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Completion of Replication Map of *Saccharomyces cerevisiae* Chromosome III

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In *Saccharomyces cerevisiae* chromosomal DNA replication initiates at intervals of ~40 kb and depends upon the activity of autonomously replicating sequence (ARS) elements. The identification of ARS elements and analysis of their function as chromosomal replication origins requires the use of functional assays because they are not sufficiently similar to identify by DNA sequence analysis. To complete the systematic identification of ARS elements on *S. cerevisiae* chromosome III, overlapping clones covering 140 kb of the right arm were tested for their ability to promote extrachromosomal maintenance of plasmids. Examination of chromosomal replication intermediates of each of the seven ARS elements identified revealed that their efficiencies of use as chromosomal replication origins varied widely, with four ARS elements active in ≤10% of cells in the population and two ARS elements active in ≥90% of the population. Together with our previous analysis of a 200-kb region of chromosome III, these data provide the first complete analysis of ARS elements and DNA replication origins on an entire eukaryotic chromosome.

INTRODUCTION

The replication of eukaryotic chromosomes initiates at multiple replication origins spaced at intervals of 40–100 kb. In the budding yeast, *Saccharomyces cerevisiae*, replication origins depend upon *cis*-acting replicators called autonomously replicating sequence (ARS) elements, which are recognized by their ability to maintain extrachromosomal plasmids. The initiation of replication at individual replicators is a tightly regulated process. Replication initiations are confined to S phase, and individual replicators initiate at reproducible times during S phase, some early and some late (Reynolds *et al.*, 1989, and references therein; Friedman *et al.*, 1997; Donaldson *et al.*, 1998b). Moreover, the efficiencies of initiation vary from one replicator to another, the extreme case being ARS elements that are not active as replication origins in their normal chromosomal positions (Dubey *et al.*, 1991; Newlon *et al.*, 1993; Friedman *et al.*, 1997; Yamashita *et al.*, 1997).

Replicators become competent to initiate replication during the G1 phase of the cell cycle through the stepwise assembly of prereplicative complexes on replicators that are bound by origin recognition complex (ORC) (reviewed by Dutta and Bell, 1997). The actual initiation events require the activities of at least two protein kinases, the cyclin-dependent kinase (CDK) Cdc28p associated with cyclin B (Clb5p or Clb6p) and the Cdc7p kinase associated with its regulatory subunit Dbf4p. The assembly of prereplicative complexes is prevented by the activity of cyclin B-associated CDK, effectively preventing reinitiation at origins during a single S phase (reviewed by Diffley, 1996). Timing determinants also appear to be specified during G1 of the cell cycle (Raghuraman *et al.*, 1997), although the relationship between establishment of the prereplication complex and the specification of initiation timing is unclear. In the case of a late-replicating region of chromosome XIV, DNA sequences surrounding the small replicators influence the timing of initiation in plasmids, demonstrating that timing determinants are not restricted to the replicator sequences per se (Friedman *et al.*, 1996). However, initiation at late origins requires the activity of both the Cdc7 kinase and the S-phase CDK (Cdc28p/Clb5p or Cdc28p/Clb6p) whose targets presumably include one or more components of the prereplication complex (Donaldson *et al.*, 1998a,b). The relationship between replication timing and the efficiency of replicator use also is not clearly understood.

Further elucidation of the mechanisms underlying the regulation of the timing and efficiency of use of replicators on a chromosomal level will require the systematic identification of ARS elements on entire chromosomes and analysis of their activity as chromosomal replicators. Despite years of study, including the detailed analysis of several ARS ele-

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ments, it is not yet possible to predict the locations of ARS elements by DNA sequence analysis. Nevertheless, ARS elements share a number of features. They typically are 100–200 bp in length and are located in intergenic regions. The paradigm ARS element contains a single essential match to a degenerate 11-bp sequence [5′-(A/T)TTTA(T/C)(A/G)TTT(A/T)-3′], the ARS consensus sequence (ACS), which is the core of the binding site for the six-subunit initiator protein, the ORC (reviewed by Newlon, 1996). However, it appears that 20–30% of ARS elements contain multiple, redundant matches to the ACS, all of which must be mutated to inactivate ARS activity (Theis and Newlon, 2001). Because the ACS is degenerate and the yeast genome has a high A + T content, matches to the ACS occur far more frequently than ARS elements, making the occurrence of the sequence a poor predictor of the presence of an ARS element (Newlon and Theis, 1993). For example, although only 19 ARS elements have been identified on chromosome III, this chromosome contains ~3800 sequences that match the ACS at nine or more positions, ~60% of which contain the three highly conserved Ts at positions 8, 9, and 10 of the ACS (Newlon, unpublished data). In addition to the ACS, ARS elements contain a variable number of small elements that contribute to activity (Marahrens and Stillman, 1992; Rao *et al*., 1994; Theis and Newlon, 1994; Huang and Kowalski, 1996). However, with the exception of binding sites for Abf1p, which are not present in all ARS elements, there is no sequence homology between the functional elements found in different ARSs. Thus, despite the availability of the entire yeast genome sequence, ARS elements and replication origins have to be identified by functional assays.

Only a small fraction of the yeast genome has been analyzed for chromosomal replicator activity. One of the largest chromosomal regions that has been tested is a 200-kb region that comprises ~65% of chromosome III (Newlon *et al*., 1991, 1993). In this article, we report the systematic identification and localization of ARS elements in the 140-kb region of chromosome III that was not previously studied, as well as studies of the efficiencies with which each of these ARS elements functions as a chromosomal replicator. Together with our previous analysis of the left two-thirds of chromosome III (Newlon *et al*., 1991, 1993), these studies provide the first complete analysis of the replication of an entire eukaryotic chromosome.

MATERIALS AND METHODS

Plasmids, Strains, and Media

The *URA3* shuttle vector pRS306 (Sikorski and Hieter, 1989) was used for subcloning fragments of chromosome III. *Escherichia coli* strain HB101 (Boyer and Roulland-Dussoix, 1969) was used for propagation of plasmids. *S. cerevisiae* strain YPH45 (*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-1*; Sikorski and Hieter, 1989) was used for plasmid stability measurements. *S. cerevisiae* strains YPH45, YPH47 (*MATα ura3-52 lys2-801 ade2-101 trp1-1*; Sikorski and Hieter, 1989), and CF4-16B (*MAT***a** *his4-280/MATα his4-290, ade2-101 ura3-52*), a haploid strain disomic for chromosome III (Dershowitz and Newlon, 1993), were used for the analysis of replication intermediates. *S. cerevisiae* strain YPH45-7 was constructed by integrating plasmid pYND95 near the right telomere of chromosome III of strain YPH45 (Figure 4).

Yeast strains were propagated on YEPD or color assay medium (Dershowitz and Newlon, 1993). Transformants carrying *URA3* plasmids were selected and maintained on −Ura medium (Van Houten and Newlon, 1990).

