Conservation of ARS Elements and Chromosomal DNA Replication Origins on Chromosomes III of *Saccharomyces cerevisiae* and *S. carlsbergensis*

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ABSTRACT

DNA replication origins, specified by ARS elements in *Saccharomyces cerevisiae*, play an essential role in the stable transmission of chromosomes. Little is known about the evolution of ARS elements. We have isolated and characterized ARS elements from a chromosome III recovered from an alloploid Carlsberg brewing yeast that has diverged from its *S. cerevisiae* homeologue. The positions of seven ARS elements identified in this *S. carlsbergensis* chromosome are conserved: they are located in intergenic regions flanked by open reading frames homologous to those that flank seven ARS elements of the *S. cerevisiae* chromosome. The *S. carlsbergensis* ARS elements were active both in *S. cerevisiae* and *S. monacensis*, which has been proposed to be the source of the diverged genome present in brewing yeast. Moreover, their function as chromosomal replication origins correlated strongly with the activity of *S. cerevisiae* ARS elements, demonstrating the conservation of ARS activity and replication origin function in these two species.

LITTLE is known about the evolution of noncoding elements of chromosomes. One class of these noncoding elements, the origins of DNA replication, is well characterized in the budding yeast, *Saccharomyces cerevisiae*. They are dependent upon ARS elements, sequences of 100–200 bp that were identified by their ability to promote the extrachromosomal maintenance of plasmids (reviewed by Newlon 1996). As is the case for centromeres, fission yeast ARS elements are larger than those of *S. cerevisiae*, and replication appears to initiate in broad zones in metazoans (reviewed by DePamphilis 1996).

ARS elements have been identified systematically on chromosome III (Newlon *et al.* 1993; A. Poloumienko, A. Dershowitz, J. Shah and C. S. Newlon, unpublished results), chromosome V (Tanaka *et al.* 1996), chromosome VI (Shirahige *et al.* 1993), and in a 140-kb region of chromosome XIV (Friedman *et al.* 1995). Many, but not all, of the ARS elements function detectably as chromosomal replicators (Deshpande and Newlon 1992; Greenfeder and Newlon 1992b; Rivier and Rine 1992; Zhu *et al.* 1992; Newlon *et al.* 1993; Friedman *et al.* 1995, 1997; Theis and Newlon 1997; Yamashita *et al.* 1997).

Several observations make the question of whether the positions of replication origins are conserved an interesting one. Brewer (1988) has noted that bacterial and viral genomes are organized so that replication forks traverse heavily transcribed genes in the same direction as RNA polymerase, suggesting that there may be constraints on the location of replication origins in chromosomes. However, the analysis of origin deletions on chromosome III has shown that the direction of fork movement has little or no effect on chromosome stability (Dershowitz and Newlon 1993; Newlon *et al.* 1993; A. Dershowitz and C. S. Newlon, unpublished results). Moreover, the observation that deletion of several origins had no effect on chromosome stability suggests that more origins are present than are needed to replicate the chromosome and that some could be lost during evolution. One explanation of inactive replication origins is that they represent origins that are active in other species, but no longer function in *S. cerevisiae*. Alternatively, the inactive or inefficient ARS elements might represent fortuitous binding sites for the replication initiator protein, origin recognition complex (ORC; reviewed by Newlon 1996) that allows plasmid, but not chromosomal, replication origin activity. A third explanation is that inactive ARS elements are used for some function other than replication. Two inactive origins, *ARS301* and *ARS302*, are associated with the silent mating type locus, *HML*, where they function as transcriptional silencers. ORC clearly plays a role in silencing because mutations in two different subunits of the complex result in derepression of *HML* (Foss *et al.* 1993; Loo *et al.* 1995). In addition, mutations in ORC have been found that separate its role in silencing from its role in DNA replication, indicating that the two functions are independent (Fox *et al.* 1995).

As a first approach toward addressing these questions, we chose to isolate and characterize ARS elements from a chromosome III of a brewing yeast that has diverged

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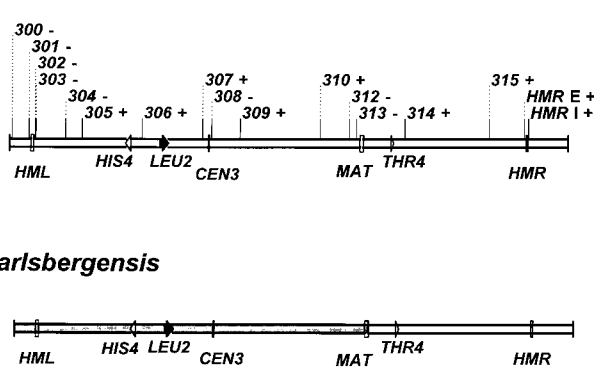


