labeled ARS*1501* DNA. This analysis has shown that ARS*1501* is located on chromosome *XV* (unpublished observations). We then used standard procedures to generate a partial restriction map of chromosome *XV* in the region containing the ARS*1501*, as shown in Figure 3A. While this work was in progress, the database for the *S.cerevisiae* genome was reported. The ARS*1501* DNA sequence (coordinates 437191–436575) was identified on chromosome *XV* in an intergenic region downstream to the YORCdelta11 open reading frame and upstream to the ASE1 gene encoding a microtubule-associated protein necessary for anaphase spindle elongation (29).

To determine if ARS*1501* is an active origin of DNA replication on chromosome *XV*, we used the Brewer and Fangman 2D agarose

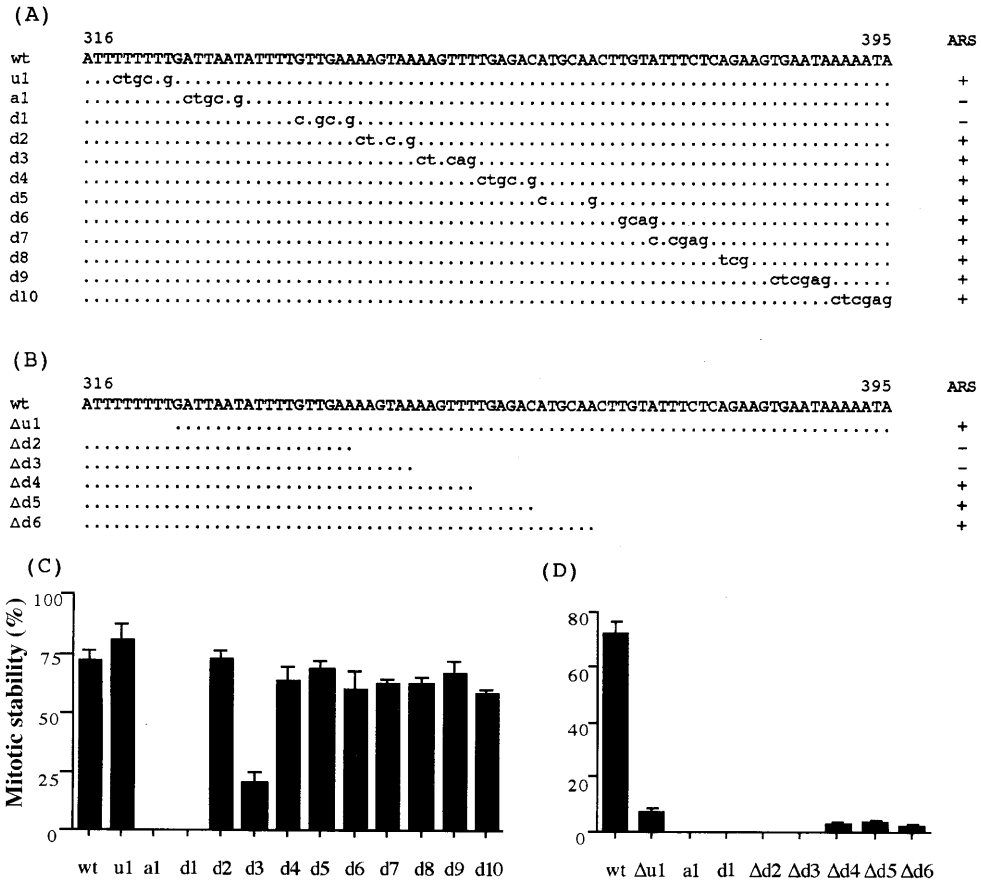

Figure 2. Definition of the essential core sequence (minimal origin of replication) by linker substitution and deletion mutagenesis. **(A)** A segment of the ARS*1501* (nt 316–395) that was mutagenized by linker substitutions as described in Materials and Methods. Lower case letters indicate the change in the DNA sequence resulting from the *Pst*I linker substitution; dots represent nucleotides in the sequence. ARS function was determined by yeast transformation. **(B)** Deletions generated as described in Materials and Methods. **(C)** and **(D)** Mitotic stability of plasmids bearing the linker substitution and deletion mutations described in **(A)** and **(B)**, respectively. + indicates the ability of the plasmid bearing the mutated origin to transform yeast at a high frequency; – indicates the inability of the plasmid to transform yeast. Mitotic stability measurements were performed as described in Materials and Methods.

gel electrophoresis procedure for the analysis of replicating intermediates (2). Yeast genomic DNA was digested with either *Bgl*II or *Hin*cII restriction enzymes, which generated a 5.7 and a 3.2 kb DNA fragment, respectively, containing the ARS*1501*. This restriction digest positioned the ARS*1501* sequence off the center in the *Bgl*II fragment and near a terminus of the *Hin*cII fragment. Each of these digests was subjected to 2D electrophoresis in agarose gels. Following electrophoresis, the DNA was probed with a radioactively labeled ARS*1501* DNA shown in Figure 3.

