The EMBO Journal vol.13 no.14 pp.3395–3400, 1994

Replicator dominance in a eukaryotic chromosome

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Communicated by T.Lindahl

Replicators are genetic elements that control initiation at an origin of DNA replication (*ori*). They were first identified in the yeast *Saccharomyces cerevisiae* as autonomously replicating sequences (ARSs) that confer on a plasmid the ability to replicate in the S phase of the cell cycle. The DNA sequences required for ARS function on a plasmid have been defined, but because many sequences that participate in ARS activity are not components of chromosomal replicators, a mutational analysis of the *ARS1* replicator located on chromosome IV of *S.cerevisiae* was performed. The results of this analysis indicate that four DNA elements (A, B1, B2 and B3) are either essential or important for *ori* activation in the chromosome. In a yeast strain containing two closely spaced and identical copies of the *ARS1* replicator in the chromosome, only one is active. The mechanism of replicator repression requires the essential A element of the active replicator. This element is the binding site for the origin recognition complex (ORC), a putative initiator protein. The process that determines which replicator is used, however, depends entirely upon flanking DNA sequences.

Key words: ARS structure/DNA replication/initiator proteins/*S.cerevisiae*

Introduction

The initiation of DNA replication has been studied in considerable detail in bacteria, bacteriophages and eukaryotic viruses. These organisms replicate their genomes from distinct sites called origins of DNA replication (*ori*s) and *ori* activity is controlled by a genetic element called a replicator (Jacob *et al.*, 1963; Stillman, 1993). Many bacterial and viral replicators function correctly when cloned onto plasmids. This has facilitated their study particularly when the replicators normally occur in large chromosomes that would be difficult to manipulate. The structures of a number of prokaryotic and viral replicators have been defined in great detail and found to consist of complex configurations of highly conserved sequences (Kornberg and Baker, 1992). Initiator proteins that interact with important sequences have been identified and some replicators have been induced to generate *ori*s *in vitro* with purified proteins (Kornberg and Baker, 1992).

Replicators have also been identified in the chromosomes of a number of eukaryotes including the yeast *Saccharomyces cerevisiae* (Fangman and Brewer, 1991), *Drosophila* (Orr-Weaver, 1991) and human (Kitsberg *et al.*, 1993). In contrast to the replicators of prokaryotes and viruses, the structures of eukaryotic replicators are poorly understood and the initiation mechanism is not known. One critical obstacle is that attempts to establish plasmid replication systems have failed in most eukaryotic cells. In the cells where extrachromosomal plasmid replication has been established, it is unclear to what degree the plasmid replication mirrors the replication in the chromosome. For example, DNA introduced into *Xenopus* oocytes is replicated regardless of its source (Harland and Laskey, 1980; Mechali and Kearsey, 1984). In *S.cerevisiae*, sequences from unrelated organisms as well as yeast sequences that do not represent chromosomal replicators will function as autonomously replicating sequences (ARSs) (Newlon, 1988; Newlon and Theis, 1993). In contrast to the complex and highly conserved replicators present in prokaryotes and viruses, no conserved sequences have been detected in the sequences that replicate autonomously in yeast with the exception of a single 11 bp element called the ARS consensus sequence (ACS) (Broach *et al.*, 1983). This element is essential but not sufficient for replicator function (Campbell and Newlon, 1991; Deshpande and Newlon, 1992; Rivier and Rine, 1992; Huang and Kowalski, 1993). The sequence requirements for autonomous replication vary according to the plasmid context (Bouton and Smith, 1986) and portions of an ARS can functionally be substituted by seemingly unrelated sequences (Marahrens and Stillman, 1992 and unpublished observations).

