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A Replication Map of a 61-kb Circular Derivative of *Saccharomyces cerevisiae* Chromosome *III*

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Using two-dimensional agarose gel electrophoresis, we determined the replication map of a 61-kb circular derivative of *Saccharomyces cerevisiae* chromosome *III*. The three sites of DNA replication initiation on the ring chromosome are specific and coincide with *ARS* elements. The three origins are active to different degrees; two are used >90% of the time, whereas the third is used only 10–20% of the time. The specificity of these origins is shown by the fact that only *ARS* elements were competent for origin function, and deletion of one of the *ARS* elements removed the corresponding replication origin. The activity of the least active origin was not increased by deletion of the nearby highly active origin, demonstrating that the highly active origin does not repress function of the relatively inactive origin.

Replication termination on the ring chromosome does not occur at specific sites but rather occurs over stretches of DNA ranging from 3 to 10 kb. A new region of termination was created by altering the sites of initiation. The position of the new termination site indicates that termination is not controlled by specific *cis*-acting DNA sequences, but rather that replication termination is determined primarily by the positions at which replication initiates. In addition, two sites on the ring chromosome were found to slow the progression of replication forks through the molecule: one is at the centromere and one at the 3′ end of a yeast transposable element.

INTRODUCTION

Eukaryotic chromosomal DNA replication initiates at multiple sites and replication forks move bidirectionally through the genome from these sites. There is little detailed information available pertaining to the replication of any specific segment of eukaryotic chromosomal DNA. Important questions include whether replication initiates at specific sites, whether replication terminates at specific sites, and whether replication forks move at constant rates through the chromosome.

The development of two-dimensional agarose gel techniques for mapping replication intermediates (Brewer and Fangman, 1987; Nowatka and Huberman, 1988) has made it possible to map several chromosomal origins of replication to specific sites in the yeast, *Saccharomyces cerevisiae* (Brewer and Fangman, 1988; Huberman *et al.*, 1988; Linskens and Huberman, 1988; Ferguson *et al.*, 1991; Walker *et al.*, 1991). In every case studied, chromosomal replication origins coincided with *ARS* elements, which were identified by their ability to promote high-frequency transformation and extrachromosomal replication of collinear DNA (Struhl *et al.*, 1979; reviewed by Campbell and Newlon, 1991). Nonetheless, not all *ARS* elements function as chromosomal replication origins (Dubey *et al.*, 1991), and thus a map of *ARS* elements does not directly yield a map of yeast chromosomal replication origins.

Replication termination in eukaryotic chromosomes is largely uncharacterized. In the *Escherichia coli* chromosome, replication terminates in a DNA region defined by two pairs of termination sequences that stop replication forks in a polar manner. Replication forks approaching these sequences from one direction are stopped, but forks approaching from the opposite direction pass freely. These pairs of sites are oriented so that the termination region forms a trap for replication forks and is located ~180° from the origin on the cir-

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cular chromosome (reviewed by Kuempel *et al.*, 1989). Similarly, in the Epstein-Barr virus genome, a replication fork barrier near the replication origin determines the position of replication termination. However, its asymmetric position makes the replication of the genome essentially unidirectional (Gahn and Schildkraut, 1989). In the circular genome of the simian virus, SV40, there are no specific termination sequences (Griffin and Fried, 1975; Martin *et al.*, 1975). A polar replication fork barrier has been found at the 3′ end of the 35S ribosomal RNA genes in the tandem rDNA repeats of yeast (Brewer and Fangman, 1988; Linskens and Huberman, 1988). In contrast, replication appears to terminate over a broad region of chromosome *III* between *ARS305* and *ARS306* (Zhu *et al.*, 1992).

We have used the two-dimensional agarose gel technique of Brewer and Fangman (1987) to map replication in a 61-kb circular derivative of yeast chromosome *III*. The sequences of this 61-kb derivative are also contained within the 200-kb region of chromosome *III* that has been cloned, mapped, and analyzed for *ARS* elements (Newlon *et al.*, 1991). The experiments reported here demonstrate that bidirectional DNA replication initiates only at specific sites on the ring chromosome that coincide with *ARS* elements. Three regions of replication termination were found, two of which are in regions midway between the two highly active origins and the third in the region near the centromere. We found that the position of termination could be moved to a new position by removing one of the highly active replication origins, demonstrating that the sites of termination are not fixed. Two replication fork pause sites were identified. One is associated with the 3′ end of a yeast transposable element and the other associated with the centromere of the ring chromosome.

MATERIALS AND METHODS

Strains and Enzymes

E. coli strain JA226 was used as host strain for plasmid DNA isolation. *S. cerevisiae* strain SG3-47A (*MAT*a *his6 trp1-289*) was used for wild-type DNA isolations. SG3-47A was constructed by crossing strain ICH2 (*MATα trp1-289*) (Surosky and Tye, 1985) with strain RM14-3A (*MAT*a *cdc7-1 bar1 his6 trp1-289 ura3-52 leu2-3,112*) (McCarroll and Fangman, 1988), sporulating the resultant diploid (SG3) and isolating the appropriate haploid spore. In both ICH2 and SG3-47A, chromosome *III* is rearranged. Sequences between Ty1-17 on the left arm and Ty1-161 on the right arm are present as a 61-kb circular molecule, and the remainder of the chromosome is linear. The linear portion of chromosome *III* is stabilized by a copy of *CEN5* integrated near the *HIS4* locus (Surosky and Tye, 1985).

Strain SG4-10A (*MATα his4-S8 ura3-52 ade1*) was used for construction of the *ARS307* deletion in the 61-kb ring chromosome and was made by crossing SG3-47A (described above) with strain M2984 (*MATα arg4-17 trp1-1 tyr7-1 ade1*). M2984 carries a copy of chromosome *III* derived from the Carlsberg brewing strain. This chromosome appears to be a hybrid between *S. carlsbergensis* and *S. cerevisiae* chromosomes *III*. It shows normal levels of recombination and high homology with *S. cerevisiae* in the region distal to *MAT* on the right arm. The region to the left of *MAT* shows no recombination and limited hybridization with *S. cerevisiae* probes, indicating considerable divergence in this interval. Nonetheless, this recombinant chromosome retains all of the essential functions on chromosome *III* (Nilsson-Tillgren *et al.*, 1981; Holmberg, 1982). SG4-10A is a haploid strain containing the hybrid *S. carlsbergensis* chromosome *III* together with the 61-kb ring chromosome derivative. This strain was used because the brewing yeast chromosome could complement the essential function deleted from the ring chromosome by the *ARS307* deletion without interfering with specific detection of the ring by hybridization. The 522 bp *ARS307* deletion was made by integrating an *ARS307* deletion plasmid described elsewhere (Dershowitz and Newlon, unpublished data) in the ring chromosome *III* of strain SG4-10A. Ura^+ integrants were selected on -ura medium. Strains that had lost the integrated copy of the plasmid by homologous recombination between duplicated sequences (Ura^- pop-outs) were selected by growth on 5-fluoro-orotic acid–containing plates (Boeke *et al.*, 1987). The pop-outs were then screened for those carrying the *ARS307* deletion by probing blots of *Eco*RV-digested DNA with probes adjacent to the region. Strain SG4-10A-Δ307 is the deletion strain used in subsequent studies.

Strain XJ24-24a (*MAT*a, *ade6, arg4-17, trp1-1, aro7-1*) was used to examine the replication of the full-length linear chromosome *III* (Strathern *et al.*, 1979).

Restriction endonucleases were purchased from New England Biolabs, Inc. (Beverly, MA). [α-^{32}P]-dATP was purchased from Amersham Corp (Arlington Heights, IL). ^{32}P-labeled DNA probes were made using the Amersham Multiprime Random Primer DNA Labeling kit. Fragments used for hybridization probes were isolated by agarose gel electrophoresis of restriction digests of the chromosome *III* plasmids listed in the legend to Figure 2 (Newlon *et al.*, 1991).

Plasmids

Plasmid DNA was prepared from *E. coli* by the alkaline lysis procedure (Maniatis *et al.*, 1982).

Determination of Plasmid Stabilities

Plasmid stabilities were determined as the percentage of plasmid-bearing cells (mitotic stability) in a culture grown in selective medium (Palzkill and Newlon, 1988; Van Houten and Newlon, 1990). Cells were grown in selective medium and plated onto both selective and nonselective medium. The mitotic stability was determined as the ratio of colonies on the selective plates versus nonselective plates.

Transformations

E. coli transformations were performed by the method of Davis *et al.* (1980). Yeast transformations were carried out using the lithium acetate transformation procedure (Ito *et al.*, 1983).

Isolation of Replicating Yeast DNA

Yeast cultures were grown to mid-log phase ($1–5 \times 10^7$ cells/ml) and mixed with an equal volume of Toluene stop solution (95% EtOH, 3% Toluene, 20 mM tris(hydroxymethyl)aminomethane [Tris], pH 7.4) followed immediately by the addition of 0.25M EDTA to a final concentration of 10 mM (Johnston and Williamson, 1978). The suspension was swirled for 1 min and placed on ice or immediately centrifuged at 4°C. The cells were then washed three times with sterile water and DNA was prepared by the method of Huberman *et al.* (1987).

Restriction Enzyme Digestion

Restriction enzyme digestion of yeast genomic DNA was carried out by sequential addition of 2 U/μg of restriction enzyme at intervals of 1.5 h to DNA in buffer containing 20 mM Tris pH 7.4, 50 μm spermine, 125 μm spermidine, 20 mM KCl, 70 mM NaCl, 10 mM MgCl, 2.5KIU/ml aprotinin, and 0.1% digitonin (Mirkovitch *et al.*, 1984).