Construction of Chromosome III Plasmids

Because the complete DNA sequence of chromosome III was known at the time we initiated this project (Oliver *et al*., 1992), we made use of the overlapping bacteriophage λ and cosmid clones isolated from *S. cerevisiae* strain AB972 that were used in the initial chromosome III sequencing project as the primary source of DNA for subcloning (Riles *et al*., 1993). To facilitate analysis of the chromosome III fragments for ARS activity subclones were constructed in the integrating *URA3* vector pRS306 (Sikorski and Hieter, 1989). The choice of restriction enzymes used for subcloning was based on the predicted restriction map of the region in question. Subclones derived from the Riles *et al.* (1993) clones cover 120.6 kb of the 131.5-kb region examined. A 4.7-kb gap containing *THR4* was covered with DNA subcloned from pSG315 (Goldway *et al*., 1993) and pYthr4 (Mannhaupt *et al*., 1990), and a 1.6-kb gap containing *RAD18* was covered with DNA subcloned from pJJ192 (Jones *et al*., 1988). Each of these plasmids carried DNA isolated from yeast strains closely related to AB972. The 4.5 kb of DNA from the right end of the chromosome was isolated from bacteriophage λ 2H4 (Yoshikawa and Isono, 1990), the clone used in the chromosome III sequencing project (Oliver *et al*., 1992). Finally, two fragments, CN20 and CN21, covering regions of chromosome III in which the overlaps between clones were ≤100 bp were amplified by polymerase chain reaction (PCR) with the use of strain AB972 genomic DNA as template and cloned in pRS306. Primer sequences are available on request.

Plasmid Stability Measurements

Mitotic stabilities of plasmids were measured as the fraction of plasmid-bearing cells in a culture growing under selection for the plasmid as described previously (Palzkill and Newlon, 1988). The values reported represent the ratio of the number of colonies on selective (−Ura) plates divided by the number of colonies on nonselective plates (YEPD). At least three independent transformants were analyzed. In the case of plasmids carrying fragments generated by PCR (CN20 and CN21), at least two independent clones were tested for plasmid stability.

Analysis of Replication Intermediates

Preparation of genomic DNA and two-dimensional (2D) gel electrophoresis were performed as described previously (Theis and Newlon, 1997). For some experiments, benzoylated naphthoylated DEAE cellulose chromatography was used to enrich replication intermediates after restriction digestion of genomic DNA (Liang *et al*., 1995). DNA was transferred to nylon membranes and probed with radioactive probes prepared with the use of a Multiprime kit (Amersham Pharmacia Biotech, Piscataway, NJ). Images were obtained with the use of a Molecular Dynamics (Sunnyvale, CA) PhosphorImager and analyzed with the use of ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Fork direction gels were quantitated as described by Friedman *et al.* (1997). The averages reported are based on the analysis of at two independent DNA preparations, and the analysis of at least three independent gels.

RESULTS

Subcloning the Right Arm of Chromosome III

We previously described the cloning and identification of ARS elements in the 200-kb region of chromosome III extending from the left telomere to the *MAT* locus, which is located near the middle of the right arm (Newlon *et al*.,