The image of two DNA arcs was visible in the *Bgl*II digest (Fig. 3B). The most leftward arc (upper arrow) is characteristic of intermediates containing replication bubbles, while the second arc is characteristic of replicated Y-shaped molecules (lower arrow). The presence of the bubble arc indicates that initiation of DNA replication has occurred within the 5.7 kb *Bgl*II DNA fragment.

The Y arc appears to be enriched for intermediates containing large Y molecules (>1.5× the mass of the monomer fragment), as indicated by the relatively high intensity of radioactive label in the area concentrated between the inflection point and the tip of the arc. If replication proceeds in a bidirectional manner with both forks moving at similar rates, then the origin appears to be asymmetrically placed, in agreement with the location of the ARS in the *Bgl*II DNA fragment (Fig. 3A). If this is true, then placing the ARS near a terminus of a restriction fragment should yield predominantly Y-shaped molecules. Indeed, Y-shaped molecules are exclusively present in a *Hin*cII digest (Fig. 3C), consistent with the bidirectional movement of replication forks initiated at the ARS*1501* origin.

In addition, to show that ARS*1501* is the origin of replication on chromosome *XV* responsible for the 2D pattern shown in Figure 3, we constructed a strain in which the genomic ARS*1501* sequence was replaced by the a1 mutant ARS. The requirement of the ACS for origin firing in the chromosome has been shown (8). This ARS derivative contains a linker substitution in the ACS that completely inactivates ARS function (Fig. 2). As shown in Figure 3D, the *Bgl*II genomic digest of DNA isolated from a strain containing the a1 mutation yielded only Y-shaped molecules, indicating that initiation failed to occur at the mutated origin. The observed Y-shaped molecules presumably originated from incoming forks which were initiated outside the boundaries of the *Bgl*II fragment.

An important stimulatory DNA region is located downstream to the essential core sequence

Deletion analysis described in Figure 2D identified DNA sequences important for origin function flanking the essential core. For

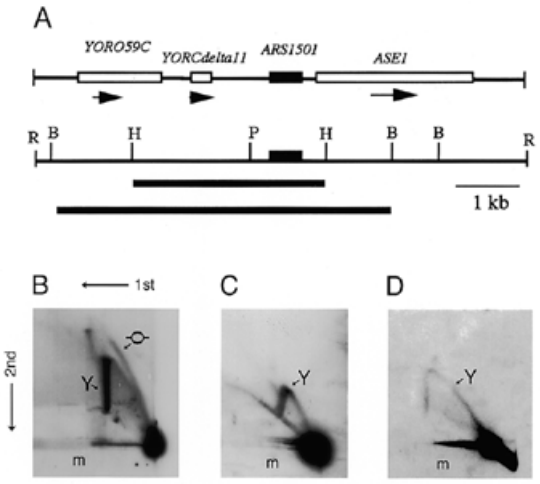

Figure 3. ARS*1501* is an origin of replication on chromosome *XV*. **(A)** Schematic presentation of a genetic and restriction map of the region in chromosome *XV* containing ARS*1501*. The information used to depict the genetic locus of ARS*1501* was reproduced from the yeast genomic DNA sequence database. The open boxes and the arrows designate the direction of the open reading frames. The ARS*1501* DNA sequence, whose chromosomal coordinates are 437191–436575, is represented by the filled-in box. The letters R, B, H and P designate the following restriction enzymes *Eco*RV, *Bgl*II, *Hinc*II and *Pst*I, respectively. **(B)** Autoradiogram of genomic DNA digested with *Bgl*II and subjected to electrophoresis in two dimensions as described in Materials and Methods. The arrows point to a 'bubble' arc and a 'Y' arc, the letter m designates the position of the monomeric *Bgl*II fragment. **(C)** Same as **(B)** except that the radioactive profile represents a *Hinc*II digest. **(D)** Same as **(B)** except that the yeast strain used for isolating the DNA was FY251(a1) in which the genomic ARS*1501* was inactivated by the a1 linker substitution mutation in the ACS.