Two-Dimensional Agarose Gel Electrophoresis

The method of Brewer and Fangman (1987) was used for two-dimensional gel electrophoresis.

Quantitation of Hybridization Signals

Densitometric analysis of autoradiograms was carried out using a Molecular Dynamics (Sunnyvale, CA) computing densitometer.

Determination of the Direction of Replication Fork Movement

The determination of the direction of replication fork movement was carried out as previously described (Fangman and Brewer, 1991; Brewer, personal communication).

RESULTS

Our approach to preparing a replication map of a portion of *S. cerevisiae* chromosome *III* was to analyze the replication intermediates found for a set of overlapping restriction fragments that cover the 61-kb region of the chromosome extending from Ty1-17 on the left arm to Ty1-161 on the right arm. The small ring chromosome carrying this region, isolated by Surosky and Tye (1985), is diagrammed in Figure 1A, which shows the positions of the centromere (*CEN3*), the three *ARS* elements (*ARS307, ARS308,* and *ARS309*) and the hybrid Ty element that resulted from the intrachromosomal recombination that created the ring chromosome (Newlon *et al.*, 1991). In addition, the positions of the *Bam*HI clones containing this region are indicated (Newlon *et al.*, 1991). In the strain used for this analysis, SG3-47A, chromosome *III* is in two parts, the 61-kb ring chromosome and a linear chromosome carrying the remainder of chromosome *III* stabilized by an insertion of *CEN5* near the *HIS4* locus (Surosky and Tye, 1985). Thus the ring chromosome *III* sequences are present in only a single copy per cell.

For the analysis of replication intermediates, DNA was prepared from asynchronous cultures, cut with one or more restriction enzymes, and then separated in a first dimension gel under conditions designed to separate molecules on the basis of mass. The first dimension track was cut out, rotated 90°, embedded in a second-dimension gel, and DNA was separated under conditions designed to maximize the separation based on differences in the shapes of molecules. The DNA was then transferred to a nitrocellulose membrane, and the blot was hybridized with a radioactive probe complementary to the genomic DNA fragment of interest. Figure 1B shows a diagram of the patterns that are expected to arise from this two-dimensional gel analysis along with the shapes of molecules that migrate in these arcs. The interpretation of these patterns has been validated by a number of studies (Brewer and Fangman, 1987, 1988; Brewer *et al.*, 1988; Huberman *et al.*, 1988; Gahn and

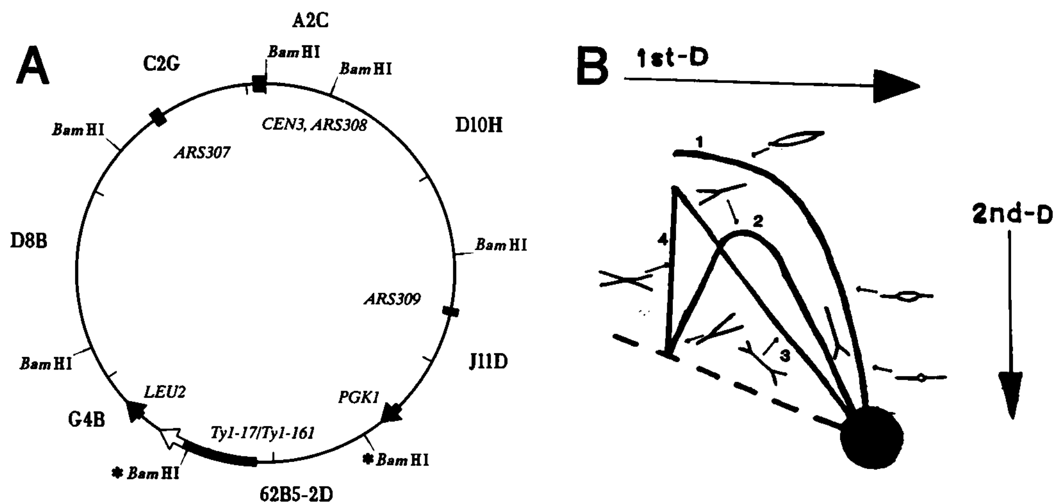

Figure 1. (A) Diagram of the 61-kb ring derivative of chromosome *III*. The positions of *CEN3*, the three *ARS* elements (*ARS307, ARS308,* and *ARS309*), the hybrid Ty element, *LEU2* and *PGK1* are indicated. The approximate position of the recombination that produced the hybrid Ty element is indicated by the boundary between the filled and open portion of the Ty. The positions of the *Bam*HI sites that define the boundaries of each cloned fragment are indicated by tick marks on the outside of the circle and the names of the cloned fragments are shown. The *Bam*HI site between J11D and 62B5-2D and the *Bam*HI site in Ty1-17 (marked by asterisks) are missing in this ring chromosome. The tick marks on the inside of the map are at 10-kb intervals. (B) Expected patterns from two-dimensional gel analysis. The arcs that are expected from two-dimensional gel analysis of replicating DNA are shown along with diagrams of the molecules that migrate in each of the arcs. The spot in the lower right corner depicts the position at which monomer length molecules migrate. The dashed line depicts the line formed by all of the linear molecules in the population. Arc 1 contains bubble-shaped replication intermediates resulting from replication initiation in the center of a fragment. Arc 2 contains Y-shaped replication intermediates resulting from a single replication fork moving through a fragment from one side. Arc 3 contains double-Y-shaped replication intermediates resulting from two replication forks entering a fragment simultaneously from both ends. Arc 4 contains X-shaped molecules resulting from recombination.

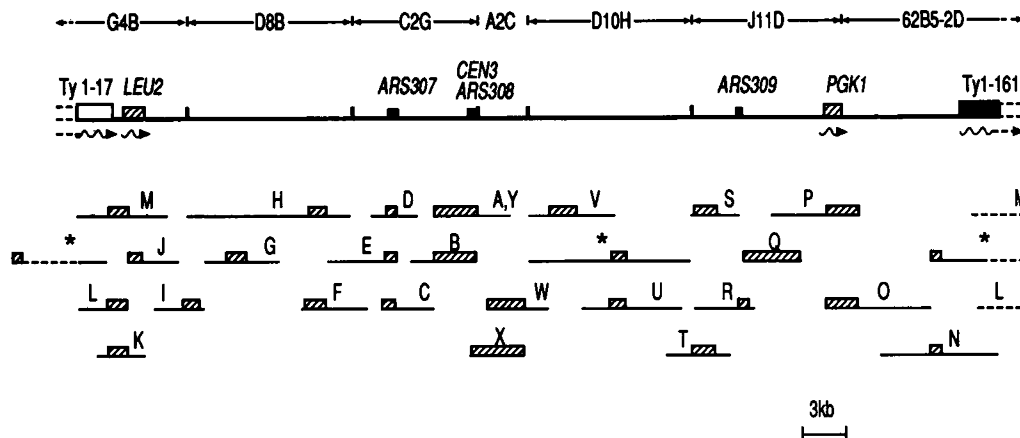

Figure 2. Fragments of the 61-kb ring chromosome analyzed by two-dimensional electrophoresis. A diagram of the 61-kb ring chromosome linearized at the hybrid Ty element is shown with the positions of *CEN3*, the three *ARS* elements, the *LEU2* and *PGK1* genes indicated. The right half of Ty1-17 and the left half of Ty1-161 are shown. The directions of transcription are indicated by arrows beneath the genes. The names and boundaries of cloned *Bam*HI fragments (Newlon *et al.*, 1991) are shown above the linear map. Each of the DNA fragments analyzed by two-dimensional gel analysis is shown as a line below the map of the chromosome with a letter corresponding to the panels in Figure 3. The fragments marked by asterisks were analyzed but the patterns obtained from these are not shown in Figure 3. The restriction sites that define the ends of each fragment are as follows and are indicated with the leftmost site first: A, *Eco*RI-*Eco*RI; B, *Xba* I-*Bam*HI; C, *Eco*RI-*Eco*RI; D, *Eco*RV-*Eco*RV; E, *Cla* I-*Cla* I; F, *Hin*dIII-*Hin*dIII; G, *Bgl* II-*Bgl* II; H, *Bam*HI-*Bam*HI; I, *Bgl* II-*Bgl* II; J, *Eco*RI-*Eco*RI; K, *Sal* I-*Sal* I; L, *Pst* I-*Eco*RI; M, *Pst* I-*Pst* I; N, *Eco*RI-*Pst* I; O, *Xho* I-*Xho* I; P, *Eco*RI-*Eco*RI; Q, *Hin*dIII-*Hin*dIII; R, *Bgl* II-*Bgl* II; S, *Eco*RI-*Bam*HI; T, *Pst* I-*Pst* I; U, *Pvu* II-*Pvu* II; V, *Xho* I-*Xho* I; W, *Cla* I-*Pvu* II; X, *Bam*HI-*Bam*HI; and Y, *CEN3*-containing *Eco*RI-*Eco*RI fragment from the *ARS307* deletion strain. The DNA fragments used as hybridization probes are indicated by the hatched boxes. The following is a list of the probe fragments and the DNA fragments detected by each probe: 2.6 kb *Bam*HI-*Eco*RV from C2G (A, B, and Y), 0.52 kb *Eco*RI-*Cla* I from C2G (C, D, and E), 1.1 kb *Eco*RV from D8B (F and H), 1.3 kb *Eco*RI from D8B (G), 0.9 kb *Bam*HI-*Eco*RV from G4B (I), 0.9 kb *Sal* I-*Eco*RI from G4B (J), 1.4 kb *Eco*RI-*Xho* I from G4B (K, L, and M), 0.7 kb *Bgl* II-*Xho* I from 62B5-2D (N), 2.0 kb *Bgl* II-*Xho* I from 62B5-2D (O and P), 3.5 kb *Hin*dIII from J11D (Q), 0.8 kb *Sal* I-*Eco*RI from J11D (R), 1.3 kb *Hin*dIII from J11D (S and T), 0.8 kb *Hin*dIII-*Xho* I from D10H (U), 1.7 kb *Pvu* II from D10H (V), 2.3 kb *Bam*HI-*Cla* I from A2C (W), and 3.2 kb *Bam*HI from A2C (X).