Figure 1.—Physical maps of chromosomes *III* of *S. cerevisiae* and *S. carlsbergensis*: All numbers represent ARS elements on the chromosome. A "+" following the number denotes that the ARS element functions as a chromosomal replication origin, while a "−" indicates that it does not. The region from *MAT* to the right telomere is homologous between *S. cerevisiae* and *S. carlsbergensis*, *i.e.*, the sequences are similar, and recombination occurs between the two chromosomes, while the region from the left telomere to *MAT* is homeologous, *i.e.*, the sequences have diverged and there is little recombination between the two chromosomes. Despite the sequence divergence, all the essential genes in this region are functional, as indicated by the fact that the *S. carlsbergensis* chromosome III can substitute for its *S. cerevisiae* counterpart. The homeologous region is shaded gray.

from *S. cerevisiae*. The brewing yeast is an alloploid that carries genomes from two different Saccharomyces species: one is like *S. cerevisiae*; the other, referred to as *S. carlsbergensis*, has diverged significantly at the DNA sequence level. Six chromosomes of the brewing strain have been transferred into *S. cerevisiae* (reviewed by Kielland-Brandt *et al.* 1995). Two or more copies of each chromosome were recovered. For some chromosomes, one copy was indistinguishable from *S. cerevisiae*, and the other was *S. carlsbergensis*-like, hybridizing with *S. cerevisiae* probes at low stringency and having a different restriction map. Other chromosomes were mosaic, having segments from both *S. cerevisiae* and *S. carlsbergensis*, suggesting that they were products of rare recombination events between the homeologous chromosomes in the alloploid brewing strain.

The *S. carlsbergensis* chromosome III can substitute for the *S. cerevisiae* chromosome III (Nilsson-Tillgren *et al.* 1981). The *S. carlsbergensis* chromosome III is actually a mosaic chromosome (Figure 1). The region between the left telomere and the *MAT* locus, in the middle of the right arm, presumably was derived from the *S. carlsbergensis* genome and has diverged from the corresponding region in *S. cerevisiae* sufficiently that meiotic recombination between the homeologues is reduced at least 100-fold (Nilsson-Tillgren *et al.* 1981; Priebe *et al.* 1994). Despite these DNA sequence differences, equivalent genes appear to be present in the same order on both chromosomes. In contrast, the region distal to *MAT* on the right arm is homologous to *S. cerevisiae* with normal levels of meiotic recombination (Nilsson-Tillgren *et al.* 1981).

In this article, we present data demonstrating that ARS elements are located in the brewing yeast chromosome within the same intergenic intervals that they occupy in *S. cerevisiae*. Moreover, their ability to function as replication origins is conserved both in *S. cerevisiae* and in *S. monacensis*, which has been proposed to be the source of the *S. carlsbergensis* genome in the Carlsberg brewing strain (Hansen and Kielland-Brandt 1994).

MATERIALS AND METHODS

Strains and plasmids: The *S. cerevisiae* strain YP45 (Sikorski and Hieter 1989) was utilized for transformations to examine ARS activity. The *S. carlsbergensis* chromosome III, initially recovered from Carlsberg production strain 244, was maintained in the *S. cerevisiae* chromosome III substitution strain M1253 (C80-1253; Nilsson-Tillgren *et al.* 1981). The *S. monacensis* type strain was obtained from the American Type Culture Collection (#76670, an isolate of CBS1503). The *Escherichia coli* strains JA226 (Devenish and Newlon 1982) and DH5α (Life Technologies, Grand Rapids, NY) were used for routine plasmid manipulations. The shuttle vector pRS306 (Sikorski and Hieter 1989) was used for constructing of *S. carlsbergensis* chromosome III libraries and for subcloning the ARS elements.

A plasmid pRS333 was constructed to test for ARS activity in *S. monacensis*. $CEN3^{carl}$ and a G418 resistance cassette were inserted into the *S. cerevisiae-E. coli* shuttle vector, pRS303 (Sikorski and Hieter 1989). The 0.5-kb *Xba*I-*Pst*I fragment of 19EG1, containing the centromere (T. H. Andersen and T. Nilsson-Tillgren, personal communication), was treated with Mung Bean Nuclease (New England Biolabs, Beverly, MA) and cloned into pFA-KanMX4 (Wach *et al.* 1994) that had been digested with *Bgl*II and treated with Mung Bean Nuclease to generate blunt ends. This construct was digested with *Asc*I plus *Sac*I, then treated with T4 polymerase (New England Biolabs) plus dNTPs; the 1.9-kb fragment containing the centromere and the kanamycin-resistant cassette was isolated. This fragment was ligated to pRS303, which had been digested with *Aat*II and treated with T4 DNA polymerase and dNTPs to generate blunt ends. The plasmid pRS333 was recovered in which the kanamycin-resistant cassette is transcribed in the same direction as the ampicillin-resistant gene.