A subset of ARSs colocalizes with *ori*s in the chromosome (Fangman and Brewer, 1991) suggesting a functional link between chromosomal replicators and the autonomous replication property of these sequences. A detailed mutational analysis of one such plasmid-borne sequence, the autonomously replicating sequence 1 (*ARS1*), led to the identification of four short sequences (A, B1, B2 and B3) that participate in plasmid replication and collectively are sufficient for efficient ARS function (Marahrens and Stillman, 1992). Element A contains the conserved ACS and is bound by the origin recognition complex (ORC), a candidate initiator protein (Bell and Stillman, 1992). Short functional sequences equivalent to B1 and B2 have been identified at another autonomously replicating sequence, ARS307 at very similar positions relative to element A, as found in *ARS1* (H.Rao, Y.Marahrens and B.Stillman, in preparation). The sequences of the B1 and B2 elements are remarkably dissimilar in the two ARSs. Element B3 has been identified at a subset of ARSs and is a protein

binding site for ARS binding factor 1 (ABF1) which functions as a transcription factor at a large variety of promotors. Element B3 can be substituted by the binding sites of other transcription factors (Marahrens and Stillman, 1992). It is not known whether elements B1, B2 or B3 are components of the replicator in the chromosome.

In the current study we have examined the requirements for replicator function in a eukaryotic chromosome. A mutational analysis was performed at the *ARS1* locus on chromosome IV of *S.cerevisiae* which colocalizes with a chromosomal *ori* (Ferguson *et al.*, 1991). The results of this analysis indicate that the four ARS elements (B3, B2, B1 and A) are components of the replicator that controls the *ori*. Unexpectedly, we also find that a functional replicator can inactivate a second replicator located nearby in the same chromosome. A protein binding site is identified in the active replicator that is essential for replicator dominance.

Results

Two essential components of a chromosomal replicator

ARS1 is derived from a region in yeast chromosome IV that is flanked by the *TRP1* and *GAL3* genes (Figure 1A). Four short sequences (A, B1, B2 and B3) have been identified in an *ARS1* clone that, collectively, support plasmid replication in *S.cerevisiae* (Marahrens and Stillman, 1992). Although sequences that support or stimulate plasmid replication are considerably more abundant than chromosomal origins, these sequences are attractive candidates for elements of a chromosomal replicator since the *ARS1* clone colocalizes with a chromosomal *ori* (Ferguson *et al.*, 1991). To determine whether any of these sequences correspond to components of the chromosomal replicator, we used homologous recombination to introduce mutations into the *ARS1* locus in chromosome IV which disrupt these sequences singly or in combination. The *ori* activity of the mutant loci was investigated by a two-dimensional (2-D) gel electrophoresis technique that identifies replication intermediates (Brewer and Fangman, 1987). In this assay restriction fragments bearing replication intermediates are electrophoretically separated from the rest of the DNA by their mass and shape. DNA blot hybridization identifies *ori*-containing fragments by a characteristic 'bubble arc' which can easily be distinguished from the 'Y-arc' signal characteristic of restriction fragments that do not contain an *ori* and are therefore replicated by a single externally derived replication fork (Figure 1C).

ori activity was assayed in a 5 kb *Nco*I fragment containing *ARS1* (Figure 1A). This fragment was excised from chromosome IV in wild type strains and in strains containing mutations in either the A element or the three B elements at the *ARS1* locus. The fragment containing wild type *ARS1* produced a strong bubble arc and an incomplete Y-arc indicative of a highly efficient origin of replication (Figure 1D). The incomplete Y-arc arises because the replication bubbles are not centered on this particular restriction fragment. Therefore, as the bubbles become larger, one of the two replication forks will run off one end of the restriction fragment before the other. This bubble to Y transition produces an incomplete Y-arc