Schildkraut, 1989; Krysan and Calos, 1991; Martin-Parras *et al.*, 1991).

The two-dimensional patterns shown in Figure 1B are oriented so that the first dimension separation is from left to right and the second dimension separation is from top to bottom. This places the fastest migrating DNA fragments in the lower right corner of the figures and the slowest migrating in the upper left. The spot in the lower right corner depicts the spot of linear, monomer-length, nonreplicating DNA fragments detected by the hybridization probe (the monomer spot). The dashed line ascending diagonally from the monomer spot depicts the line containing all of the linear molecules in the population (the line of linears).

The patterns typically obtained are composites of the arcs shown in Figure 1B. For example, if replication initiates off-center within a fragment, early replication intermediates will contain bubbles, whereas late replication intermediates will be Y-shaped. This results in a discontinuous pattern that begins as a bubble arc and ends as a Y arc. Similarly, a transition from a Y arc to a double-Y arc suggests that a termination site is located near one end of the fragment of interest. Additional information can be obtained by examining the density of hybridization signal along the arcs of replication intermediates. Discrete sites at which replication forks pause or stop are revealed by the accumulation of replication intermediates of a particular size, which is manifested as an intense spot on a Y arc.

If replication initiates near one end of a DNA fragment, then the short arc of bubble-containing replication intermediates is difficult to distinguish from the early Y arc and can also be hidden in the intense hybridization to the monomer spot. Thus, to avoid missing origins, it is necessary to examine overlapping DNA fragments so that each DNA sequence is analyzed at least once when it is in the middle 70% of a fragment. Figure 2 summarizes the overlapping restriction fragments of the 61-kb ring chromosome that were analyzed. The fragments are indicated by thin lines below the linearized map of the ring chromosome, and the probes used to visualize the patterns are shown by hatched boxes on the lines. The letters on the fragments correspond to the autoradiograms of the two-dimensional gels shown in Figure 3.

Initiation of DNA Replication

We found evidence of replication initiation in three fragments, each containing one of the *ARS* elements (*ARS307, ARS308,* and *ARS309*). We found no evidence of replication initiation in any regions that did not con-

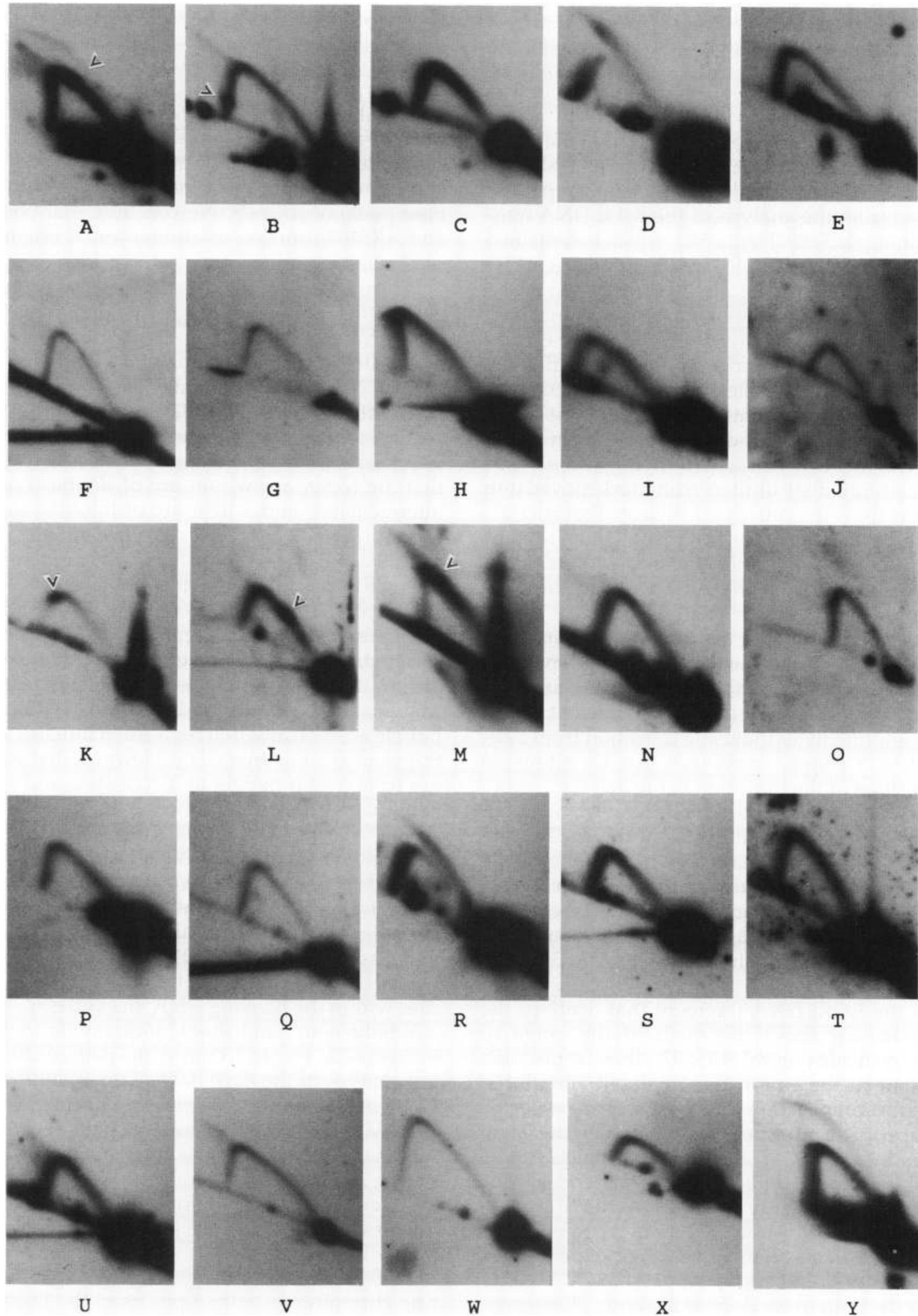

Figure 3. Two-dimensional patterns obtained from analysis of the ring chromosome. (A–X) Patterns obtained from the overlapping DNA fragments analyzed from the 61-kb ring chromosome in strain SG3-47A. Each pattern corresponds to one of the fragments diagrammed in Figure 2. The pattern in Y was obtained from the analysis of the *Eco*RI *CEN3*-containing DNA fragment from the *ARS307* deletion ring chromosome in strain SG4-10A-Δ307.

tain *ARS* elements. *ARS307* is contained within a 522-bp *Eco*RI-*Cla* I fragment from the C2G *Bam*HI fragment (Palzkill *et al.*, 1986; Newlon *et al.*, 1991). The analysis of the 3.2-kb *Eco*RV fragment containing *ARS307* is shown in Figure 3D. This pattern contains an arc of bubble-shaped replication intermediates in the early part of the pattern (right side) and an arc of Y-shaped intermediates in the late part (left side). A similar pattern was obtained from the analysis of the *Bgl* II DNA fragment containing *ARS309* (Figure 3R), which is contained within a 340-bp *Hpa* I-*Eco*RI fragment of the J11D *Bam*HI fragment (Palzkill *et al.*, 1986; Newlon *et al.*, 1991). This discontinuous bubble-to-Y pattern indicates that replication initiates off-center within these fragments and that replication forks proceed bidirectionally from the initiation site. If replication were unidirectional, as has been described for plasmids carrying the ColE1 origin, a single fork would move from the origin to one end of the fragment, which would then accumulate as a Y-shaped intermediate until a second fork moved into the fragment from the other end (Martin-Parras *et al.*, 1991). Thus, replication of the fragment would be completed as a double-Y intermediate, and a termination arc, which is absent from the patterns in Figure 3, D and R, would be present.

The presence of a bubble arc in a two-dimensional gel pattern indicates the presence of a replication origin within the fragment, but further analysis is required to establish the position of the origin. The mass at which replication intermediates make the transition from bubble-shaped to Y-shaped molecules can be estimated from the position of the transition in the first dimension. Assuming equal rates of replication fork movement, the position of initiation relative to one of the ends of the fragment can be estimated. A second, overlapping, DNA fragment must be then analyzed to determine which end is closest to the initiation site. In the case of the *ARS307*-containing *Eco*RV fragment, the origin maps 1.1 kb from one end, either coincident with *ARS307* or 1 kb away. Figure 3C shows the pattern obtained from analysis of the *Eco*RI DNA fragment that overlaps the *ARS307*-containing *Eco*RV fragment. If the origin of replication coincides with *ARS307*, then replication should initiate at the end of this fragment and all but the very earliest replication intermediates are expected to be Y-shaped. The other possible position for the origin is in the middle of the *Eco*RI fragment, which should yield bubble-shaped replication intermediates. The simple-Y arc seen in Figure 3C indicates that the *Eco*RI fragment is replicated by a single fork and demonstrates that the replication origin must be within experimental error (several hundred base pairs) of *ARS307*.