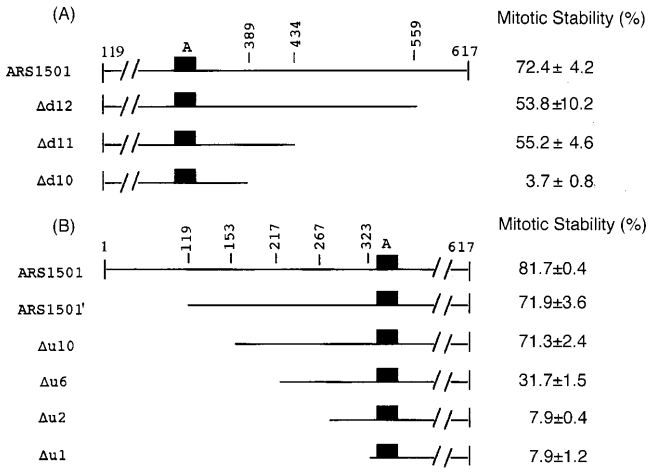

Figure 4. Identification of DNA elements important for origin function located downstream and upstream to the ACS. **(A)** Schematic presentation of the deletions introduced in the ARS. The horizontal numbers above the top line designate first and last nucleotides of ARS*1501'*. The vertical numbers designate the ARS*1501* nucleotide joined to the 5′ end of the linker mutation. **(B)** Schematic presentation of the deletions introduced in the ARS. The horizontal numbers above the top line designate the first and last nucleotides of ARS*1501*. The vertical numbers designate the ARS*1501'* nucleotide joined to the 3′ end of the linker mutation. The filled-in box designates the ACS labeled as A. Plasmid mitotic stability was determined as described in Materials and Methods.

example, the deletion Δd6 caused a drastic reduction in plasmid mitotic stability, suggesting that a DNA sequence 3′ to nt 372 (Fig. 1) is necessary for normal origin activity. To determine the 3′ boundary of this region, a series of deletions from the 3′ terminus were generated. As shown in Figure 4A, a deletion created from a *Xho*I linker substitution mutation at nt 435–440 (Δd11) had a minor effect on the mitotic stability of the ARS*1501*-containing plasmid. In contrast, deletion of an additional 49 nt (Δd10) rendered the origin ineffective. This indicated that an important DNA sequence is located between nt 386 and 435. Examination of the DNA sequence in this region revealed that A+T nucleotides comprise 88% of the DNA content, thus resembling the DUE/B2/ATR region of other ARSs (6,9,10,30).

Identification of a new DNA element, REN1501, required for ARS function

With the exception of the Abf1p and Rap1p DNA-binding sites, all auxiliary elements important for origin function have been located downstream to the ACS. The first indication that ARS*1501* may contain an important DNA sequence upstream to the ACS came from the analysis of the Δu1 deletion described in Figure 2. This deletion, which eliminated the entire upstream flanking sequence (nt 1–324), considerably decreased plasmid mitotic stability.

To localize the DNA sequence that affects plasmid mitotic stability, deletion analyses described in Figure 4B were performed. *Pst*I linker substitution mutations have been introduced in the upstream region at intervals of ~50 nt. Deletions were created utilizing the newly formed *Pst*I sites as described in Materials and Methods. As shown in Figure 4B, the Δu2 and Δu10 deletions defined the boundaries of a region (nt 153–267) important for origin activity. In contrast to the deletions, the linker substitution mutations u1, u2 and u10 had no effect on the plasmid mitotic stability. However, the mitotic stability of plasmids containing the u6 linker substitution at nt 213–218 was similar to that of the Δu6 deletion (Fig. 5), suggesting that the u6 mutation lies within a DNA element important for origin function.

To further delineate this region, single linker substitution mutations were introduced in the DNA sequences surrounding nt 213–218. As shown in Figure 5B, mutations u4 and u9 define the boundaries of a 24 bp nucleotide sequence (CAAACCATACA-TAGCAATATACTA), termed REN1501 (replication enhancer 1501), within which linker substitution mutations caused a significant reduction in the ARS*1501* activity.

The analysis of the u7 mutation was particularly interesting since it altered only three bases of the wild type sequence. This mutation, however, appeared to have the largest effect on origin function. Guided by the results of the u7 mutation, we have extended this analysis to single point mutations within the REN1501 element, as shown in Figure 5C. While the alteration of nucleotide 207A to a C had no effect on origin function, the change of 209A and 212T to a G reduced plasmid stability to levels comparable to a deletion of 24 nt (Δ207/230), which spans most of the REN1501 element. These results strongly suggest that REN1501 serves as a recognition site for a protein that functions in initiation of DNA replication.