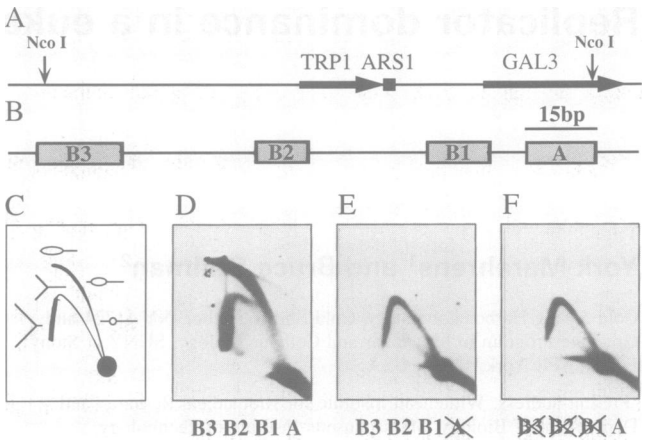

Fig. 1. Two essential components in the replicator at the yeast *ARS1/TRP1* locus. (**A**) The region in *S.cerevisiae* chromosome IV that contains *ARS1*. In this diagram, drawn approximately to scale, *ARS1* is designated as a black box and the protein coding regions of the *TRP1* and *GAL3* genes as black arrows. The direction of the arrows indicates the direction in which the genes are transcribed. The *Nco*I sites shown are 5 kb apart and represent the sites cleaved in the 2-D gel analysis. (**B**) Sequences important for the maintenance of *ARS1*-containing plasmids (Marahrens and Stillman, 1992). In this figure, which is also drawn approximately to scale, the functional sequences are marked by boxes and the line represents the surrounding sequence. The *TRP1* gene is located to the left of *ARS1* and its protein coding region terminates between the B3 and B2 elements. (**C**) Diagram depicting the patterns produced by bubble-containing and Y-shaped DNA replication intermediates in the 2-D gel assay (Ferguson *et al.*, 1991). *ori* formation is responsible for the top bubble arc and, if the replication bubble is not centered on the restriction fragment, also for the thick left-most portion in the lower Y-arc. In the absence of an *ori*, a complete lower Y-arc pattern is observed and the bubble arc is absent. The large black dot represents the position of linear, non-replicating DNA. Chromosomal *ARS1* replication intermediates generated by the parental *ARS1*$^+$TRP$^-$ *S.cerevisiae* strain SP1 (**D**) and derivative *ARS1*$^-$TRP$^+$ strains that were defective in the A element (**E**) and contained a mutation in each of the elements B1, B2 and B3 (**F**). The *ARS1* elements are listed at the bottom of (D), (E) and (F). A cross through an element represents a mutation in that element. Mutations that disable elements A, B1, B2 or B3 are, respectively, a point mutation 860 T→G (Bell and Stillman, 1992), *Xho*I linker substitutions 835–842, 798–805 and a double point mutation: a C to G transversion at position 756 and a T to C transition at position 758 (Marahrens and Stillman, 1992).

if the replicator is active in every, or nearly every, passage through S phase. The presence of a point mutation in element A, which eliminates the autonomous replication of plasmid-borne *ARS1*, completely inactivates origin function in the chromosome (Figure 1E). A similar result has been reported for the chromosomal loci of other ARSs (Deshpande and Newlon, 1992; Rivier and Rine, 1992; Huang and Kowalski, 1993). Previous results demonstrated that the simultaneous disruption of the three B elements in a plasmid-borne *ARS1* abolished autonomous replication of the plasmid (Marahrens and Stillman, 1992). We therefore simultaneously introducted three mutations into the B1, B2 and B3 elements in the chromosome. This group of mutations similarly inactivated *ori* function in the chromosome (Figure 1F). Thus, the mutational analysis has located a replicator in the yeast chromosome IV and identified two essential components of the replicator.