Similarly the pattern for *ARS309* in Figure 3R suggests that the origin is 1 kb from the end, either coincident with *ARS309* or 1.2 kb away. Figure 3S shows the pattern obtained from the analysis of the *Eco*RI-*Bam*HI fragment that overlaps the *ARS309*-containing *Bgl* II DNA fragment. If the origin is coincident with *ARS309* then all but the very earliest replication intermediates are expected to be Y-shaped. The other position for the origin is in the middle of the *Eco*RI-*Bam*HI fragment, which should yield bubble-shaped replication intermediates. Again, this pattern reveals Y-shaped replication intermediates, indicating that the replication origin must be within experimental error (several hundred base pairs) of *ARS309.* We conclude that both *ARS307* and *ARS309* function as chromosomal origins of bidirectional replication.

Figure 3A shows the pattern obtained from the analysis of the *Eco*RI DNA fragment containing *CEN3* and the *CEN3*-associated *ARS, ARS308. CEN3* and *ARS308* are located within a 624-bp *Sau* 3A fragment contained in the C2G *Bam*HI fragment (Clarke and Carbon, 1980; Fitzgerald-Hayes *et al.*, 1982; Newlon *et al.*, 1991). This pattern contains a complete arc of Y-shaped replication intermediates, a spot along the ascending portion of that arc (open arrow), an arc of double-Y replication intermediates, and a light arc of bubble-shaped replication intermediates. The complete simple-Y arc indicates that this fragment is replicated predominantly by a single replication fork that initiates outside of the fragment. The spot of accumulated replication intermediates results from a replication fork pause site associated with the centromere and the double-Y arc indicates that some replication termination occurs in this fragment (Greenfeder and Newlon, 1992). The light bubble arc indicates that replication initiates within this fragment at a low frequency. However, the pattern does not have a clear bubble-to-Y transition and so does not allow us to determine whether initiation occurs at a specific site or at multiple sites within the fragment (Krysan and Calos, 1990; Vaughn *et al.*, 1990). To distinguish between these two possibilities, we analyzed DNA fragments that contain the *CEN3* flanking sequences. If replication initiates at *ARS308* (*CEN3*), the patterns for the flanking DNA should not contain bubble arcs. If, however, replication initiates at multiple sites within the flanking DNA, the patterns should contain bubble arcs and should look similar to the pattern in Figure 3A. Figure 3B shows the pattern obtained from the analysis of the *Bam*HI-*Xba* I fragment that contains *CEN3* at the extreme *Bam*HI end and extends toward *ARS307,* and Figure 3X shows the pattern obtained from the analysis of the *Bam*HI fragment that flanks *CEN3* on the *ARS309* side. There is no evidence of a bubble arc in either of these patterns, indicating that replication initiates at or near *ARS308* and not in the regions flanking it.

To be certain that the pattern of origin usage of the ring chromosome is the same as in the linear chromosome, we analyzed the three chromosomal origins in a yeast strain, XJ24-24a, that carries a full-length linear chromosome *III* (Strathern *et al.*, 1979). No differences were found when the same DNA fragments that were

analyzed in Figure 3, A, D, and R, were analyzed in the linear chromosome, indicating that origin usage in this interval of the linear chromosome is the same as in the 61-kb ring chromosome.

Frequency of Origin Usage

We were interested not only in identifying the locations of replication origins but also in their efficiency of use. If origins are not used in every S phase, then an origin-containing fragment will be replicated by a fork from an adjacent origin in some fraction of the population, resulting in Y-shaped replication intermediates. Examination of the two-dimensional gel patterns containing bubble arcs (Figure 3, A, D, and R) revealed the presence not only of bubble arcs, but also of complete Y arcs. However, breakage of bubble-containing intermediates at one of the replication forks can give rise to molecules that approximate Y-shaped replication intermediates in structure and result in a Y arc (Linskens and Huberman, 1990; Fangman and Brewer, 1991). Thus, the simple ratio of bubble-containing intermediates to Y-shaped intermediates cannot be used alone to determine the efficiency of origin usage.

A better way to determine the efficiency of origin usage is to determine the direction of replication fork movement in the regions surrounding the replication origin (Fangman and Brewer, 1991). If the origins function in all of the ring molecules in every S phase, then only replication forks that arise at the *ARS* elements and move bidirectionally away from them would be expected. If, however, the origins fail to function in a fraction of the population, then replication forks moving into the *ARS*s from one direction or the other would be expected.

For fork direction analysis, DNA fragments produced by restriction enzyme digestion are separated in a first dimension gel (see MATERIALS AND METHODS) and then digested with a second restriction endonuclease in the gel before the second-dimension separation. The second restriction enzyme is chosen to cut toward one end of the fragment of interest. A probe specific for the larger fragment can then be used to detect the replication intermediates. Different patterns are produced depending on the direction of replication through the fragment.

When replication forks enter the fragment from the end proximal to the second restriction cleavage, early Y-shaped replication intermediates will be cleaved to yield short Y-shaped molecules not detected by the probe and monomer length linear fragments that are detected (Figure 4A). Late Y-shaped replication intermediates will retain the Y-shape but will be smaller. Therefore, the simple-Y pattern is retained but starts further to the left along the line of linear molecules, and the early replication intermediates are detected as a band of monomer-sized fragments in the lower right of the pattern extending leftward from the spot of monomer length nonreplicating molecules (Figure 4B).

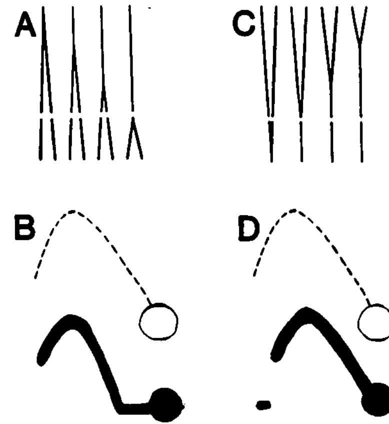

Figure 4. The replication intermediates and patterns expected from fork direction analysis. (A and B) Shapes of replication intermediates and the patterns expected from fork direction analysis of the larger DNA fragment produced by the second restriction digestion in the gel if forks move into the molecule from the side proximal to the in gel cleavage site. (C and D) Shapes of replication intermediates and the patterns expected from fork direction analysis of the larger fragment if forks move into the molecules from the side distal to the in gel cleavage site.

In contrast, when forks move in from the side distal to the second restriction site, early replication intermediates will be cleaved to yield Y-shaped molecules detected by the probe and small linear fragments that are not detected (Figure 4C). Cleavage of late Y-shaped replication intermediates yields monomer-sized linear molecules detected by the probe. This results in a simple-Y pattern that starts from the monomer spot and a band of monomer-sized linear molecules in the late-Y region (Figure 4D).

Direction of Replication around ARS307

We used fork direction analysis to determine the efficiency of usage of the origins coincident with *ARS307* and *ARS309* and to confirm that forks move bidirectionally from both origins. The pattern in Figure 5A was obtained from analysis of the *Bam*HI-*Xho* I fragment centromere-distal to *ARS307*. The restriction enzyme used to cleave in the gel was *Hin*dIII and the DNA probe used for hybridization detected the *Bam*HI-*Hin*dIII fragment. There are two patterns in Figure 5A. The lower pattern is the result of cleavage in the gel and the upper pattern is the remnant of the uncleaved pattern resulting from partial digestion in the gel. In the lower pattern a simple-Y arc ascends directly from the cleaved monomer spot. This pattern indicates that replication forks move through this region away from *ARS307*.

The pattern in Figure 5B was obtained from analysis of the *ARS307*-containing *Eco*RI fragment. The restriction enzyme used to cleave in the gel was *Eco*RV and the DNA fragment used for hybridization detected the larger *Eco*RI-*Eco*RV fragment. In this pattern a simple-

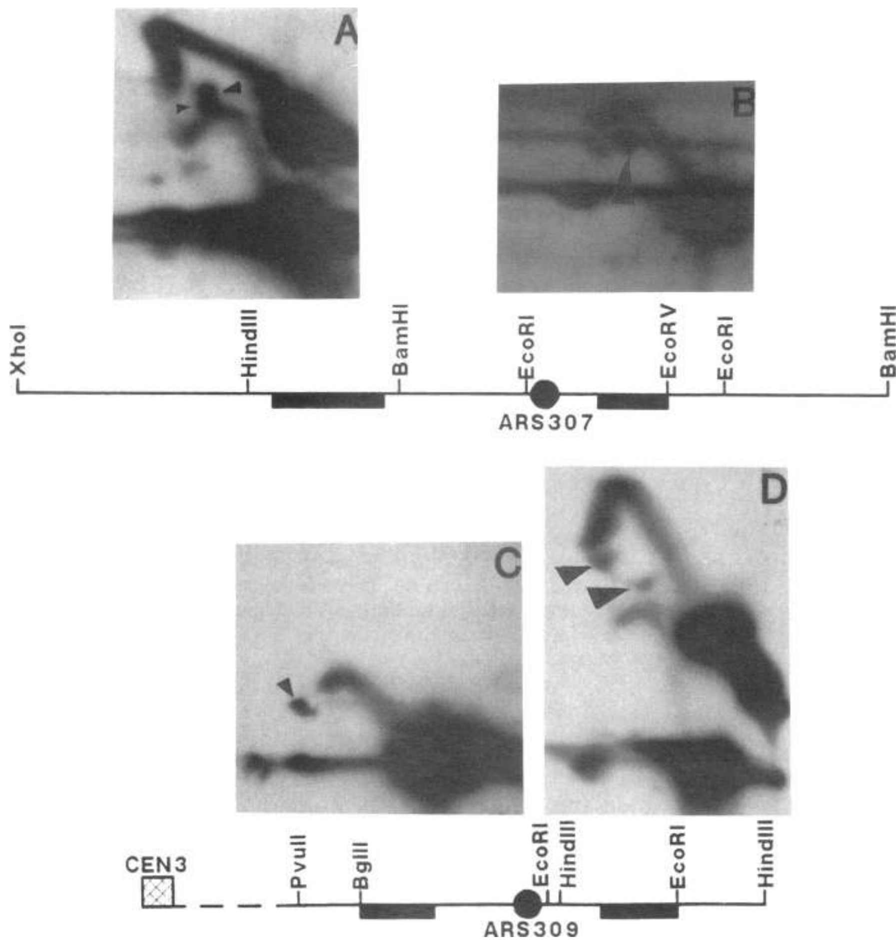

Figure 5. Fork direction analysis of the regions flanking *ARS307* and *ARS309*. A diagram of the chromosomal region is shown below each pair of patterns. The black rectangles below each diagram show the positions of the DNA fragments used as hybridization probes for this analysis. The small black arrows indicate spots resulting from partial restriction enzyme digestion. (A) The *Bam*HI-*Xho* I DNA fragment centromere-distal to *ARS307* cleaved in the gel with *Hin*dIII. (B) The *Eco*RI DNA fragment centromere-proximal to *ARS307* cleaved in the gel with *Eco*RV. (C) The *Eco*RI-*Pvu* II DNA fragment centromere-proximal to *ARS309* cleaved in the gel with *Bgl* II. (D) The *Hin*dIII DNA fragment centromere-distal to *ARS309* cleaved in the gel with *Eco*RI.