Abf1p DNA-binding sites have a minor role in the function of ARS*1501*

The DNA sequence of ARS*1501* (Fig. 1) revealed the presence of two Abf1p DNA-binding sites flanking the ACS. Direct DNA

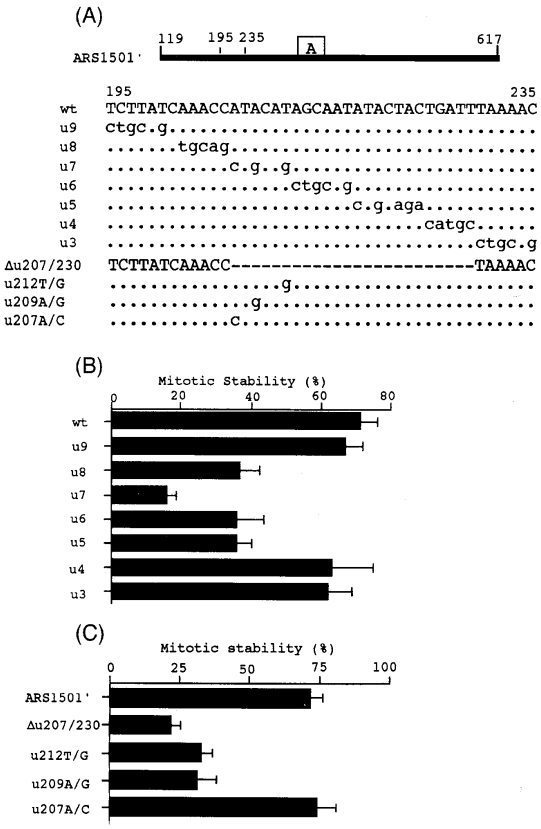

Figure 5. Definition of the REN1501 element. **(A)** Schematic presentation of ARS*1501′*. The filled-in box designates the ACS labeled as A. The relative position of nt 195 and 236 is also shown. The DNA sequence encompassed by nt 195–236 is designated in upper case letters. Lower case letters show the change in the DNA sequence resulting from linker substitution mutations. The dots represent nucleotides. The mutagenesis that yielded the u5 mutation introduced an additional alteration of a C to an A nucleotide 3′ to the linker. The mutation u4 was generated using a *Sph*I linker. This mutagenesis altered only 5 nt. Single base alterations 212T/G, 209A/G, 207A/C and the internal deletion Δu201/223 were introduced by PCR mutagenesis as described in Materials and Methods. **(B)** Histogram describing the mitotic stability of plasmids bearing ARS*1501′* containing the corresponding linker substitution mutations. **(C)** Histogram describing the mitotic stability of plasmids bearing single base substitution mutations.

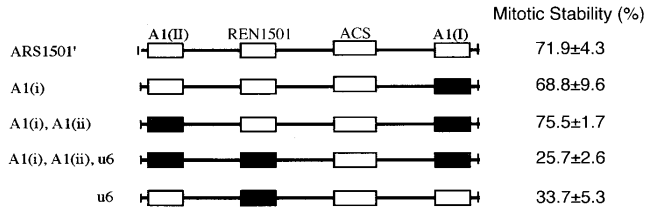

Figure 6. DNA-binding sites do not contribute appreciably to the ARS*1501* function. **(A)** Schematic presentation of the Abf1p DNA-binding sites [A1(I) and A1(II)], the ACS and the REN1501 element. The open box represents the wild type sequence. The filled-in box represents the mutated element. A1(i) designates an internal deletion at nt 560–574. A1(ii) designates a *Xho*I linker substitution mutation at nt 117–122. Both mutations eliminated the binding of Abf1p to ARS*1501*, as judged by a DNA mobility shift assay in agarose gels (unpublished observations). u6 is a linker substitution mutation described in Figure 5. Plasmid mitotic stability was determined as described in Materials and Methods.

binding studies have shown that Abf1p has a high affinity for site (I) and low affinity for site (II) (unpublished observations).