Role of individual B elements in replicator function

The experiment in Figure 1 establishes an essential role for the B elements in the function of the chromosomal

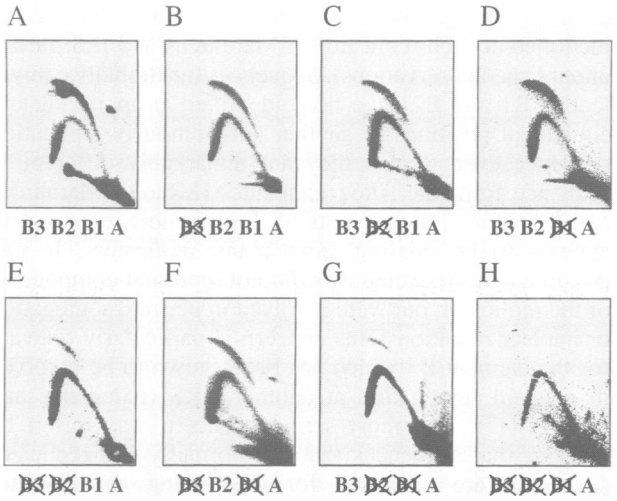

Fig. 2. B1, B2 and B3 elements function in the chromosome. 2-D gel patterns produced by replication intermediates of TRP$^+$ *S.cerevisiae* strains derived from SP1 were obtained three or more times for each strain and the patterns of representative blots are shown. The *ARS1* elements are listed at the bottom of each figure. A cross through an element represents a mutation in that element. Mutations that disable elements B1, B2 and B3 are as listed previously (Figure 1). Filters were exposed to Fuji imaging plates.

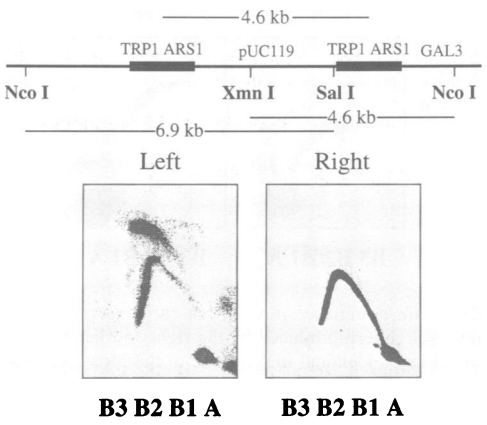

Fig. 3. Replicator inactivation following insertion of a second replicator. (Top) Organization of a locus containing two direct repeats of a 1.45 kb sequence containing the *TRP1* gene and wild type *ARS1* sequence in chromosome IV. The left *TRP1/ARS1* repeat and the pUC119 sequence in between the two repeats are plasmid-derived. The distance separating corresponding positions in the two repeats is indicated at the top of the figure, which is drawn approximately to scale. The restriction fragments used to assay the left and right replicators for *ori* function are indicated at the bottom of the map. (Bottom) Two-dimensional gel patterns produced by the right and left copies of *ARS1* are aligned below the representations of the replicators. The elements present in each copy of *ARS1* are listed below each pattern. A very low level of *ori* activity has been detected at the right replicator (not shown).

replicator but does not indicate which of the B elements is involved. To determine which of the B elements is a component of the replicator, the 2-D gel assay was performed on yeast strains that carry a disruption in a single B element. All three mutant strains produced arcs that resembled the pattern produced by wild type *ARS1* in TRP$^-$ (Figure 1D) or TRP$^+$ (Figure 2A) strains. The wild type strains reproducibly yielded incomplete or extremely faint Y-arcs. In contrast, the strains bearing mutations in a single B element consistently generated complete Y-arcs (Figure 2B, C and D). Because the bubble arcs were still present, however, the mutagenesis of any single B element appears to only slightly impair the function of the replicator in the chromosome. This is consistent with the results of our earlier plasmid studies where plasmid loss rates where higher than the wild type plasmid by 11.9 ± 4.2% for the B1 mutant, 8.8 ± 2.7% for B2 mutants and 6.4 ± 3.3% for the B3 mutant (Marahrens and Stillman, 1992).