Y arc ascends directly from the cleaved monomer spot. There is also a band of monomer length fragments in the lower left of the pattern. The spot below the apex of the Y arc (small arrow) is the result of partial cleavage of nonreplicating DNA before the first-dimension and subsequent cleavage in the gel. The lines across the entire pattern result from hybridization to DNA that spread along the first-dimension gel slice before the second-dimension. There is no evidence of replication forks moving toward *ARS307* through this region. This pattern indicates that replication forks move through this region from *ARS307* toward the centromere. These data indicate that, in the regions surrounding *ARS307*, the majority of replication forks move away from the *ARS*, demonstrating that *ARS307* is an origin of bidirectional replication active in most cells in every S phase.

Direction of Replication around ARS309

Figure 5, C and D, shows the patterns obtained from fork direction analysis of fragments that flank *ARS309*. The pattern in Figure 5C was obtained from analysis of the *Eco*RI-*Pvu* II fragment centromere-proximal to *ARS309*. The restriction enzyme used to cleave in the gel was *Bgl* II and the DNA fragment used for hybridization detected the *Eco*RI-*Bgl* II fragment. In this pattern a simple-Y arc ascends from the cleaved monomer spot. There is also a band of monomer-length fragments in the lower left of the pattern. This pattern indicates that the majority of replication forks move through this region from *ARS309* toward the centromere. Although there is no evidence of replication forks moving into the *ARS* through this region, this exposure is lighter than those in Figure 5, A and B, and therefore a second light arc might not be visible.

The pattern in Figure 5D was obtained from analysis of the *Hin*dIII fragment centromere distal to *ARS309*. The restriction enzyme used to cleave in the gel was *Eco*RI, and the DNA fragment used for hybridization detected the *Hin*dIII-*Eco*RI fragment closest to *ARS309*. As in Figure 5A there are two patterns present in this figure, with the upper pattern resulting from partial digestion in the gel. In the lower pattern a simple-Y arc ascends from the cleaved monomer spot indicating that replication forks move away from *ARS309*. These data indicate that *ARS309* is an origin of bidirectional replication active in most cells in every S phase and together

with the data from *ARS307* suggest that these two replication origins function in greater than 90% of the ring molecules.

Initiation Frequency at the ARS308 Associated Origin

The frequency of initiation at *ARS308* was estimated by densitometric analysis of the autoradiogram shown in Figure 3A to determine the fraction of bubble-shaped replication intermediates in the population. Analysis of different exposures indicated that ~5–10% of the replicating molecules were present in the bubble arc. Because they are replicated by two forks, bubble-shaped intermediates are underrepresented by a factor of two compared to Y-shaped intermediates; thus, replication initiates at *ARS308* in ~10–20% of the ring molecules.

It is unclear why *ARS308* has limited origin function. The *ARS* itself may lack elements that are necessary for efficient origin function, or origin activity may be influenced by its association with *CEN3* or its proximity to *ARS307*. These *ARS* elements are separated by only 7 kb, and it is possible that initiation at one of the elements represses initiation at the other. If *ARS307* were the preferred site of interaction of the replication apparatus, then *ARS308* might be less accessible because of limiting components of the replication machinery or because of other constraints such as changes in local DNA topology.

The possibility that *ARS308* functions poorly as a replication origin because it is repressed by the proximity of *ARS307* was explored. A strain in which *ARS307* was deleted from the ring chromosome was constructed (see MATERIALS AND METHODS), and the replication of *ARS308* was analyzed. Because the 522-bp *ARS307* deletion partially deletes an essential gene, it was necessary to have sequences in the strain that complement this essential function. For this purpose, we constructed a strain carrying the 61-kb ring chromosome *III* and a full-length chromosome *III* recovered from the Carlsberg brewing strain (Nilsson-Tillgren, 1981; Holmberg, 1982). The Carlsberg chromosome *III* is homeologous to the *S. cerevisiae* chromosome *III* in this region and can substitute for *S. cerevisiae* chromosome *III*. However, sequence divergence makes it possible to detect only the *S. cerevisiae* chromosome *III* sequences under high-stringency hybridization conditions.

To be certain that origin function was eliminated by the deletion of *ARS307* from the ring chromosome, the replication of the *Eco*RV fragment carrying the deletion that was analyzed previously in the wild-type ring chromosome (Figure 3D) was examined (Figure 6). The absence of a bubble arc and the presence of an intense Y arc indicate that the replication origin in this fragment was removed by the deletion. This result demonstrates directly that DNA sequences essential for chromosomal origin function are contained within the 522-bp fragment containing *ARS307*.

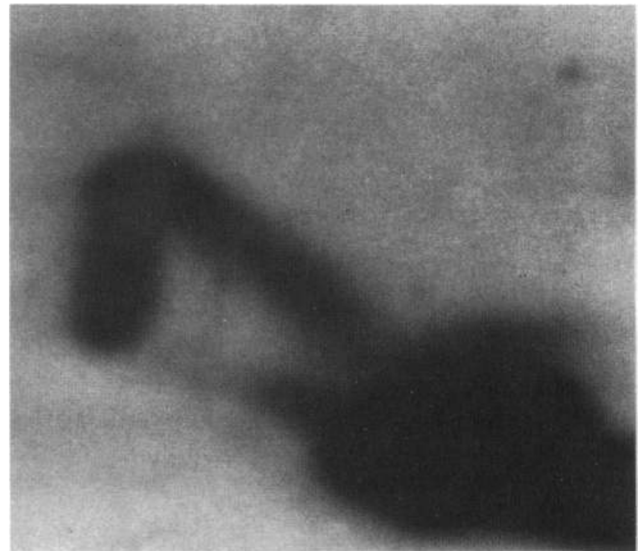

Figure 6. Deletion of the 522 bp *Eco*RI-*Cla* I *ARS307*-containing fragment eliminates chromosomal origin function. The pattern obtained from the analysis of the 2.7-kb *Eco*RV DNA fragment from the *ARS307* deletion strain, corresponding to fragment D in the wild-type strain is shown. The 1.2-kb *Eco*RV-*Pst* I DNA fragment from the C2G region was used as a hybridization probe.

Figure 3Y shows the pattern obtained from analysis of the *ARS308*- (*CEN3*-) containing *Eco*RI fragment from the *ARS307* deletion strain. No qualitative difference between this pattern and that from the nondeletion strain is apparent (Figure 3A). In addition, densitometry revealed no significant quantitative difference in the frequency of initiation at *ARS308* in the two strains (wild-type ~20%, *ARS307* deletion ~18%). These data demonstrate that the low level of origin activity of *ARS308* is not due to the proximity of *ARS307*.

Termination of DNA Replication and Replication Fork Pause Sites

We found three regions of the ring chromosome in which replication terminates. The first is located in the D10H region to the right of *CEN3* between the two highly active replication origins. The other two are associated with replication fork pause sites. One pause site is in the *CEN3* region and the second is in the region containing the hybrid Ty element, halfway between the two highly active origins.

The patterns that arise from replication termination will differ according to whether termination occurs at a specific site or nonspecifically within a region and also whether converging replication forks enter a fragment synchronously or asynchronously. The simplest case is one in which converging replication forks enter a fragment synchronously and meet in the middle, resulting in the double-Y arc shown in Figure 1B. Figure 7 shows the two-dimensional patterns expected for specific and

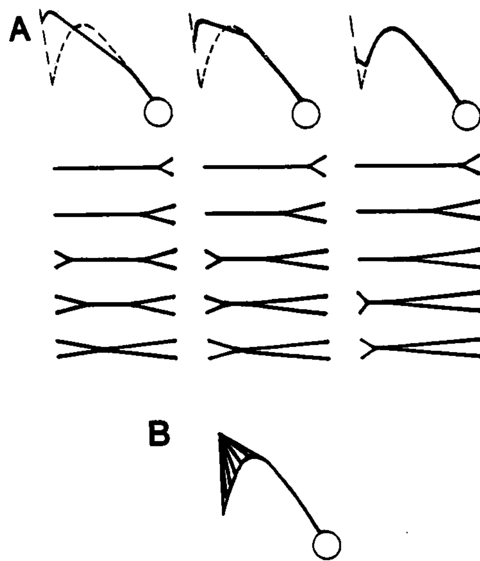

Figure 7. The patterns expected from specific and nonspecific replication termination. (A) Representative patterns expected to arise due to three different specific sites of termination within a fragment are shown. These patterns are expected if double-Y replication intermediates arise form simple-Y intermediates at specific masses. This would occur if the movement of one of the replication forks was blocked, at a specific site, so that a second converging fork could enter the fragment. This would result in a spike of hybridization signal arising from a particular point on the Y arc. (B) The pattern expected to arise due to nonspecific termination is shown. This pattern is expected if termination occurs randomly within a DNA fragment and would contain a collection of all of the spikes expected from the patterns in A. This would result in a diffuse triangle of hybridization extending from the late part of the Y arc.

non-specific termination in the more complex situations in which converging forks enter the fragment asynchronously (Martin-Parras *et al.*, 1991).