Previous deletion analysis described in Figure 4 suggested that the Abf1p DNA-binding sites may have a minor role in ARS*1501* function. For example, the Δd11 deletion caused a minor decrease in plasmid mitotic stability, although it eliminated the high affinity site (I). Similar effects were observed with the Δu10 which deleted site (II). Since such minor effects on plasmid stability would be consistent with the sites fulfilling a redundant function, multiple mutations were introduced into the ARS*1501* sequence (Fig. 6). It is evident that eliminating Abf1p DNA binding does not result in a significant decrease of plasmid mitotic stability. A substantial decrease in plasmid mitotic stability was obtained only when the REN1501 element was also mutated. This effect can be ascribed exclusively to the REN1501 element since mutagenesis of REN1501 alone resulted in a similar loss of plasmid mitotic stability (Fig. 5).

REN1501 element is a DNA replication enhancer

Previous studies have shown that the Abf1p DNA-binding site in ARS*121* works as a replication enhancer in an orientation and distance independent manner (20). We examined whether REN1501 exhibited similar properties. As shown in Figure 7C, the inverted sequence of REN1501 (nt 201–223) is capable of fully stimulating the origin. In addition, REN1501 stimulated the origin when placed at a distance of >220 nt on either side of the ACS. In its native context REN1501 is located 130 nt upstream to the ACS. The insertion of REN1501 at new locations was carried out in a plasmid containing the Δ203–230 deletion which eliminated REN1501 function. Construction of newly containing REN1501 ARS resulted in the concomitant inactivation of the Abf1p DNA-binding sites.

Since mitotic stability conferred by the recombinant origins was lower relative to the wild type ARS (Fig. 7C), some effect of distance from the ACS on the function of REN1501 cannot be excluded. It is also possible that the REN1501 flanking DNA sequences influence the functional efficiency of this element. Nonetheless, the results clearly show that REN1501 works in either orientation and at variable distances from the ACS (Fig. 7C), defining REN1501 as a replication enhancer.

Other yeast enhancers cannot replace REN1501 function

REN1501 represents the third enhancer element identified in ARSs. Other known enhancers include the Abf1p and Rap1p DNA-binding sites (9,20,31). To test if these enhancers can functionally replace REN1501, the REN1501 sequence was replaced by the Abf1p and Rap1p sites, as shown in Figure 7A. Similarly, the recognition sequence for the transcription factor Gal4p was tested for its ability to substitute for REN1501 (Fig. 7B). The results described in Figure 7A and B show that both the Rap1p and Gal4p DNA-binding sites failed to stimulate the origin. The stability of the plasmids containing these elements equalled that of a plasmid bearing an origin containing a REN1501 deletion.

Substitution of REN1501 with an Abf1p DNA-binding site only partially stimulated the origin (Fig. 7A). In this experiment the Abf1p site of ARS*121* was used. Although these results suggest a possible role for Abf1p at ARS*1501*, it is evident that