Because a mutation in any single B element produced only a small reduction in replicator use, strains with mutations in two B elements were examined to confirm that these elements are indeed components of the replicator. 2-D gel analyses revealed that the simultaneous disruption of elements B3 and B1 also resulted in only a partial reduction in *ori* efficiency compared with the wild type level (Figure 2F). The simultaneous disruption of B3 and B2 (Figure 2E) or B2 and B1 (Figure 2G) resulted in dramatic reductions in *ori* efficiency. These analyses indicated that, as observed in earlier plasmid studies, the mutation of any single B element shows surprisingly little effect, but collectively the B elements are essential in the chromosome. Therefore, although many ARS sequences do not appear to function as a replicator in yeast chromosomes, the functional organization of a chromosomal replicator is faithfully reproduced in the plasmid context.

The finding that *ori* formation occurs in a relatively high proportion of S phases with either B2 present or with both B1 and B3 present in addition to element A indicates that the B elements can be divided into two partially redundant groups. Our findings also reveal that the relative importance of the B1 and B2 elements changes according to their context. B2 is the most important B element at the native *ARS1* locus in the chromosome, whereas B1 is the most important B element in the plasmid (Marahrens and Stillman, 1992). This suggests that replicator context can strengthen or weaken the role of individual elements. Alternatively, one or more of the B elements may have an effect on the segregation or nuclear retention of plasmids and therefore display a different relative role in the plasmid retention assay. For example, a B3 element (an ABF1 binding site) at the *HMR E* ARS is known to affect plasmid segregation (Kimmerly and Rine, 1987).

Replicator dominance in a yeast chromosome

Having examined the structure of a replicator in the yeast chromosome, we then tested whether two identical replicator sequences placed near one another in the chromosome would control *ori* function. We expected that both replicators would be active because the closely spaced *ori*s in the early cleavage embryos of *Drosophila* (Blumenthal *et al.*, 1973) and adjacent *ori*s in mammalian cells detected by DNA fiber autoradiography appear to be activated at the same time in S phase (Huberman and Riggs, 1968). A second copy of *ARS1* was inserted 4.6 kb away from the original copy by the site-directed integration of a linearized plasmid (Figure 3). The function of each replicator was examined by the 2-D gel assay. Surprisingly, the centromere proximal (left) *ori* was fully active while the centromere distal (right) *ori* was inactive (Figure 3).

Since the sequences of the two replicators are identical,

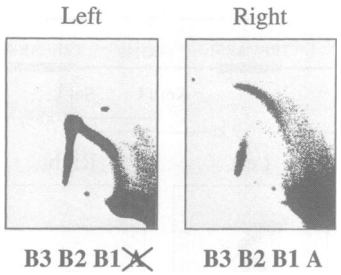

Fig. 4. Replicator dominance in the yeast chromosome. Two-dimensional gel pattern produced by the right and left replicators in a chromosomal locus identical in sequence to the locus previously pictured (Figure 3) with the exception of a point mutation designed to disable the A element in the left replicator (860 T→G). The elements present in each copy of *ARS1* are listed below each pattern. A cross through an element represents a mutation in that element. This strain was constructed as previously described (Figure 3) except that plasmid pTA1/860T→G was integrated. The complete Y-arc generated by the left replicator of this figure has a different shape from the complete Y-arc produced by the right replicator in figure 3 because of a difference in the size of the restriction fragments assayed.

the above result demonstrates that an unidentified sequence(s) adjacent to the replicators selects one, but not the other, to be inactivated. One possibility is that the inactive right replicator is both selected and repressed by this unidentified sequence. For example, it has been shown that a nucleosome, positioned by a protein binding site adjacent to a replicator, can interfere with replicator function (Simpson, 1990). A second possibility is that although flanking sequence selects a replicator to be repressed, the active replicator participates in the repression mechanism. To determine whether the active left replicator is involved in the repression of the right replicator, a strain was constructed with a point mutation in the A element of the left replicator. The corresponding single base substitution completely eliminated *ori* function at the single chromosomal copy of *ARS1* (Figure 1E). Examination of the *ori* activities in the strains with two copies of *ARS1* revealed that, as expected, this single base change in the left *ARS1* replicator similarly completely abolished the activity of the left *ori* (Figure 4, left; this Y-arc is distorted due to the large size of the 6.9 kb fragment). In addition, the presence of this point mutation resulted in the right *ori* being fully active (Figure 4, right). Since the left and right replicators contain identical sequences, flanking DNA sequences must determine that the left replicator will function. These results also demonstrate, however, that the mechanism that suppresses the right replicator depends upon an intact A element in the left replicator.