Figure 7A shows three examples of patterns that are expected to arise if a fragment is initially replicated by a single replication fork and a second converging fork always enters the fragment at a precise time after the first fork. This results in a transition from Y-shaped to double-Y-shaped replication intermediates at a particular mass, determined by the time between the entry of the first fork and the entry of the second converging fork, and produces a pattern with a spike of double-Y-shaped intermediates emanating from a single point on the Y arc.

Similar patterns will be obtained if the first fork to enter the fragment encounters a barrier to replication fork movement at a specific site (Martin-Parras *et al.*, 1991). This can also result in a spot of accumulated Y-shaped replication intermediates if the converging fork enters the fragment substantially later than the first fork.

Figure 7B shows the pattern expected if replication terminates nonspecifically within a fragment. In this example, the time at which the second converging fork enters the fragment is not constant with regard to the first fork, and in some of the population a second fork never enters the fragment. This results in a complete Y arc (arising from molecules in which replication does not terminate) and a triangle of replication intermediates (arising from the random population of double-Y replication intermediates) extending leftward from the late Y arc.

Termination in the D10H Region

The region of replication termination to the right of *CEN3*, shown in Figure 3, U and V, were obtained from the analysis of overlapping *Pvu* II and *Xho* I fragments from the 10.5-kb D10H region. The patterns in both of these figures contain complete Y arcs with light triangles of double-Y replication intermediates, consistent with the pattern diagrammed in Figure 7B and indicating that replication terminates nonspecifically throughout the region. These fragments define a zone of termination because there is no evidence of double-Y replication intermediates in adjacent fragments (Figure 3, T and W). Forks should enter this region from *ARS307* and/or *ARS308* on the left and *ARS309* on the right. The observation that termination occurs over a large (10.5 kb) region suggests that termination occurs simply wherever replication forks meet.

Termination and Replication Fork Pausing in the CEN3 Region

The second region of termination is in the area of *CEN3*. As discussed above, Figure 3A is the pattern obtained from the analysis of the *CEN3*-containing *Eco*RI fragment, and Figure 3B is the pattern obtained from the analysis of the overlapping *CEN3*-containing *Bam*HI-*Xba* I fragment. The predominant feature of these patterns is a complete Y arc, indicating that these two fragments are replicated primarily by single replication forks that initiate outside of the fragment. However, both patterns also contain light triangles of double-Y replication intermediates that emanate from the Y arcs, indicating that replication terminates nonspecifically in this region. (Darker exposures of the autoradiogram in Figure 3B reveal the light triangle.) Termination in this region is limited to these fragments because there is no evidence of double-Y arcs in the patterns obtained from the analysis of adjacent fragments (Figure 3, C and X). The termination region is ~7 kb in length.

The patterns in Figure 3, A and B, also reveal spots of increased hybridization signal along the Y arc, indicating that Y-shaped replication intermediates of a particular size are accumulating. The complete Y arcs indicate that replication forks are able to move completely through the fragments, and the absence of single spikes of double-Y replication intermediates suggests that there is not an absolute barrier to replication fork movement, but rather a site at which replication forks pause. The mass of the accumulated replication inter-

mediates in these patterns indicates that the position of the pause site is coincident with *CEN3*. We have shown that the ability to cause replication forks to pause is a general property of yeast centromeres and that the pause site is dependent on the protein-DNA structure that the centromere normally adopts (Greenfeder and Newlon, 1992). The termination in this region might occur because some forks initiated at *ARS307* pause long enough at *CEN3* to allow forks to enter the fragment from the opposite end, or because forks initiated at *ARS307* meet forks initiated at *ARS308*. Densitometric analysis revealed that ~15% of the replication intermediates of the *CEN3*-containing *Eco*RI fragment are termination intermediates. This is very close to the estimate of the frequency of origin activity at *ARS308* (10–20%), suggesting that all of the termination in this region might be due to the low level of origin activity at *ARS308*. It is, however, possible that the *CEN3*-associated pause site contributes to termination and further exploration of this will require the removal of the *ARS308* replication origin.

Termination and Replication Fork Pausing in the Ty, LEU2 Region

The third region of termination is in the sequences around and including part of the hybrid Ty element and part of the *LEU2* gene, midway between *ARS307* and *ARS309* on the ring chromosome. This region is also associated with a replication fork pause site. Figure 3L shows the pattern obtained from the analysis of the *Pst* I-*Eco*RI fragment that contains most of the hybrid Ty element and the 5′ half of the *LEU2* gene. This pattern contains a complete Y arc, a triangle of double-Y-shaped replication intermediates (revealed in darker exposures), and a region of intense hybridization signal along the ascending portion of the Y arc. The triangle of double-Y-shaped intermediates indicates that replication terminates nonspecifically within this fragment. The intense signal of accumulated Y-shaped replication intermediates along the complete Y arc indicates that there is a site in this region at which replication forks pause (open arrow). The mass of the accumulated replication intermediates indicates that replication forks pause either in the middle or at the 3′ end of the hybrid Ty element. To determine the position of the pause site, we analyzed overlapping DNA fragments.

The pattern obtained from the analysis of the *Pst* I fragment that overlaps the fragment in Figure 3L (Figure 3M) contains a complete Y arc, of varying intensity, and a triangle of double-Y replication intermediates that emanates from the Y arc. The early part of this Y arc is more intense than the late part, indicating that early Y-shaped replication intermediates are more abundant than late Y-shaped intermediates. A spot of accumulated replication intermediates is present near the apex of the Y arc but is difficult to see in this exposure because of the intense hybridization signal. Figure 3K is the pattern obtained from the overlapping *Sal* I fragment. This pattern contains a complete Y arc and a spot on the late part of the Y arc. The mass of the accumulated replication intermediates in these three fragments indicates that replication forks that initiate at *ARS307* pause near the 3′ end of the hybrid Ty element. These three overlapping fragments define a third zone of termination because there is no evidence of double-Y replication intermediates in adjacent fragments (Figure 3, J and N).

The varying intensity of hybridization signal in the pattern in Figure 3M suggests that forks initiated at *ARS307* reach this *Pst* I fragment and replicate it up to the pause site. These forks then slow near the 3′ end of the hybrid Ty giving rise to the intense hybridization signal in the early part of the Y arc, and allowing forks to enter the fragment from the other end to complete replication. The pattern of double-Y intermediates is less diffuse than the patterns seen in other regions, and there is a paucity of late Y-shaped intermediates, suggesting that termination occurs within a narrower area in the *Pst* I fragment and that most of the late replication intermediates are double-Y shaped. The small amount of late Y-shaped replication intermediates could arise in at least three ways. First, they could result from molecules in which replication failed to initiate at either *ARS307* or *ARS309*, in which case this fragment would be replicated by a single fork originating at the remaining active origin. Second, they could result from forks that initiate at *ARS307* that enter the fragment, pause transiently at the 3′ end of the Ty element, and then continue to move through the fragment. Third, late Y-shaped intermediates could be produced by the breakage of X shaped molecules that have the crossover near one end (Martin-Parras *et al.*, 1991).

Are the Sites of Termination Fixed?

The patterns of replication termination on the ring chromosome demonstrate that replication does not terminate at specific sites. However, this observation does not eliminate the possibility that these termination regions are fixed and controlled by *cis*-acting sequences that function in a manner similar to the termination sequences in the *E. coli* chromosome (reviewed by Kuempel *et al.*, 1989). We analyzed replication termination in the derivative of the ring chromosome in which *ARS307* was deleted to determine if the positions of termination could be altered by changing the pattern of initiation. If the regions of termination are determined by replication fork traps, as in *E. coli*, the sites of termination should not be altered. However, if termination simply occurs where replication forks meet, then a new region of termination should be created.

Figure 8, A and B, shows the patterns obtained from the analysis of the *Bam*HI fragment containing the D8B region of the ring chromosome isolated from the wild-

type and *ARS307* deletion ring chromosomes, respectively. This fragment is directly opposite *ARS309* and is the region in which termination would be expected if replication initiated only at *ARS309* and forks moved around the ring chromosome unimpeded. Figure 8A is an enlargement and darker exposure of the pattern in Figure 3H and contains a very small amount of hybridization signal in the area of double-Y replication intermediates. The pattern from the *ARS307* deletion chromosome (Figure 8B) contains a complete simple-Y arc with a prominent hybridization signal in the region of termination intermediates. Densitometric analysis of these two patterns indicated that the frequency of double-Y replication intermediates was 1 ± 0.09% in the wild-type ring chromosome and 18 ± 3% in the chromosome containing the *ARS307* deletion. This indicates that the position of replication termination is altered by the removal of *ARS307* and demonstrates that replication termination is not restricted to a limited region of the ring chromosome. This suggests that the positions of termination are not controlled by specific *cis*-acting termination sequences that block the movement of replication forks, but, rather that replication generally terminates wherever replication forks meet.