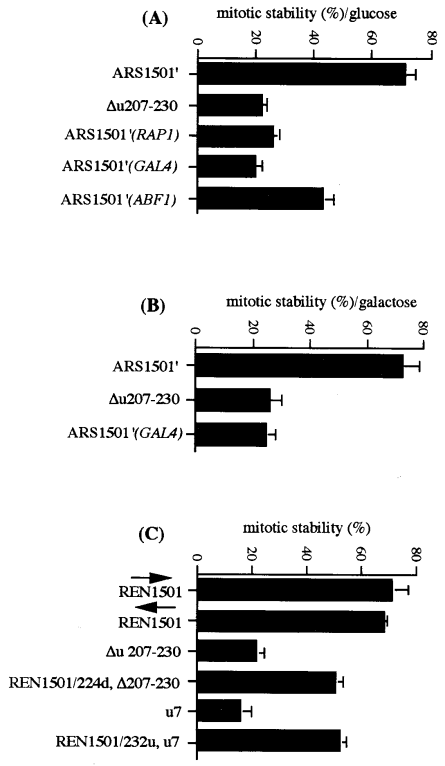

Figure 7. REN1501 is a DNA replication enhancer that has a unique function at ARS*1501*. **(A)** The REN1501 element, nt 201–224 (Fig. 1), has been deleted and replaced by DNA sequences that recognize the Rap1p, Gal4p and Abf1p. All sequences were inserted at the same location between nt 200 and 224 of ARS*1501*. The histogram shows the mitotic stabilities of plasmids bearing the modified ARS*1501*. Δu207–230 is ARS*1501* from which nt 207–230, which represent most of the REN1501 element, have been deleted. Cells harboring the various plasmids were grown in a glucose containing medium. **(B)** As **(**A**)**, except that the plasmid containing cells were grown in a galactose containing medium. **(C)** The REN1501 sequence was deleted and inserted in an inverted orientation (indicated by the arrows) at the same location. The origin (REN1501/224d, Δ207–230) contained (i) the REN1501 sequence cloned downstream to the ACS at a distance of 224 nt (224d) and (ii) a deletion of nt 207–230, which inactivates the function of REN1501 in the wild type ARS. The insertion of REN1501 sequence was accompanied by a deletion of nt 556–579, which eliminated the Abf1p DNA-binding site I. The origin (REN1501/232u, u7) contained (i) the REN1501 sequence cloned upstream to the ACS at a distance of 232 nt (232u) and (ii) a u7 linker substitution mutation in the REN1501 sequence, which also inactivates the function of this element in the wild type ARS. The insertion of the REN1501 sequence resulted in a deletion of nt 117–122, which eliminated the Abf1p DNA-binding site II. The distance was measured from the first (5′) nucleotide of the REN1501 sequence to the centrally located T residue (nucleotide number 6) of the ACS. The histogram shows mitotic stabilities of plasmids bearing the modified ARS*1501* origins. For REN1501 replacements, which were performed by PCR as described in Materials and Methods, the following DNA sequences were used: the DNA sequence of the Rap1p site was GCAAAAACCCATCAACCTTGAA identified by DNase I footprinting at the *HMRE* ARS (41); the DNA sequence of the Gal4p site was the synthetic consensus sequence CGGAAGACTCTCCTCCG (42); the DNA sequence of the Abf1p site was the sequence TTTTCGTATTTAGTGATTA-TAATA identified by DNase I footprinting at ARS*121* (40).

even a third Abf1p DNA binding sequence (two sites are already present in the native ARS) cannot fully restore the function of REN1501. Taken together these results suggest a specific function for REN1501 at the ARS*1501* origin of replication.

DISCUSSION

We have described a molecular dissection of a newly isolated origin from *S.cerevisiae*, the ARS*1501*, and showed that this ARS functions as an efficient origin of replication on chromosome *XV*. ARS*1501* represents the first origin of chromosome *XV* that has been isolated and characterized. The results of the chromosomal analysis are consistent with initiation of DNA replication occurring at the ARS*1501* origin and the bidirectional progression of replication through flanking sequences.

Molecular dissection of ARS*1501* involved extensive analysis of the ARS by linker scanning and deletion mutagenesis. Deletions identified the functional regions of the ARS and the subsequent linker scanning provided a more precise definition of the elements involved in replication. For convenience this analysis was performed using a plasmid model system. The effect on replication elicited by genetic alterations of the origin sequence was measured by high frequency transformation of yeast and mitotic stability analysis of plasmids bearing mutated ARS*1501*. The relevancy of this system for a functional dissection of replication origins in yeast has been established by a large body of work reported from several laboratories (13). Analysis of ARSs which function as chromosomal origins has shown that all DNA elements found necessary for ARS function in a plasmid are also important for the activation of the origin in its native chromosomal context.

The mutagenesis analysis of ARS*1501* has shown that this origin has a modular arrangement of functional DNA elements, similar to other well characterized ARSs. ARS*1501* consists of an essential core sequence containing an ACS flanked by auxiliary elements that stimulate origin activity. However, the unique feature of ARS*1501* is the presence of a new DNA replication enhancer designated REN1501. Although the DNA-binding sequences of another enhancer, Abf1p, are also present within ARS*1501*, these sites appear to minimally affect the ARS*1501* activity. We cannot exclude, however, the possibility of a more significant role for Abf1p in stimulating this origin in its genomic location on chromosome *XV*. Work is in progress to examine the role of the ABF1p DNA-binding sites, as well as the function of the REN1501 enhancer, in the chromosomal context.

REN1501 is located at a distance of 100 nt 5′ to the T-rich strand of the ACS. The mutagenesis analysis delimited a region of ~24 nt comprising the REN1501 element. When moved to locations, on opposite sides of the ACS, this element exhibited replication stimulatory activity. REN1501 was also functional when inverted relative to the ACS in its native location. Hence, REN1501 is a DNA replication enhancer that functions in an orientation and distance independent manner. Most significantly, point mutations within REN1501 abolished its stimulatory activity, strongly suggesting that the REN1501 sequence binds an enhancer protein. The identification of such a protein is critical for further analysis of the role of REN1501 in origin activation.