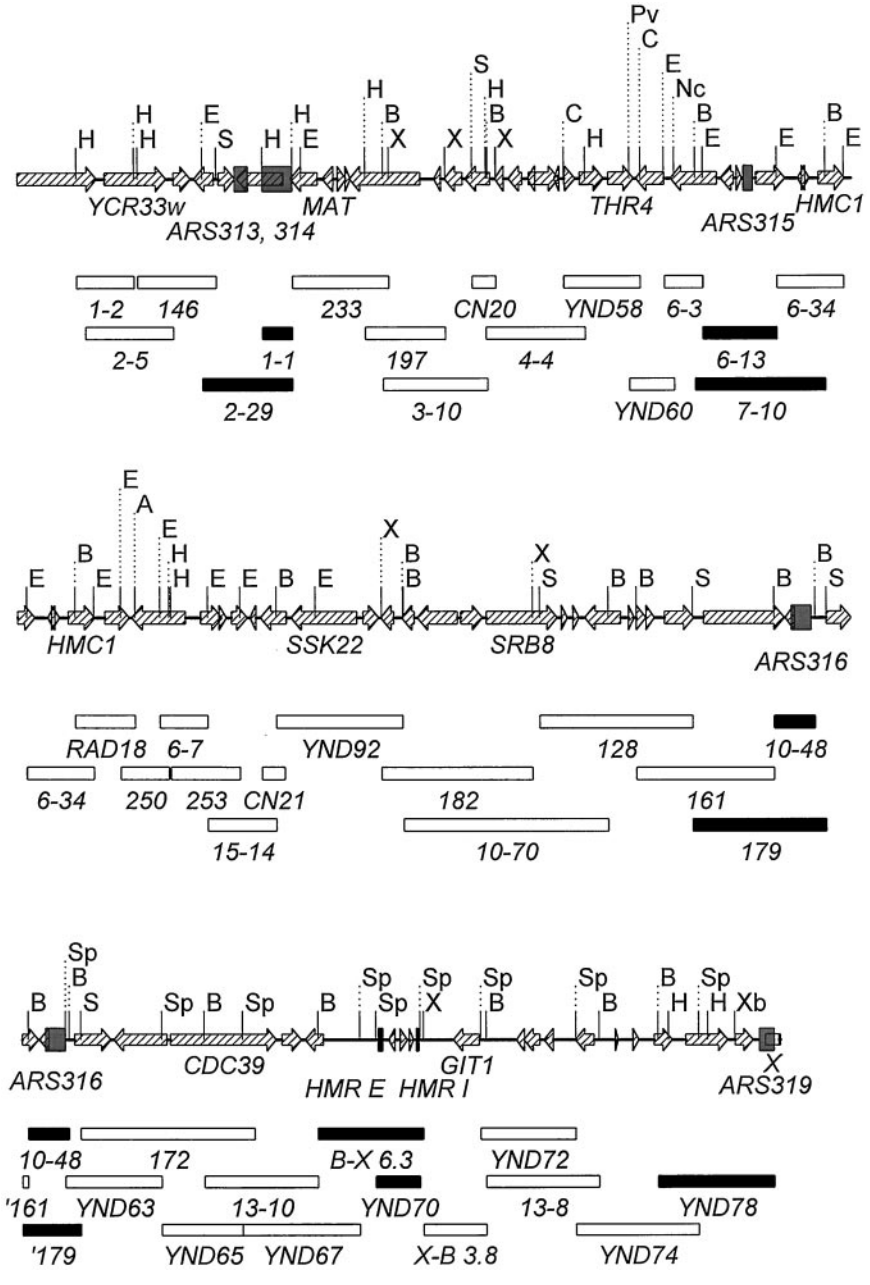

Figure 1. Map of the rightmost 136 kb of chromosome III. Three overlapping segments of this region are shown with open reading frames indicated by arrows on the map lines and restriction sites used for subcloning indicated above the map lines. Open reading frames are based on the September 2000 release of the chromosome III sequence, and names indicated are from the Saccharomyces Genome Database (February 2001). Only restriction sites that mark the boundaries of subclones are shown; additional sites exist for each of these enzymes. The top segment extends from nucleotide 181,264-231,267, the middle segment from 226,264-276,191, and the bottom segment from 271,271 to the right end of the chromosome at nucleotide 316,613. The positions of ARS elements, identified by the subclones shown in this figure and further refined by subcloning shown in Figure 2, are indicated by shaded boxes. The open box at the right end of the lower segment indicates the position of the subtelomeric X, A, B, C, and D sequences (Louis *et al.*, 1994). These subtelomeric sequences are followed by 93 bp of the $G_{1-3}T$ telomeric repeat. Boxes below the map represent subclones prepared in the vector pRS306 and analyzed for ARS activity, with open boxes representing Ars^- subclones and filled boxes representing Ars^+ subclones. With the exception of subclones YND58, YND60, RAD18, YND78, and YND79, described in Table 1, all subclones were derived from chromosome III inserts in bacteriophage λ or cosmid vectors prepared from strain AB972 by Riles and Olson (1993) and used in the chromosome III sequencing project (Oliver *et al.*, 1992). Restriction enzyme abbreviations: A, *Aat*II; B, *Bam*HI; Bn, *Bst*NI; Bs, *Bst*1107; Bw, *Bsi*WI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; Pv, *Pvu*II; Nc, *Nco*I; Rv, *Eco*RV; S, *Sal*I; Sc, *Sac*I; Sh, *Sph*I; Sp, *Spe*I; Ss, *Ssp*I; X, *Xho*I; and Xb, *Xba*I.

1991). To complete the identification of ARS elements on chromosome III required isolating subclones of the ~115-kb region distal to *MAT*. Our goal was to analyze subclones that overlapped by at least the length of a typical ARS element (~100 bp) to be sure that the description of ARS elements was complete. In addition, because the ARS elements centromere-proximal to *MAT* were only coarsely defined (Newlon *et al.*, 1991), we undertook further subcloning of the 20 kb of DNA in which these ARS elements are located.

The set of overlapping subclones chosen for further analysis covers the region of chromosome III between bp 184,788 of the nucleotide sequence and the middle of the right end subtelomeric X element at 316,232 (Figure 1). The subcloned fragments and the sources of the chromosome III DNA that they contain are detailed in Table 1 and Figure 1. In general, the overlap between subclones was a kilobase or more (Figure 1; and redundent subclones not shown), with no overlaps of <350 bp. Because our previous analysis of chromosome III ARS elements had not included a plasmid that overlapped the junction between the clones C2G and D8B (Newlon *et al.*, 1991), we also used DNA from these two clones to construct a plasmid, pCN22, carrying a 4.9-kb fragment spanning this junction that extends from the *Hin*dIII site at position 103,447 to the *Xba*I site at position 108,347 of the chromosome III sequence (Table 1).

Table 1. Chromosome III subclones

Subclone	Source of DNA	Hft	Mitotic Stability (%)[a]
1-2	λPM7121	−	100
2-5	″	−	100
146	″	−	100
2-29	″	+	9
1-1	″	+	32
233	λPM5240	−	100
197	″	−	100
3-10	″	−	100
CN20	PCR of AB972 DNA	−	100
4-4	λPM5240	−	100
YND58	pSG315	−	100
YND60	pYthr4	−	100
6-3	λPM7181	−	100
7-10	″	+	79
6-13	″	+	34
6-34	″	−	100
RAD18	pJJ192	−	100
250	λPM7181	−	100
6-7	″	−	100
253	″	−	100
15-14	λPM6589	−	100
CN21	PCR of AB972 DNA	−	100
YND92	cPM9176	−	100
182	″	−	100
10-70	″	−	100
128	″	−	100
161	″	−	100
179	″	+	5
10-48	″	+	4
YND63	cPM9189	−	100
172	cPM9176	−	100
YND65	cPM9189	−	100
13-10	″	−	100
YND67	″	−	100
B-X 6.3	″	+	78
YND70	″	+	93
X-B 3.8	″	−	100
YND72	″	−	100
13-8	″	−	100
YND74	″	−	100
YND78	λ2H4	+	61
CN22	C2G, D8B[b]	−	100

[a] Mitotic stabilities were measured as described in MATERIALS AND METHODS.

[b] Clones described by Newlon *et al.* (1991).

Identification and Localization of ARS Elements

S. cerevisiae ARS elements are defined by their ability to promote the high-frequency transformation (Hft) and extrachromosomal maintenance of plasmids. The subclones shown in Figure 1 were tested for their ability to transform *S. cerevisiae* strain YP45 at high frequency compared with the plasmid vector pRS306, which lacks an ARS element. Plasmid pRS316, a derivative of pRS306 that carries *CEN6* and *ARSH4* (Sikorski and Hieter, 1989) was used as a positive control. As expected, the subclones fell into two classes, those that yielded 1–20 Ura^+ transformants per microgram of DNA (Hft^-), and those that yielded 100 to several thousand transformants per microgram of DNA (Hft^+) (Table 1). ARS plasmids segregate poorly during cell growth, with both copies of the plasmid often retained in the mother cell (Murray and Szostak, 1983). As a result, even in cultures maintained under selection, a significant fraction of cells lack plasmids. In contrast, plasmids that have integrated into a chromosome are stable. Therefore, the mitotic stability, the fraction of plasmid-bearing cells in colonies grown under selection for the plasmid, of each of the subclones was determined as described in MATERIALS AND METHODS. The data in Table 1 demonstrate that the Hft^+ subclones all exhibited mitotic stabilities of <100%, as expected of ARS-containing plasmids, whereas the Hft^- subclones were all 100% stable, as expected of integrated plasmids. This initial screening of the subclones revealed the presence of five ARS-containing regions (Figure 1, subclones 2-29, 6-13, 10-48, YND70, and YND78).

Based on our previous analysis of the region to the left of the *MAT* locus (Newlon *et al.*, 1991), we expected that subclones 146, 2-5, and possibly 1-2 would have ARS activity (Figure 1). Because our previous subcloning used plasmids from a different yeast strain, it was of interest to determine whether the failure to find ARS activity in these subclones was the result of strain differences. Therefore, we retested our original subclones of plasmid E5F (Newlon *et al.*, 1991) and also constructed additional subclones of this plasmid. The results indicated that neither the 5.2-kb *Eco*RI fragment previously reported to contain *ARS311* (R5.2) nor the 1.5-kb *Eco*RI fragment previously reported to contain *ARS312* (R1.5) had ARS activity (Figure 2A). We do not understand the basis for this discrepancy with our previous results (Newlon *et al.*, 1991). Nevertheless, it seems clear from these results that *ARS311* and *ARS312* do not exist.