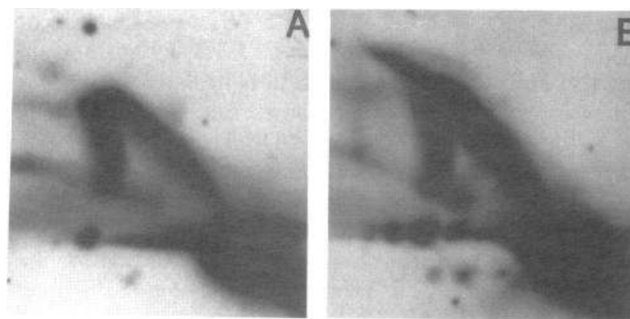

Figure 8. Termination in the D8B region in the wild-type and *ARS307* deletion ring chromosomes. The patterns obtained from the analysis of the D8B *Bam*HI DNA fragment from the wild-type and *ARS307* deletion ring chromosomes are shown. (A) Wild-type. (B) *ARS307* deletion. The hybridization probe in both cases was a 1.1-kb *Eco*RV fragment from the D8B region.

DISCUSSION

Replication Initiation

Our analysis of the 61-kb ring chromosome shows that there are three sites at which DNA replication initiates on the ring chromosome, each coincident with one of the *ARS* elements. In addition, the rDNA *ARS*, *ARS1*, *ARS305*, *ARS501*, the *HMRE ARS*, *ARS121*, and *ARS306* have all been shown to function as chromosomal replication origins (Brewer and Fangman, 1988; Huberman *et al.*, 1988; Linskens and Huberman, 1988; Ferguson *et al.*, 1991; Walker *et al.*, 1991; Deshpande and Newlon, 1992; Rivier and Rine, 1992; Zhu *et al.*, 1992). These results indicate that replication initiates at specific DNA sequences in yeast chromosomes that have been identified by their ability to function as *ARS* elements on plasmids. These results lead to two further questions.

First, do all chromosomal replication origins have *ARS* function on plasmids? Including the 61 kb analyzed in this study, ~120 kb of chromosome *III* have been analyzed by two-dimensional electrophoresis, and *ARS* elements are the only DNA sequences that have been found to function as chromosomal replication origins (Huberman *et al.*, 1988; Dubey *et al.*, 1991; Rivier and Rine, 1992; Zhu *et al.*, 1992). These results suggest that *ARS* elements are the only sequences capable of functioning as chromosomal replication origins. Examination of additional origin sequences will provide the information needed to substantiate this conclusion. Second, do all *ARS* elements function efficiently as chromosomal replication origins? The answer is clearly no. We previously showed that the *ARS* elements at the left end of chromosome *III*, associated with the silent-mating-type locus *HML*, have no detectable chromosomal origin function (Dubey *et al.*, 1991). Here we demonstrate that the three ring chromosome *ARS* elements differ quantitatively in their origin function; *ARS307* and *ARS309* function >90% of the time, whereas *ARS308* functions only 10–20% of the time. Thus, all chromosomal replication origins identified to date coincide with *ARS* elements, but several *ARS* elements have little or no chromosomal origin function.

The low level of origin function at *ARS308* is not due to the proximity of *ARS307* because the removal of *ARS307* had no effect. This result contrasts with the results of other studies that examined origin use in circular plasmid molecules containing two or more copies of the same origin. In multimers of the yeast 2 μm plasmid (Brewer and Fangman, 1987), SV40 (Brockman *et al.*, 1975), and ColE1 (Schvartzman, personal communication) only one of the available origins appears to be used.

The weak origin function of the centromere-associated *ARS308* correlates with its weak *ARS* activity in plasmid stability assays (Clarke and Carbon, 1980; Newlon *et al.*, 1986), suggesting either that centromere function interferes with origin activity or that *ARS308* lacks sequences necessary to function as an efficient origin. Each *ARS* element studied to date contains at least one perfect or near-perfect match to the *ARS* core consensus sequence (reviewed by Newlon, 1988). It is interesting to note that the only matches to the core consensus sequence within the 624-bp *Bam*HI-*Sau* 3A DNA fragment that contains *ARS308* and *CEN3* (Clarke and Carbon, 1980; Fitzgerald-Hayes *et al.*, 1982; Newlon *et al.*, 1991) are in the central A+T rich region of *CEN3* (centromere DNA element II) (Van Houten and Newlon, unpublished results). Thus, it is possible that the centromere-binding proteins, which are associated with the centromere during S phase, inhibit origin activity of *ARS308* (Greenfeder and Newlon, 1992).

In other studies we have also analyzed the effects of nearby telomere sequences, chromosomal position, and transcriptional silencing on the ability of the *HML*-associated *ARS* elements to function as chromosomal replication origins (Dubey *et al.*, 1991). These *ARS* elements have strong *ARS* function in plasmid stability assays, and *ARS301* has been directly demonstrated to have origin function on plasmids. There was no evidence of origin function associated with *ARS302* or *ARS303* when these *ARS* elements were analyzed in a yeast strain in which chromosome *III* was circularized, suggesting that the nearby telomere of chromosome *III* is not responsible for repressing origin function. Moreover, we have found that creating a new telomere within 2 kb of *ARS307* does not alter the efficiency with which *ARS307* functions as a chromosomal origin (Ong and Newlon, unpublished results), and Ferguson and Fangman (1992) have reported that *ARS501* functions within ~10 kb of telomeric sequences on a linear plasmid. There was also no evidence of chromosomal origin function at the *HML*-associated *ARS*s in a strain in which the silent-mating-type locus was derepressed by *sir1* or *sir4* mutations, suggesting that the transcriptional silencing machinery was not responsible for repressing origin function (Dubey *et al.*, 1991).

Replication Termination and Replication Fork Pausing

Our results demonstrate that termination occurs nonspecifically over large regions of DNA rather than at specific sites in the ring chromosome. This indicates that the positions of termination on the ring chromosome are not controlled by DNA sequences that function as absolute barriers to replication fork movement. Because removal of *ARS307* created a new region of termination, we also conclude that efficient replication fork traps must not be present on the ring chromosome. However, termination in the *ARS307* deletion chromosome was not completely eliminated from the two regions midway between *ARS307* and *ARS309* (Greenfeder and Newlon, unpublished results). The interpretation of these results is confounded by the weak origin activity of *ARS308*. Because *ARS308* is only 7 kb away from *ARS307*, forks that initiate at *ARS308* and *ARS309* in the deletion chromosome would meet in the same areas as forks that initiate at *ARS307* and *ARS309* meet in the wild-type chromosome. Alternatively, the low level of termination in these regions could result from inefficient replication fork traps. Inactivation of *ARS308* origin function in conjunction with the *ARS307* deletion could rule out replication fork traps more definitively.

The two replication fork pause sites on the 61-kb ring chromosome are the second and third examples of replication fork barriers in the yeast genome. A replication fork barrier that defines a termination site has been found at the end of the yeast 35S rDNA transcription unit (Brewer and Fangman, 1988; Linskens and Huberman, 1988). Yeast rDNA is arranged as a 9-kb repeating unit that contains the genes that encode rRNA (Bell *et al.*, 1977). There is an *ARS* element in the nontranscribed spacer region (Szostak and Wu, 1979; Kouprina and Larianov, 1983) that functions inefficiently as a chromosomal replication origin (Saffer and Miller, 1986; Brewer and Fangman, 1988; Linskens and Huberman, 1988). Replication forks initiate at the rDNA *ARS* and those that move toward the 35S transcript are halted by a replication fork barrier. It is not yet clear whether the rDNA replication fork barrier is the result of a *cis*-acting DNA sequence or the result of the transcription machinery impeding the replication machinery. It has been suggested that this barrier serves to stop the collision of transcription machinery moving through the 35S gene with the replication machinery moving in the opposite direction, an arrangement analogous to that in *E. coli* (reviewed by Brewer, 1988).

A detailed analysis of the *CEN3* pause site, which is dependent upon the protein-DNA interactions of centromere binding proteins, is reported elsewhere (Greenfeder and Newlon, 1992). The termination that occurs in the region of *CEN3* is due to the pause site, to the low level of initiation at *ARS308*, or to both.

The Ty-associated pause site is midway between *ARS307* and *ARS309* on the 61-kb ring chromosome. The two-dimensional gel patterns found for fragments from this region indicate that the pause site slows or stops replication forks that initiate at *ARS307*.

Our analysis of overlapping fragments of the ring chromosome suggests that replication forks move at a relatively uniform rate through the chromosome. The complete DNA sequence of chromosome *III* (Oliver *et al.*, 1992) predicts that there are 35 open reading frames at least 100 amino acids in length in the 61-kb ring chromosome, each open reading frame presumably having a promoter where specific regulatory proteins interact. Identification of only two sites in this chromosome associated with detectable slowing of replication forks suggests either that the replication apparatus has no difficulty passing through most sites of protein-DNA interaction associated with regulation of gene expression or that these interactions are sufficiently short lived that they do not produce significant accumulations of replication intermediates.

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REFERENCES

Bell, B.I., DeGennero, L.J., Gelfand, D.H., Bishop, R.J., Valenzuela, P., and Rutter, W.J. (1977). Ribosomal RNA genes of *Saccharomyces*

cerevisiae: physical map of the repeating unit and identification of the region coding for the 5S, 5.8S, 18S and 25S ribosomal RNAs. J. Biol. Chem. *252*, 8118–8125.

Boeke, J.D., Trueheart, J., Natsoulis, G., and Fink, G.R. (1987). 5-fluoroorotic acid as a selective agent in yeast molecular genetics. Methods Enzymol. *154*, 164–175.

Brewer, B.J. (1988). When polymerases collide: replication and the transcriptional organization of the *E. coli* chromosome. Cell *53*, 679–686.