Only two other enhancer elements were found to be associated with origins. The Abf1p enhancer was identified at ARS*121* and ARS*1*, and the Rap1p was identified at the *HMRE* ARS. The DNA sequence of REN1501 differs from the sequences of the Abf1p and Rap1p DNA-binding sites. Moreover, purified Abf1p does not bind the REN1501 DNA (unpublished observations) and the Rap1p site cannot substitute for Abf1p at ARS*121*. Interestingly, using the TRANSFAC (transcription factor search) database (32) we were unable to match with high fidelity the 24 bp

REN1501 nucleotide sequence to any of the consensus sequences of the known transcription factors in yeast.

The properties of REN1501 clearly differ from any other stimulatory elements residing 3′ to the T-rich strand of the ACS of well characterized ARSs. For example, (i) the distance separating the B1 element from the ACS is critical for B1 function (6,10,33); (ii) the B3 element of ARS*1* is merely the Abf1p DNA-binding site (9); (iii) point mutations do not affect the function of B2/DUE (9,11). Moreover, the correct positioning of the DUE element (3′ to the ACS) is necessary to elicit DUE function (6). We thus infer that REN1501 is a new genetic element that has not been observed before in other ARSs.

How yeast DNA replication enhancers work is unknown. Replication enhancers may influence chromatin structure at the origin and thus facilitate the assembly of a replication initiation complex at the essential sequences (34,35). Alternatively, the enhancer proteins may interact directly with the replication initiation machinery (36). Evidence consistent with this latter possibility has been reported for the Abf1p enhancer at the ARS*121* (22,25). In addition, since the identified enhancer proteins also function *in vivo* in transcription, a transcriptional event at the origin influencing replication initiation cannot be excluded. A replication stimulatory transcriptional event has been observed at the *E.coli* and λ phage replication origins (37,38). This multitude of potential mechanisms for enhancer-dependent stimulation of replication initiation raises the possibility that the mode of action of enhancers at yeast origins may differ depending on the origin in which they reside.

Indeed, evidence has been accumulating to suggest such a possibility. For example, it has been reported that the stimulatory function of Abf1p at ARS*1* can be replaced by Rap1p or Gal4p DNA-binding sites (9). The stimulatory activity of Gal4p, a transcriptional factor not naturally associated with origins of replication, has suggested that the transcriptional activation domain of the enhancer proteins is necessary and sufficient to stimulate the ARS*1* replication origin. In contrast to ARS*1*, neither the Rap1p nor the Gal4p DNA-binding sites could replace the REN1501 function at ARS*1501*. Even a functional Abf1p DNA-binding site was only partially effective in replacing REN1501. These results suggest that REN1501 may fulfill a specific role at ARS*1501* and that its mode of action may differ from the mechanism of other enhancers at other ARSs. Recent experiments demonstrating that the REN1501 element cannot function at a heterologous origin are consistent with this notion (39).

Our results suggest a specific REN1501/ARS*1501* relationship. This situation is not unique to ARS*1501* since a similar specific relationship has been demonstrated for the Abf1p enhancer at ARS*121* (39). In addition, studies with a synthetic *HMRE* ARS containing both the Rap1p and Abf1p DNA-binding sites have shown that only the Rap1p DNA-binding site stimulates this origin in the chromosome (31). Taken together, these observations suggest that the initiation of replication in yeast may be regulated by different enhancer proteins each functioning at a limited number of active origins. Hence, unidentified enhancer elements might be associated with other uncharacterized origins. Their potential existence awaits the continued genetic dissection of ARS elements.