Discussion

We previously examined the autonomous replication property (ARS activity) of a cloned restriction fragment derived from a chromosomal locus with *ori* activity and identified four short sequence elements (A, B1, B2 and B3) that were either essential or important for the extrachromosomal maintenance of plasmids (Marahrens and Stillman, 1992). With the exception of element A, it remained unclear whether these elements represented components of a yeast replicator in the chromosome. Elements B1, B2 and B3 do not represent conserved sequence motifs that can be identified at each replicator (Y.Marahrens and B.Stillman, unpublished observations). Sequences functionally equivalent to B1 and B2 have been identified at approximately equivalent positions in another autonomously replicating sequence even though they are remarkably different in sequence from the *ARS1* elements (H.Rao, Y.Marahrens and B.Stillman, in preparation). Furthermore, element B3 appears to be missing from many replicators. It was possible that these elements did not represent components of the replicator, but were involved in plasmid segregation or nuclear retention. This concern is particularly relevant for the element B3 which has been shown to be involved in plasmid segregation at another ARS (Kimmerly and Rine, 1987). The most serious concern was raised by the fact that sequences that are normally not replicator sequences are notorious for functioning as replicator sequences in yeast in non-native contexts. Indeed, a large portion of the sequences that function as autonomously replicating sequences in plasmids do not correspond to replicators in the chromosome (Newlon and Theis, 1993). The current study indicates that, in the case of an ARS derived from a locus with a chromosomal *ori*, all four sequences that participate in plasmid replication are components of a replicator in the yeast chromosome.

Consistent with previous results at other loci, element A was essential for replicator function in the chromosome (Deshpande and Newlon, 1992; Rivier and Rine, 1992; Huang and Kowalski, 1993). This element is specifically bound *in vitro* by the multisubunit ORC which is a strong candidate initiator protein (Bell and Stillman, 1992). A similarity between the *in vitro* footprint produced by the ORC and the genomic footprint seen at *ARS1* in permeabilized cells, as well as recent genetic evidence, suggests that the ORC binds the A element *in vivo* and indeed functions as an initiator (Diffley and Cocker, 1992; Bell *et al.*, 1993; Foss *et al.*, 1993; Li and Herskowitz, 1993; Micklem *et al.*, 1993). The demonstration that element B3 is a replicator element in the chromosome is consistent with previous studies implicating the ABF1, which binds to this sequence, in the initiation reaction (Diffley and Stillman, 1988; Rhode *et al.*, 1992). ABF1 is known to function also as a transcription factor at a wide variety of promotors. The functions of the other two elements of the replicator, B1 and B2, are not known. B1 and B2 are thought to be contacted by the ORC, which appears to wrap the region containing the three B elements around it (Bell and Stillman, 1992). Specific interactions between B1 or B2 and the ORC might therefore occur that have not yet been detected. One possibility is that B1 or B2 functions in a manner analogous to the 13mers of the *E.coli* replicator. The 13mers are specifically contacted by the *E.coli* initiator protein and serve as the sites of localized melting by an initiation complex (Bramhill and Kornberg, 1988). B1 or B2 may also represent binding sequences for other proteins. One of these elements may function as an easily unwound sequence, called a DNA unwinding element (DUE) (Umek and Kowalski, 1988; Kowalski and Eddy, 1989), at which the initial stages of duplex opening and unwinding have been postulated to occur. These possible roles for B1 and B2 are not mutually exclusive. It will be informative to determine the location

at which the DNA strands separate for the initiation of synthesis.