Brewer, B.J., and Fangman, W.L. (1987). The localization of the replication origins on *ARS* plasmids in *S. cerevisiae.* Cell *51*, 463–471.

Brewer, B.J., and Fangman, W.L. (1988). A replication fork barrier at the 3′ end of yeast ribosomal RNA genes. Cell *55*, 637–643.

Brewer, B.J., Sena, E.P., and Fangman, W.L. (1988). Analysis of replication intermediates by two-dimensional agarose gel electrophoresis. In: Cancer Cells. Eukaryotic DNA Replication, vol. 6, ed. B. Stillman and T.J. Kelly. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 229–234.

Brockman, W.W., Gutai, M.W., Nathans, D. (1975). Evolutionary variants of simian virus 40: characterization of cloned complementing variants. Virology *66*, 36–52.

Campbell, J.L., and Newlon, C.S. (1991). Chromosomal DNA replication. In: The Molecular and Cellular Biology of the Yeast *Saccharomyces:* Genome Dynamics, Protein Synthesis, and Energetics, ed: J.R. Broaob, J.R. Pringle, and E.W. Jones. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 41–146.

Clarke, L., and Carbon, J. (1980). Isolation of a yeast centromere and construction of small circular minichromosomes. Nature *287*, 504–509.

Davis, R.W., Botstein, D., and Roth, J.R. (1980). Advanced Bacterial Genetics, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Deshpande, A.M., and Newlon, C.S. (1992). The *ARS* consensus sequence is required for chromosomal origin function in *Saccharomyces cerevisiae.* Mol. Cell. Biol. (*in press*).

Dubey, D.D., Davis, L.R., Greenfeder, S.A., Ong, L.Y., Zhu, J., Broach, J.R., Newlon, C.S., and Huberman, J.A. (1991). Evidence suggesting that the *ARS* elements associated with silencers of the yeast mating-type locus *HML* do not function as chromosomal replication origins. Mol. Cell. Biol. *11*, 5346–5355.

Fangman, W.L., and Brewer, B.J. (1991). Activation of replication origins within yeast chromosomes. Annu. Rev. Cell Biol. *7*, 375–402.

Ferguson, B.M., Brewer, B.J., Reynolds, A.E., and Fangman, W.L. (1991). A yeast origin of replication is activated late in S phase. Cell *65*, 507–515.

Ferguson, B.M., and Fangman, W.L. (1992). A position effect on the time of replication origin activation in yeast. Cell *68*, 333–339.

Fitzgerald-Hayes, M., Clarke, L., and Carbon, J. (1982). Nucleotide sequence comparison and functional analysis of yeast centromere DNAs. Cell *29*, 235–244.

Gahn, T.A., and Schildkraut, C.L. (1989). The Epstein-Barr virus origin of plasmid replication, *oriP*, contains both the initiation and termination sites of DNA replication. Cell *58*, 527–535.

Greenfeder, S.A., and Newlon, C.S. (1992). Replication forks pause at yeast centromeres. Mol. Cell. Biol. *12*, 4056–4066.

Griffin, B.E., and Fried, M. (1975). Amplification of a specific region of the polyoma virus genome. Nature *256*, 175–177.

Holmberg, S. (1982). Genetic differences between *Saccharomyces carlsbergensis* and *Saccharomyces cerevisiae* II. Restriction endonuclease analysis of genes in chromosome *III*. Carlsberg Res. Commun. *47*, 233–244.

Huberman, J.A., Spotila, L.D., Nawotka, K.A., El-Assouli, S.M., and Davis, L.R. (1987). The *in-vivo* replication origin of the yeast 2 μm plasmid. Cell *51*, 473–481.

Huberman, J.A., Zhu, J., Davis, L.R., and Newlon, C.S. (1988). Close association of a DNA replication origin and an *ARS* element on chromosome *III* of the yeast, *Saccharomyces cerevisiae.* Nucleic Acids Res. *16*, 6373–6384.

Ito, H., Fukada, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. *153*, 163–168.

Johnston, L.H., and Williamson, D.H. (1978). An alkaline sucrose gradient analysis of the mechanism of nuclear DNA synthesis in the yeast *Saccharomyces cerevisiae.* Mol. Gen. Genet. *164*, 217–225.

Kouprina, N.Y., and Larianov, V.L. (1983). Study of rDNA replication in *Saccharomyces.* Curr. Genet. *7*, 433–438.

Krysan, P.J., and Calos, M.P. (1991). Replication initiates at multiple locations on an autonomously replicating plasmid in human cells. Mol. Cell. Biol. *11*, 1464–1472.

Kuempel, P.L., Pelletier, A.J., and Hill, T.M. (1989). Tus and the terminators: the arrest of replication in prokaryotes. Cell *59*, 581–583.

Linskens, M.H.K., and Huberman, J.A. (1988). Organization of replication of ribosomal DNA in *Saccharomyces cerevisiae.* Mol. Cell. Biol. *8*, 4927–4935.

Linskens, M.H.K., and Huberman, J.A. (1990). Ambiguities in results obtained with 2D gel replicon mapping techniques. Nucleic Acids Res. *18*, 647–652.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Martin, M.A., Khoury, G., and Fareed, G.C. (1975). Specific reiteration of viral DNA sequences in mammalian cells. Cold Spring Harbor Symp. Quant. Biol. *39*, 129–136.

Martin-Parras, L., Hernandez, P., Martinez-Robles, M.L., and Schvartzman, J.B. (1991). Unidirectional replication as visualized by two-dimensional agarose gel electrophoresis. J. Mol. Biol. *220*, 1–11.

McCarroll, R.M., and Fangman, W.L. (1988). Time of replication of yeast centromeres and telomeres. Cell *54*, 505–513.

Mirkovitch, J., Mirault, M.-E., and Laemmli, U.K. (1984). Organization of the higher-order chromatin loop: specific DNA attachment sites on nuclear scaffold. Cell *39*, 223–232.

Newlon, C.S. (1988). Yeast chromosome replication and segregation. Microbiol. Rev. *52*, 568–601.

Newlon, C., Green, R., Hardeman, K., Kim, K., Lipchitz, L., Palzkill, T., Synn, S., and Woody, S.T. (1986). Structure and organization of yeast chromosome III. UCLA Symp. Mol. Cell. Biol. *33*, 211–223.

Newlon, C.S., Lipchitz, L.R., Collins, I., Deshpande, A., Devenish, R.J., Green, R.P., Klein, H.L., Palzkill, T.G., Ren, R., Synn, S., and Woody, S.T. (1991). Analysis of a circular derivative of *Saccharomyces cerevisiae* chromosome *III:* a physical map and location of *ARS* elements. Genetics *129*, 343–357.

Nilsson-Tillgren, T. (1981). Genetic differences between *Saccharomyces carlsbergensis* and *Saccharomyces cerevisiae.* Analysis of chromosome *III* by single chromosome transfer. Carlsberg Res. Commun. *46*, 65–76.

Nowatka, K.A., and Huberman, J.A. (1988). Two-dimensional gel electrophoretic method for mapping DNA replicons. Mol. Cell. Biol. *8*, 1408–1413.

Oliver, S.G., van der Aart, Q.J.M., Agostoni-Carbone, M.L., Aigle, M., *et al.*, (1992). The complete DNA sequence of yeast chromosome III. Nature *357*, 38–46.

Palzkill, T.G., and Newlon, C.S. (1988). A yeast replication origin consists of multiple copies of a small conserved sequence. Cell *53*, 441–450.

Palzkill, T.G., Oliver, S.G., and Newlon, C.S. (1986). DNA sequence analysis of *ARS* elements from chromosome *III* of *Saccharomyces cerevisiae:* identification of a new conserved sequence. Nucleic Acids Res. *14*, 6247–6264.

Rivier, D.H., and Rine, J. (1992). An origin of DNA replication and a transcription silencer require a common element. Science *256*, 659–663.

Saffer, L.D., and Miller, O.L., Jr. (1986). Electron microscopic study of *Saccharomyces cerevisiae* rDNA chromatin replication. Mol. Cell. Biol. *6*, 1148–1157.

Strathern, J.N., Newlon, C.S., Herskowitz, I., and Hicks, J.B. (1979). Isolation of a circular derivative of yeast chromosome *III:* implications for the mechanism of mating type interconversion. Cell *18*, 309–319.

Struhl, K., Stinchcomb, D.T., Scherer, S., and Davis, R.W. (1979). High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proc. Natl. Acad. Sci. USA *76*, 1035–1039.

Surosky, R.T., and Tye, B.-K. (1985). Resolution of dicentric chromosomes by Ty-mediated recombination in yeast. Genetics *110*, 397–419.

Szostak, J.W., and Wu, R. (1979). Insertion of a genetic marker into the ribosomal DNA of yeast. Plasmid *2*, 536–554.

Van Houten, J.V., and Newlon, C.S. (1990). Mutational analysis of a replication origin from yeast chromosome *III*. Mol. Cell. Biol. *10*, 3917–3925.

Vaughn, J.P., Dijkwel, P.A., and Hamlin, J.L. (1990). Replication initiates in a broad zone in the amplified CHO dihydrofolate reductase domain. Cell *61*, 1075–1087.

Walker, S.S., Malik, A.K., and Eisenberg, S. (1991). Analysis of the interactions of functional domains of a nuclear origin of replication from *Saccharomyces cerevisiae*. Nucleic Acids Res. *19*, 6255–6262.

Zhu, J., Newlon, C.S., and Huberman, J.A. (1992). Localization of a DNA replication origin and a termination zone on chromosome *III* of *Saccharomyces cerevisiae*. Mol. Cell. Biol. (*in press*).