To further localize the ARS elements identified in the subcloning analysis, and to determine whether more than one ARS element was present in any of the ARS-containing regions, we constructed and analyzed additional subclones. The *Eco*RI fragment previously identified as *ARS313* (Newlon *et al.*, 1991) was Ars^+ in the current analysis (subclones 2–29 and R 5.9; Figures 1 and 2A). Our finding that both subclones 205 and 188 had ARS activity places *ARS313* within a 785-bp *Sph*I-*Ssp*I fragment that includes the 3′ end of the *PHO87* coding region and the intergenic region between *PHO87* and *RBK1*. These results confirm and extend the observations of Thierry *et al.* (1990), who reported an ARS element within the *Ssp*I fragment in subclone 205 (Figure 2A). The *Eco*RI fragment that carries *ARS313* also contains a second ARS element, *ARS314*, located within the 1804-bp *Hin*dIII fragment that contains the 5′ end of *PHO87* and intergenic region between *PHO87* and *BUD5* (subclones 1-1 and H 1.8; Figures 1 and 2A).

The essential region of *ARS315* was localized to the 558-bp *Bst*11071-*Sac*I fragment containing the intergenic region between YCR60w and YCR61w (Figure 2B). However, the observation that transformants carrying the 6-13-2 subclone grew more slowly than transformants carrying the 6-13-12 subclone suggests that there are sequences that stimulate the activity of *ARS315* to the left of the *Xba*I site shown in Figure 2B. The subcloning analysis shown in Figure 2C limits the position of the essential sequences of *ARS316* to the 1185-bp *Kpn*I-*Spe*I fragment containing the 5′ end of the YCR90c open reading frame and most of the intergenic region between YCR90c and YCR91w.

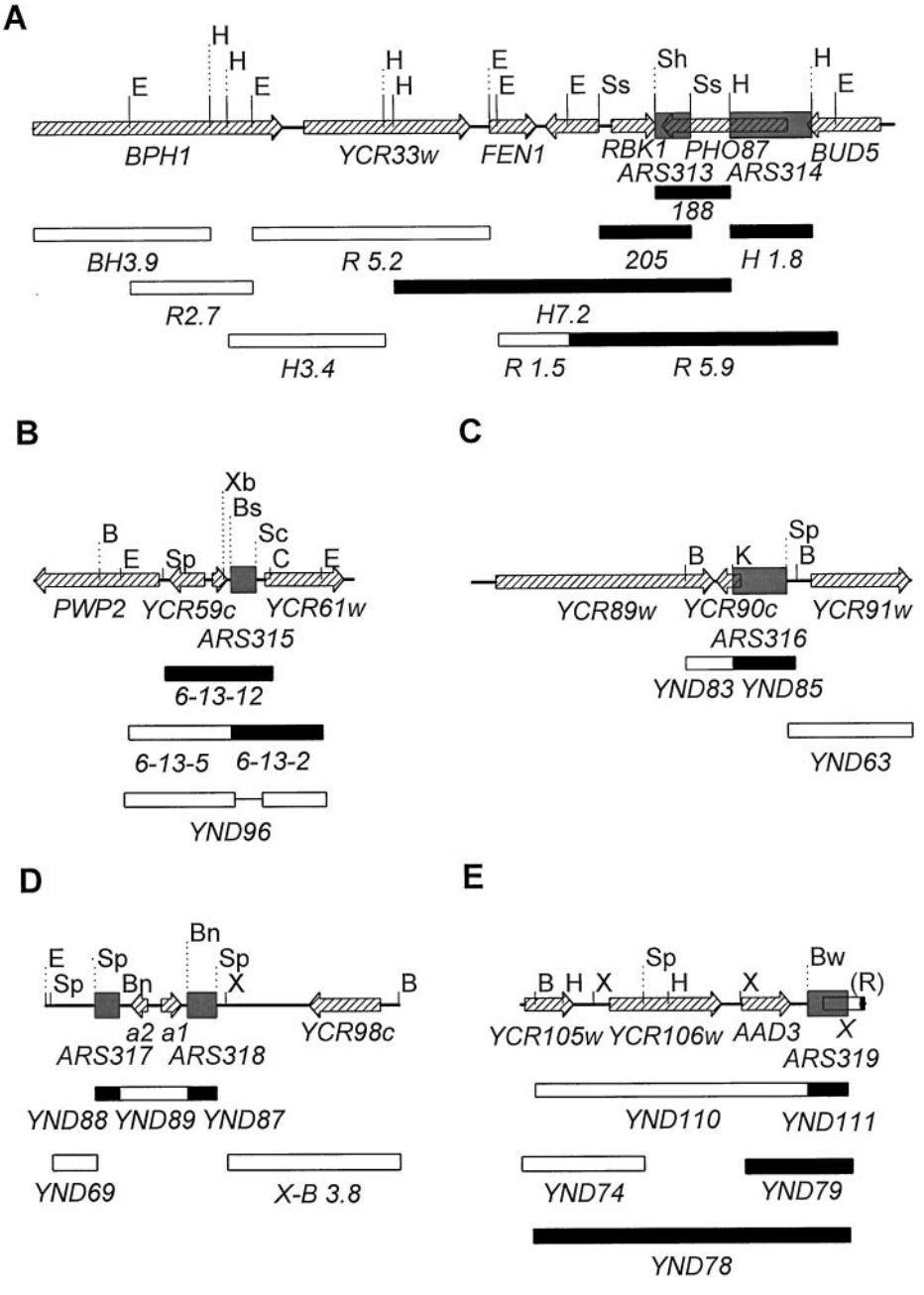

Figure 2. Subcloning of ARS-containing regions. Features and restriction enzyme abbreviations are as indicated in the legend to Figure 1. (A) The 19-kb region extending from 180515–199513. Only a portion of the *BPH1* open reading frame is shown. All subclones shown were derived from plasmid E5F (Newlon *et al.*, 1991) except 188 and 205, which are subclones of plasmid 2-29 (Figure 1). (B) The 7-kb region extending from 220448 to 227511. Plasmids are subclones of 6-13 (Figure 1). YND96 contains a deletion of the *Bst*11071-*Sac*I fragment shown. (C) The 9.7-kb region extending from 266905 to 276590. Only a portion of the YND63 subclone is shown. (D) The 7.7-kb *Eco*RI-*Bam*HI fragment extending from 291306 to 299002 and containing *HMR*. a2 and a1 refer to the *MATa2* and *MATa1* transcripts encoded at *HMR*. (E) The 7.6-kb fragment containing the right end of chromosome III. Only a portion of the YND74 subclone is shown. The *Eco*RI site shown in parentheses is within a 14-bp segment of the m13 polylinker sequence that flanks the yeast DNA insert in the bacteriophage λ 2H4 clone from which the yeast fragment in plasmid YND78 was isolated (Louis, 1994).