An additional finding in this study is that a replicator, normally active in the chromosome, can be rendered inactive through the insertion of a second replicator at a nearby position to the left of the original replicator. At least two components are involved in this form of replicator inactivation. One component is the sequence adjacent to the replicators. Since the two replicators are identical in sequence, yet only one is active, additional sequence selects a replicator to be active. A second component is the A element of the active replicator. A point mutation in this element abolishes this form of replicator inactivation. The point mutation eliminates the binding of ORC to the A element *in vitro* and therefore strongly implicates the ORC in the inactivation mechanism. Interference between two or three closely spaced replicators has also recently been reported by others (Brewer and Fangman, 1993). In this study, however, each one of the adjacent replicators was selected in a population of cells, but in any given cell, only one replicator was active and the other was inactive. We suggest, based upon these results and our own, that the selection of the active replicator is determined by sequence context, but once selected, the active replicator can suppress nearby replicators. Replicator dominance may account for the failure of some ARSs to function as replicators in the chromosome. Other forms of replicator inactivation must also exist, however, since the deletion of an active replicator failed to cause an infrequently used replicator located nearby to become active at a higher frequency (Greenfeder and Newlon, 1992).

It is possible that both unidirectional and bidirectional replicator interference are produced by a mechanism in the yeast cell which protects the genome from over-replication. It has been demonstrated that the two replicators of identical sequence, but different chromosomal position, sometimes differ in their times of activation (Ferguson and Fangman, 1992). If chromosomal context can influence replication timing over short distances in a chromosome, one replicator may be traversed, before it has fired, by a replication fork generated by an adjacent replicator. Two rounds of replication would occur in the chromosome unless the cell has a mechanism that ensures that a replicator becomes inactive if a replication fork has passed through it. The timing determinant that triggers the inactivation mechanism may be a gradient generated by specific structures of the chromosome such as centromeres or telomeres (Ferguson and Fangman, 1992), or the determinant may be derived from a more local source. According to this model, replicators would interfere with one another even over large distances provided that their times of activation are staggered sufficiently.

Alternatively, replicator interference might be caused by structural constraints. One model invokes discretely spaced sites in the nucleus with which a replicator must associate for initiation to occur. There would be replicator interference if the conjoining of one replicator with a nuclear site prevents the adjacent replicator from forming an association with another site because the nuclear sites are spaced too far apart. Which replicator first associates with a nuclear site in a given S phase may be a chance event. However, a difference in the local chromosome structure may cause one replicator to be more rapidly accepted at a nuclear site than its neighbor and therefore be active in the great majority of S phases. This model predicts that replicator-mediated inactivation is confined to closely spaced replicators.

A third possibility is that a replicator or components of a replicator establish a chromatin structure in a region that is repressive to the function of other replicators. It has been demonstrated, for example, that a replicator can be repressed by external protein binding sites that position nucleosomes over the A element (Simpson, 1990). It is also known that the A and B3 elements cooperate at a mating type locus to establish a chromatin structure in a broad region that is repressive to promotor function (Laurenson and Rine, 1992). The establishment of this form of promotor repression, called transcriptional silencing, requires a round of DNA replication but is then maintained for several generations until it is re-established. A and B3 may similarly be involved in establishing a chromatin structure repressive to other replicators. It is worth noting, however, that *ARS1* does not function as a transcriptional silencer.

Replicator dominance may occur in all eukaryotes and could contribute to an explanation as to why not all potential replicators are used every cell cycle in eukaryotic chromosomes and may also help explain why more eukaryotic sequences function extrachromosomally as replicators than in the chromosomal genome. Other forms of replicator inactivation appear to occur in the cell. Replicator-mediated inactivation may, additionally, play a role in the regulated inactivation of origins and may also ensure that the eukaryotic genome is replicated only once per S phase.