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REFERENCES

1 Stinchcomb, D. T., Struhl, K. and Davies, R. W. (1979) *Nature (London)*, **282**, 39–43.
2 Brewer, B. J. and Fangman, W. L. (1987) *Cell*, **51**, 463–471.
3 Huberman, J. L., Spotila, D. L., Nawotka, K. A., El-Assouli, S. M. and Davies, R. W. (1987) *Cell*, **51**, 473–481.
4 Huberman, J. L., Zhu, J., Davies, R. L. and Newlon, C. S. (1988) *Nucleic Acids Res.*, **16**, 6373–6383.
5 Fangman, W. L. and Brewer, B. J. (1991) *Annu. Rev. Cell. Biol.*, **7**, 375–402.
6 Walker, S. S., Malik, A. K. and Eisenberg S. (1991) *Nucleic Acids Res.*, **19**, 6255–6262.
7 Rivier, D. H. and Rine, J. (1992) *Science*, **256**, 659–663.
8 Desphande, A. M. and Newlon, C. S. (1992) *Mol. Cell. Biol.*, **12**, 4305–4313.
9 Marahrens, Y. and Stillman, B. (1992) *Science*, **255**, 817–823.
10 Theis, J. F. and Newlon, C. S. (1994) *Mol. Cell. Biol.*, **14**, 7652–7659.
11 Huang R.-Y. and Kowalski, D. (1996) *Nucleic Acids Res.*, **24**, 816–823.
12 Theis, J. H. and Newlon, C. S. (1996) In Brambl, R.R. and Marzluf, G.A. (eds), *The Mycota III, Biochemistry and Molecular Biology.* Springer-Verlag, Berlin–Heidelberg, pp. 3–28.
13 Newlon, C. S. (1996) In DePamphilis, M. L. (ed.), *DNA Replication in Eukaryotic Cells.* Cold Spring Harbor University Press. Cold Spring Harbor, NY, in press.
14 Shore, D., Stillman, D.J., Brand A. H. and Nasmyth, K. A. (1987) *EMBO J.*, **6**, 461–467.
15 Eisenberg, S., Civalier, C. and Tye B.-K. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 743–746.
16 Sweder, K. S., Rhode. and Campbell, J. L. (1988) *J. Biol. Chem.*, **263**, 17270–17277.
17 Francesconi, S. C. and Eisenberg, S. (1989) *Mol. Cell. Biol.*, **9**, 2906–2913.
18 Diffley, J. F. X. and Stillman, B. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 2120–2124.
19 Buchman, A. R., Kimmerly, W. J., Rine, J. and Kornberg, R. (1988) *Mol. Cell. Biol.*, **8**, 210–225.
20 Walker, S. S., Francesconi, C. S. and Eisenberg, S. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 4665–4669.
21 Bell, S. P. and Stillman, B. (1992) *Nature (London)*, **357**, 128–134.
22 Estes, H. G., Robinson, B. S. and Eisenberg, S. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 11156–11160.
23 Tye, B. K. (1994) *Trends Cell Biol.*, **4**, 160–166.
24 Yan, H., Merchant, M. A. and Tye B.-K. (1993) *Genes Dev.*, **7**, 2149–2160.
25 Shakibai, N., Kumar, V. J. and Eisenberg, S. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 11569–115774.
26 Boeke, J. D., Trueheart, J., Natsoulis, G., and Fink, G. R. (1987) *Methods Enzymol.*, **154**, 164–175.
27 Rose, M. D., Novick, P., Thomas, J. H., Botstein, D. and Fink, G. R. (1987) *Gene*, **60**, 237–243.
28 Rao, H. and Stillman, B. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 2224–2228.
29 Pellman, D., Bagget, M., Huei, T. and Fink, G. R. (1995) *J. Cell Biol.*, **130**, 1373–1385.
30 Umek, R. M. and Kowalski, D. (1988) *Cell*, **52**, 559–567.
31 Fox, A. C., Loo, S., Rivier, H. D., Foss, A. M. and Rine, J. (1993) *Cold Spring Harbor Symp. Quant Biol.*, **LVIII.**, 443–455.
32 Wingender,E. (1994) *J. Biotechnol.*, **35**, 273–280.
33 Rao, H., Mahrarens, Y. and Stillman, B. (1994) *Mol. Cell. Biol.*, **14**, 7643–7651.
34 DePamphilis, M.L. (1993) *Trends Cell Biol.* **3,** 161–167.
35 Pederson, D.S. and Heintz, N.H. (1994) *Transcription Factors and DNA Replication*, R. G. Landes Company, Austin, TX.
36 Ferguson, M.K. and Botchan, M.R. (1996) *J. Virol.* **70,** 4193–4199.
37 Kornberg, A.and Baker, T. (1992) *DNA Replication (second ed.)* W. H. Freeman and Co., NY. pp. 271–273.
38 Learn, B., Karzai, A.W. and McMacken, R. (1993) *Cold Spring Harbor Symp. Quant Biol.* **LVIII**, 389–402.
39 Wiltshire, S., Raychaudhuri, S. and Eisenberg, S. (1997) *Nucleic Acids Res.*, **25**, 4250–4256.
40 Walker, S. S., Francesconi, C. S., Tye, B.-K. and Eisenberg, S. (1989) *Mol. Cell. Biol.*, **9**, 2914–2921.
41 Mc Nally, J. F. and Rine, J. (1991) *Mol. Cell. Biol.*, **11**, 5648–5659.
42 Giniger, E., Varnum, M. S. and Ptashne, M. (1985) *Cell*, **40**, 767–774.