Based on the previous analysis of the ARS elements associated with the transcriptional silencers that flank the mating type information at *HMR*, *HMR*-E and *HMR*-I (Abraham *et al.*, 1984), both of these ARS elements were expected to be within the YND70 subclone (Figure 1). Further subcloning confirmed the presence of at least two ARS elements in this fragment (Figure 2D). The *HMR*-E *ARS* (*ARS317* according to the standard scheme for naming ARS elements; Campbell and Newlon, 1991) is within the 533-bp *Spe*I-*Bst*NI fragment immediately to the left of the *HMRa2* open reading frame. The *HMR*-I *ARS* (*ARS318*) is within the 632-bp *Bst*NI-*Spe*I fragment immediately to the right of the *HMRa1* open reading frame.

ARS319 was initially identified as a 6.9-kb *Bam*HI-*Eco*RI fragment containing most of the right end of chromosome III. The segment of the X element included in this clone is expected to contain an ARS element (Chan and Tye, 1983). Our subcloning analysis (Figure 2E) demonstrated that *ARS319* is within the 886-bp *Bsi*WI-*Eco*RI fragment that contains the subtelomeric X element, and that there are no additional ARS elements in the centromere-proximal region of YND78.

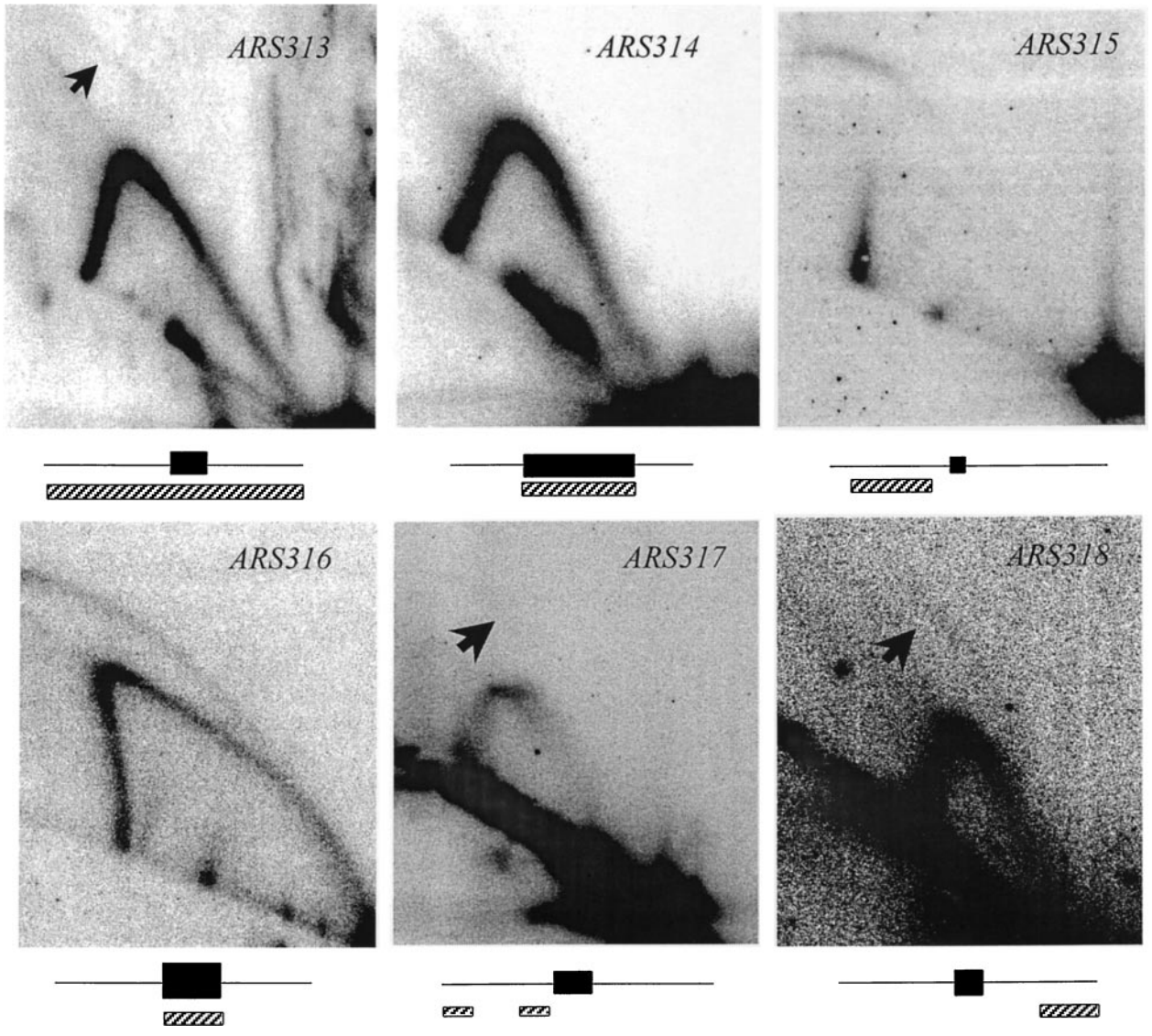

Figure 3. 2D gel analysis of chromosomal replication intermediates. *ARS313*: 3.6-kb *Eco*RI-*Hin*dIII fragment, probed with entire fragment; *ARS314*: 3.5-kb *Cla*I-*Eco*RV fragment, probed with 1.8-kb *Hin*dIII fragment. *ARS315*: 4.4-kb *Eco*RI fragment, probed with 1.3-kb *Sph*I-*Bgl*II fragment; *ARS316*: 5.9-kb *Nhe*I-*Pst*I fragment, probed with 2.5-kb *Bam*HI fragment; *ARS317* (*HMR*-E): 3.4-kb *Cla*I-*Bgl*II fragment, probed with 0.4-kb *Cla*I-*Eco*RI and 0.35-kb *Dra*I-*Xba*I fragments; and *ARS318* (*HMR*-I): 3.3-kb *Xba*I-*Eco*RV fragment, probed with 0.8-kb *Bgl*II fragment. Arrows point to faint bubble arcs. Black bars below the panels show the genomic fragments analyzed, and the boxes on the bars indicate the smallest subclone known to contain the ARS element. Hatched boxes indicate the probes used. DNA for the *ARS317* and *ARS318* patterns shown was from strain YPH45. DNA for the remainder of the patterns shown was from strain YPH47.