Materials and methods

Plasmid construction

Plasmid pTA1 is pUC119 with a 1.45 kb *Eco*RI fragment containing the *TRP1* gene and *ARS1* ligated into the *Eco*RI site such that the *TRP1* gene is adjacent to the other sites of the polylinker. Plasmids that contain one or more mutations in *ARS1* were constructed with the use of site-directed mutagenesis of pTA1. The plasmid with a point mutation in the A element of *ARS1* is pARS1/860T→G. This single base substitution corresponds to a mutation previously studied in another clone of *ARS1* (Bell and Stillman, 1992). The plasmids with one or more mutations in the B domain are pTA1/756,758; pTA1/798-805; pTA1/835-842; pTA1/756,758,798-805, pTA1/756,758,835-842; pTA1/798-805,835-842; and pTA1/756,758,798-805,835-842. These mutations correspond to mutations previously obtained in another clone of *ARS1* (Marahrens and Stillman, 1992).

Yeast strains

The parental *S.cerevisiae* strain used in this study is SP1 which is *MAT***a**, *ura3*-52, *his3*, *trp1*-289, *leu2*-2,113, *ade8*, *can1* and was a gift from Mike Wigler (Toda *et al.*, 1985). Derivatives of SP1 that are TRP^+ and contain mutations in the *ARS1* locus on chromosome IV were obtained as follows. A 1.27 kb *Xba*I to *Eco*RI restriction fragment bearing the mutation of interest in *ARS1* and a portion of the $TRP1^+$ gene, adjacent to *ARS1*, was excised from pTA1 or a mutant derivative plasmid and purified from an agarose gel. The purified restriction fragment was used to transform *S.cerevisiae* strain SP1 using standard lithium acetate procedures (Sherman *et al.*, 1986). All of the TRP^+ transformants that were recovered either carried the TRP^+ allele at the chromosomal locus or on an autonomous replicating plasmid. TRP^+ strains that had incorporated the desired mutation(s) in *ARS1* were identified by DNA blot hybridization and by direct sequencing of PCR-amplified DNA.

Strains containing two copies of wild type *ARS1* were obtained by

transforming SP1 with plasmid pTA1 that has been linearized with *Eco*RV. The desired transformants were distinguished by their growth rates on plates lacking tryptophan, by measuring the retention of the TRP^+ phenotype after growth in non-selective broth, and by probing blots of genomic DNA from selected TRP^+ colonies that had been cut with the appropriate restriction enzymes. In addition, genomic DNA was digested with the appropriate restriction enzymes, ligated, transformed into bacteria and DNA preparations of transformants were sequenced.

Analysis of replication intermediates

The 2-D gel assay was essentially as described (Brewer and Fangman, 1987; Rivier and Rine, 1992). Genomic DNA was purified from 1 l cultures of the appropriate strain grown to OD_{600} of 0.6–2.0. Genomic DNA derived from strains containing a single copy of the *ARS1* locus was cleaved with *Nco*I. Blots were probed with an *ARS1* probe derived from a 1.45 kb *Eco*RI restriction fragment that contains *ARS1* and exposed to Kodak XAR film. Genomic DNA from SP1 derivatives that contain two copies of *ARS1* in chromosome IV was purified from two 1 l cultures grown to OD_{600} of 0.6 and divided into two aliquots. One aliquot was cleaved with *Nco*I and *Sal*I and the digest was used to assay the left (centromere proximal) replicator. The second aliquot was cleaved with *Nco*I and *Xmn*I and the digest was used to assay the right replicator. Both blots were allowed to hybridize to a probe prepared from a 1.8 kb *Sal*I to *Xmn*I fragment derived from pUC119. Filters were exposed to Kodak XAR film or to Fuji imaging plates. In the latter case, images were analyzed using the MACBAS 1.01 image processing program (FUJI).

Acknowledgements

We thank F.Tanaka for many valuable discussions, D.Rivier, M.H.K. Linskens, B.Brewer and B.Futcher for technical advice during the early stages of this work and P.Kaufman for comments on the manuscript. This research was supported by a grant (AI20460) from the US National Institutes of Health.

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Received on March 30, 1994; revised on May 3, 1994