Analysis of Chromosomal Replication Origin Activity

Although the plasmid assay for ARS activity accurately reflects the ability of DNA sequences to function as plasmid replicators, not every ARS element identified in the plasmid assay is active as a chromosomal replication origin. For example, the leftmost 30 kb of chromosome III contain five ARS elements that are not detectably active as chromosomal replication origins under normal culture conditions (Dubey *et al.*, 1991; Newlon *et al.*, 1993). We therefore used 2D gel analysis to examine the replication intermediates (RIs) of chromosomal DNA fragments carrying the ARS elements identified in this study (Figure 3). Replication origin activity is revealed by the presence of an arc of replication bubble-containing RIs, and passive replication of the fragment by a fork from an origin external to the fragment by an arc of Y-shaped RIs.

The replication of the chromosomal copies of *ARS313* and *ARS314* was predominantly passive, as indicated by the arcs of Y-shaped RIs of uniform intensity. In the *ARS313* pattern shown in Figure 3A, a weak bubble arc is visible, suggesting that replication initiates at *ARS313* in a small fraction of cell

cycles. In four other DNA preparations examined, only Y-shaped RIs of the *ARS313* fragment were seen. Based on the relative intensities of the bubble and Y arcs, and our failure to detect bubble arcs in other DNA preparations, we estimate that *ARS313* is active in ≤10% of cells in the population. Bubble-shaped RIs were never detected in the analysis of *ARS314* (Figure 3B), indicating that it is less active in the chromosome than *ARS313*.

In contrast to *ARS313* and *ARS314*, *ARS315* was highly active as a chromosomal replicator. *ARS315* is located between 33 and 45% of the distance from one end of the *Eco*RI fragment examined. The discontinuous pattern of RIs, an intense bubble arc in combination with a Y arc that is light for most of its length and intense only in the region of the largest RIs, is consistent with replication initiating slightly off-center in the fragment at or near the position of *ARS315*. The absence of small Y-shaped RIs suggests that replication initiates at *ARS315* in most cells in the population.

ARS316 was also active as a chromosomal replicator. The ARS element is located between 35 and 55% of the distance from one end of the fragment examined (Figure 3D). The presence of a complete bubble arc in these patterns is consistent with replication initiating in the center of the fragment at or near the position of *ARS316*. However, the presence of a Y arc of relatively uniform intensity suggested that *ARS316* was not an efficient replicator. To obtain a more quantitative estimate of the efficiency with which *ARS316* initiated replication, we used a modification of the 2D gel procedure (Friedman and Brewer, 1995) to examine the direction of replication fork movement in regions flanking the ARS. In this procedure, replication intermediates that have been separated by size in the first dimension gel are digested in the gel slice with a restriction enzyme that cuts in the fragment of interest before the second dimension gel is run. The blot is then probed with a probe that recognizes only one of the two fragments released by the in-gel digestion. Depending on the direction of replication fork movement through the region, the Y-shaped RIs released by the in-gel digestion fall along either a Y arc that emanates from the spot of nonreplicating linear molecules, or a Y arc that is shifted to the left. The region to the left of *ARS316* predominately was replicated by a fork moving to the right toward *ARS316* (Figure 4A), whereas the region to the right appeared to be replicated exclusively by forks moving to the right, away from *ARS316*. Therefore, the frequency of replication initiation at *ARS316* is reflected by the fraction of RIs in the light arc of forks moving leftward from *ARS316* in the left flanking region, a value found to be 0.26 ± 0.04 in this and other experiments.

RIs of DNA fragments containing the *HMR* silencer ARS elements were difficult to visualize (Figure 3). In some DNA preparations, we were able to see very weak bubble arcs along with uniformly dense Y arcs in patterns obtained with *HMR*-E (*ARS317*) and *HMR*-I (*ARS318*). Therefore, neither of these ARS elements appeared to initiate replication in more than a small fraction of cell cycles. The results of fork direction analysis in regions flanking *HMR* were consistent with this conclusion (Figure 4, C and D). The 17.3-kb region that was examined was replicated predominately by rightward-moving forks. The difference between the fraction of rightward-moving forks in the region to the left of *HMR* (0.85 ± 0.036) and the fraction of rightward-moving forks in the region to the right of *HMR* (0.96 ± 0.05) suggests that *ARS317* (*HMR*-E) and *ARS318* (*HMR*-I) together are active in only ~11% of cell cycles. *HMR*-E has been studied by others, both in its native context in strains carrying deletions of

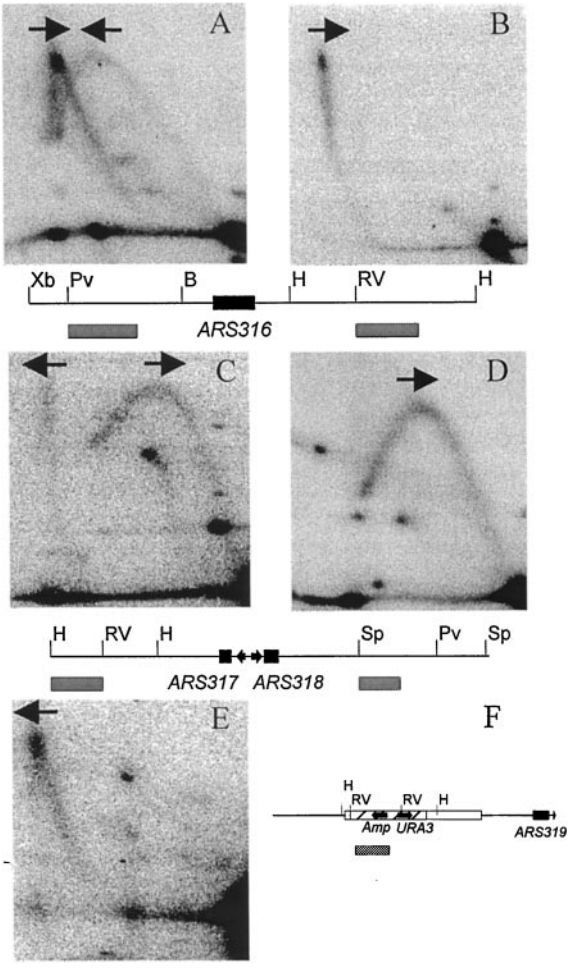

Figure 4. Direction of fork movement in chromosomal regions flanking *ARS316*, *ARS317*, *ARS318*, and *ARS319*. DNA was prepared from strain YPH45-7. Arrows above the arcs indicate the direction of fork movement. Maps below the top pairs of panels show the regions analyzed and gray boxes beneath the maps show the probes used. (A) Fragment left of *ARS316*: 5-kb *Xba*I-*Bam*HI fragment cut in gel with *Pvu*II and probed with a 2.3-kb *Pvu*II-*Hin*dIII fragment. (B) Right of *ARS316*, a 6.3-kb *Hin*dIII fragment cut in gel with *Eco*RV and probed with a 2.1-kb *Eco*RV-*Spe*I fragment. (C) Left of *HMR*, a 4.8-kb *Hin*dIII fragment cut in gel with *Eco*RV and probed with a 2.3-kb *Hin*dIII-*Eco*RV fragment. (D) Right of *HMR*, a 5.7-kb *Spe*I fragment cut in gel with *Pvu*II and probed with a 1.8-kb *Spe*I-*Sph*I fragment. Arrows on the map show the a1 and a2 transcripts. (E) Left of *ARS319*, a 5.4-kb *Hin*dIII fragment cut in gel with *Eco*RV and probed with a 1.94-kb *Aat*II-*Pvu*II fragment from pRS306 as shown in F. (F) Schematic diagram of the right end of chromosome III in strainYP45-7. The plasmid YND95 (open box), a derivative of pRS306 which includes sequences from YCR105W and YCR106W, was integrated 3.8 kb from the beginning of the right telomere sequences. The hatched box represents pRS306 vector sequences, and *ARS319* is shown as a black box. The probe used is indicated by the box below the map.

HMR-I (Hurst and Rivier, 1999; Palacios DeBeer and Fox, 1999) and in the context of a synthetic silencer (Rivier and Rine, 1992; Fox *et al.*, 1995). The 2D patterns obtained in all of these studies contain uniformly dense Y arcs and much weaker bubble arcs, suggesting that *HMR*-E is an inefficient origin. The single quantitation of activity by fork direction analysis indicated that *HMR*-E is used in 20–30% of cell cycles (Palacios DeBeer and Fox, 1999). Whether the higher efficiency of use reported in this study is related to the *HMR*-I deletion or to other differences in strain backgrounds or experimental details is not clear. *HMR*-I also has been reported to function as an inefficient chromosomal replication origin (Rivier *et al.*, 1999).

It was impossible to examine *ARS319* directly because it was not possible to find probes specific for DNA fragments carrying this ARS. *ARS319* is within a subtelomeric X element present on all natural yeast chromosomes. Moreover, the chromosome III sequences internal to the X element are duplicated on other chromosomes (Dershowitz and Newlon, unpublished data). We therefore integrated a plasmid at the *Bgl*II site in YCR106w (Figure 2E), and examined the direction of replication fork movement through plasmid sequences (Figure 4, E and F). The pattern in Figure 4E, which shows only the displaced arc of RIs, demonstrates that the integrated plasmid is replicated by forks that are moving to the left from *ARS319*. These results indicate that *ARS319* is active as a chromosomal replicator in most cells in the population.

DISCUSSION

The data presented here complete the identification of ARS elements and replication origins on yeast chromosome III, which is the first chromosome to be analyzed from end to end. Our current view of chromosome III is summarized in Figure 5. We have identified eighteen ARS elements and localized them to DNA fragments ranging from ~500 bp to 3 kb in length. One additional ARS element, *ARS320*, was identified by Vujcic *et al.* (1999). The distances between ARS elements range from ~2 kb (*ARS302*, *ARS303*, and *ARS320*; *ARS317* and *ARS318*) to 48 kb (*ARS315* and *ARS316*), with an average of 17.5 kb. A comparable analysis of a 230-kb region of chromosome VI, which excluded the subtelomeric X and Y′ ARS elements likely to be present on that chromosome, found 10 ARS elements with an average spacing of 23 kb (Shirahige *et al.*, 1993; Friedman *et al.*, 1997; Yamashita *et al.*, 1997). A 131-kb segment of chromosome XIV contained four ARS elements (Friedman *et al.*, 1996) with a spacing of 36 kb, and a lower resolution analysis of the 580-kb chromosome V revealed at least 16 ARS-containing regions, an average spacing of 36 kb. Despite the twofold difference in average spacing between ARS elements between chromosome III and the chromosome XIV segment, the average distance between active replication origins is similar on the two chromosomes, and is consistent with the 36-kb average spacing between active replication origins deduced by electron microscopic analysis of distances between adjacent replication bubbles (Newlon and Burke, 1980).

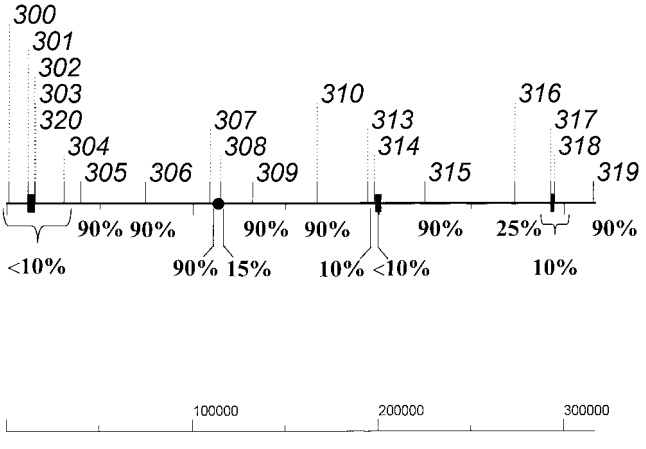

Figure 5. Summary of chromosome III replicator activity. ARS elements are numbered above the line. Rectangular boxes on the line show the positions of *HML*, *MAT*, and *HMR*. The filled circle shows the position of *CEN3*. The efficiencies of ARS elements as chromosomal replicators are indicated below the map line as the percentage of cell cycles in which they initiate replication. Values indicated as <10% identify ARS elements for which arcs of bubble-shaped replication intermediates have never been detected.

It is possible that further analysis will reveal additional ARS elements on chromosome III. Mutational analysis of the essential ACS in six of the ARS elements, *ARS304* (Theis *et al.*, 1999), *ARS305* (Huang and Kowalski, 1993), *ARS306* (Theis *et al.*, 1999), *ARS307* (Palzkill and Newlon, 1988; Van Houten and Newlon, 1990), *ARS309* (Theis and Newlon, 1997), and *ARS310* (Theis and Newlon, 2001) indicated that only a single ARS element is present in each of these subclones, although *ARS310* is unusual in the sense that its complete inactivation requires mutation of three matches to the ACS. However, it has been reported that three separable ARS elements are present in a small fragment carrying *HMR*-E (Hurst and Rivier, 1999; Palacios DeBeer and Fox, 1999), and the fragment containing *ARS303* also carries a second ARS element, *ARS320* (Vujcic *et al.*, 1999). A precedent for such closely spaced ARS elements is provided by *ARS601* and *ARS602*, which are only 241 bp apart on chromosome VI (Shirahige *et al.*, 1993). After the convention of Hurst and Rivier (1999), we have suggested that these very closely spaced ARS elements be called compound replication origins (Theis and Newlon, 2001).

The pattern of chromosomal replicator activity on chromosome III is striking, with two clusters of ARS elements that are not detectably active, i.e., used in <10% of cell division cycles. One group of five (*ARS300-ARS304*) is in the left-most 30 kb of the chromosome (Dubey *et al.*, 1991; Newlon *et al.*, 1993) and two others are just to the left of the *MAT* locus (*ARS313* and *ARS314*) (Newlon *et al.*, 1993; this study). The remaining ARS elements show a wide range of activities, with *ARS308*, *ARS317*, and *ARS318* only weakly active, *ARS316* active at intermediate levels, and *ARS305*, *ARS306*, *ARS307*, *ARS309*, *ARS310*, *ARS315*, and *ARS319* active in most cells (Huberman *et al.*, 1988; Deshpande and Newlon, 1992; Greenfeder and Newlon, 1992; Zhu *et al.*, 1992; Huang and Kowalski, 1993; Theis and Newlon, 1997; Theis and Newlon, 2001; this study).

The question of how the chromosomal activity of these ARS elements is regulated remains unresolved. As the sex chromosome of yeast, chromosome III might be special.

Indeed, the formation of a repressive chromatin structure that prevents expression of the silent mating type loci, *HML* and *HMR*, which serve as donors of mating type information in mating type switching events (reviewed by Haber, 1998b), may be unique to chromosome III. Moreover, the mating type-dependent regulation of donor preference in mating type switching, which also influences rates of mitotic recombination on chromosome III, is another feature not known to be shared by other chromosomes (reviewed by Haber, 1998a). The inactive and weakly active replicators are located near *MAT*, *HML*, and *HMR*, increasing the possibility that the regulation of mating type gene expression or mating type switching creates chromatin structures that are inconsistent with replication initiation, or that the ARS elements in these regions function primarily in these other processes. Binding of ORC to the *HMR*-E silencer (*ARS317*) plays a key role in the formation of the heterochromatin that represses gene expression at the silent mating type locus *HMR* (Triolo and Sternglanz, 1996). ORC recruits Sir1p to the silencer, and Sir1p recruits the additional proteins that mediate silencing. *Drosophila* ORC is also associated with heterochromatin (Pak *et al.*, 1997). However, the observation that the ARS elements associated with the *HML* silencers, *ARS301* and *ARS302*, are inactive in strains carrying null mutations in *sir* genes, and therefore expressing mating type genes at *HML*, indicates that repressive chromatin formation at *HML* is not a sufficient explanation for their inactivity as chromosomal replicators (Dubey *et al.*, 1991). Moreover, chromosome VI, which is not known to have heterochromatic regions except near its telomeres, like chromosome III contains ARS elements that are inactive (or at least very inefficient) as chromosomal replicators (Friedman *et al.*, 1997; Yamashita *et al.*, 1997). Thus, it is unlikely that the formation of heterochromatin inhibits the chromosomal replicator activity of the ARS elements associated with the silent mating type loci.

It is also unlikely that the regulation of donor preference in mating type-switching events is involved in repressing chromosomal replicator activity of the *HML*- and *HMR*-associated ARS elements because these ARS elements are inactive or weakly active in haploid strains of both mating types and in *MAT***a**/*MAT*α strains disomic for chromosome III, which behave as diploids (Dubey *et al.*, 1991; this study).

Potentially a late-firing replicator could be replicated by a fork from an adjacent early firing replicator before it initiates replication itself, thus decreasing its efficiency of use. Although other explanations have not been eliminated, asynchrony in initiation times of closely spaced replicators and the consequent passive replication of the later firing origin may account for the phenomenon of origin interference or replicator dominance seen in plasmids and chromosomal constructs containing two or more replicators spaced at intervals of 6 kb or less (Brewer and Fangman, 1993; Brewer and Fangman, 1994; Marahrens and Stillman, 1994). Moreover, inactive replicators near the left end of chromosome III are at least partially activated by the deletion of nearby early firing replicators, consistent with the idea that at least one of the mechanisms that contribute to the inefficient use of these replicators is passive replication by forks initiated at early firing replicators (Vujcic *et al.*, 1999; Dershowitz and Newlon, unpublished data). However, it is unlikely that replication timing is the only cause of replicator inefficiency. A systematic study of the efficiency and timing of replicator use on chromosome VI revealed that a replicator used only in 50% of cell cycles initiated earlier than either of the replicators that flank it (Friedman *et al.*, 1997).

Two situations in which the ARS elements that flank *HML* are activated as chromosomal replicators have been reported. Treatment of strains defective in the S phase checkpoint (*rad53* or *mec1*) with hydroxyurea, which slows or blocks replication fork movement by inhibiting ribonucleotide reductase, or the DNA damaging agent MMS was found to cause activation of these replicators at times later than the normal end of S phase (Shirahige *et al.*, 1998; Santocanale *et al.*, 1999). These replicators were also activated in strains in which *ARS305* and *ARS306*, or these two replicators plus additional ones, had been deleted (Vujcic *et al.*, 1999; Dershowitz and Newlon, unpublished data). These observations, together with the finding that an *ARS301* plasmid replicates late in S phase (Bousset and Diffley, 1998), provide indirect support for the idea that these replicators are programed to initiate so late in S phase that they are normally passively replicated before they have a chance to fire.

A surprising finding was that the ARS elements within the two subtelomeric X elements differ with respect to chromosomal replicator activity. These two X elements share 90% identity in sequence, but *ARS319* (the right X element ARS) is active in most cell cycles, whereas *ARS300* (the left X element ARS) is not detectably active as a chromosomal replicator (Dubey *et al.*, 1991; Dershowitz and Newlon, unpublished data). Although these ARS elements are located within 23 kb of the silent mating type loci, their pattern of activity was the same in both *MAT***a** and *MAT*α strains, indicating that the system that regulates donor preference in mating type switching is unlikely to play a role in regulating their activities as replicators.

Several observations are consistent with the idea that the differential activity of these subtelomeric replicators reflects the interaction between timing determinants and the topology of replicator use on chromosome III. The leftmost 40 kb of the chromosome is replicated by a fork that initiates at *ARS305* very early in S phase (Reynolds *et al.*, 1989; Dubey *et al.*, 1991; Newlon *et al.*, 1993). *HMR* is replicated predominantly by a fork moving rightward toward the telomere (Figure 4). Although we have not studied replication timing of the distal half of the right arm in detail, previous work demonstrated that *HML* and *HMR* replicate at about the same time (Reynolds *et al.*, 1989). Because *HML* is ~12 kb from the left telomere and *HMR* is ~23 kb from the right telomere, it would take a fork moving at an average rate (3 kb/min) at least 4 min longer to reach the subtelomeric region distal to *HMR* than the region distal to *HML*. Therefore, even if *ARS300* and *ARS319* were programmed to initiate at the same time by the Sir-dependent repressive chromatin that assembles at telomeres and appears to delay replication initiation (Stevenson and Gottschling, 1999), *ARS319* is expected to have more time to fire before it is passively replicated than *ARS300*. Experiments to directly test this timing model are in progress.

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