The ARS-containing fragments were moved into pRS333 as follows. The plasmids p305K/D0.55, p307k/s-2′/2, and p310Bg/B0.56 were digested with *Fsp*I, and the ARS-containing fragments ligated to the 4.7-kb *Fsp*I fragment of pRS326 (Theis and Newlon 1994). The $ARS305^{carl}$- and $ARS310^{carl}$-containing plasmids were digested with *Bsa*HI plus *Ngo*MI, and the ARS-containing fragments ligated to the 4.1-kb *Bsa*HI-*Ngo*MI fragment of pRS333. The $ARS307^{carl}$-containing plasmid was digested with *Bsa*I plus *Ngo*MI, and the ARS-containing fragment was ligated to the 4.5-kb *Bsa*I-*Ngo*MI fragment of pRS333.

Construction of *S. carlsbergensis* chromosome III libraries: *S. carlsbergensis* chromosome III was separated from other chromosomes by pulsed-field gel electrophoresis. Plugs from the substitution strain M1253 were prepared (Rose *et al.* 1990), and electrophoresis performed in a transverse alternating field electrophoresis apparatus (Beckman, Fullerton, CA) using the following conditions: 1% low-melting-point agarose (FMC, Rockland, ME) in 10 mm Tris, 0.4 mm EDTA, 0.025% (v/v)

acetic acid; 30 min at 200 mA switching at 4-sec intervals followed by 20 hr at 220 mA switching at 15-sec intervals. A slice containing chromosome III was excised from the gel. The DNA was digested *in situ* with restriction enzymes prior to isolation by β-agarase (New England Biolabs) digestion. The resulting *Bam*HI or *Eco*RI fragments were cloned in polylinker of pRS306. Ampicillin-resistant colonies were picked to form the *Bam*HI and *Eco*RI libraries.

A *Bgl*II library was constructed for the isolation of the *S. carlsbergensis* ARS element corresponding to *ARS309*. Genomic DNA from M1253 was digested with *Bgl*II and separated on a 0.7% agarose gel; a slice containing fragments of 4.0–4.5 kb was excised, and the DNA eluted and then cloned in *Bam*HI-digested pRS306. The ampicillin-resistant transformants represent the *Bgl*II library.

Screening of *S. carlsbergensis* chromosome III libraries for ARS-containing clones: A total of 40–48 *Bam*HI library clones were grown in patches on an LB-Amp plate overnight; the cells were pooled, and plasmid DNA prepared. The plasmid DNA pools were then used to transform *S. cerevisiae* strain YP45. The stabilities of the plasmids were examined by streaking transformants onto a nonselective plate, growing overnight at 30°, then replica plating onto a Ura plate. ARS-bearing plasmids from yeast transformants that gave >80% Ura^- colonies after nonselective growth were characterized further.

Plasmid DNA was recovered from *S. cerevisiae* by an alkaline lysis procedure adapted from Birnboim and Doly (1979). Cells from a large yeast colony (about 3 mm in diameter) were washed in 1 ml SCE (1 m sorbitol, 0.1 m sodium citrate, 60 mm sodium EDTA), then resuspended in 200 μl of SCE solution with 0.1% (v/v) β-mercaptoethanol and 0.5 mg/ml zymolyase (U.S. Biologicals, Swampscott, MA). After digestion at 37° for about an hour, 400 μl of 0.2 n NaOH/1% SDS was added, and then samples were put on ice for 5 min. A total of 300 μl of precooled 2.7 m KOAc, pH 4.8, was added, and the samples left on ice for another 5 min. The supernatants were then collected after a 1-min spin in a microfuge. The DNAs were precipitated by adding 0.6 volumes of isopropanol. The DNA pellets were washed with 70% ethanol, dried, and dissolved in TE. These DNAs were used to transform *E. coli* strain JA226. Plasmids from ampicillin-resistant transformants were analyzed by restriction digestion. ARS activity was confirmed by transformation of yeast strain YP45.

The *S. carlsbergensis* libraries were also screened by hybridization to *S. cerevisiae* chromosome III probes. Different ARS-containing fragments (see legend of Figure 3) were used to probe the *S. carlsbergensis Bam*HI, *Eco*RI, and *Bgl*II libraries at low stringency. Positive clones were recovered and tested for ARS activity by transformation of yeast strain YP45.

The Ars^+ plasmids were labeled with [α-^{32}P]dCTP (Multiprime kit; Amersham, Arlington Heights, IL) and hybridized to several blots: (1) pulsed-field gel-separated chromosomes from *S. cerevisiae* strain YP45 and the substitution strain M1253, (2) phage plaques of the ordered *S. cerevisiae* chromosome III λ library (Riles *et al.* 1993; low stringency), and (3) *Bam*HI-, *Eco*RI-, and/or *Hin*dIII-digested DNA of *S. cerevisiae* strain YP45 (high stringency). The results allowed us to identify the regions of *S. cerevisiae* chromosome III to which the *S. carlsbergensis* ARS elements were homologous.

Hybridization conditions: High-stringency hybridizations were performed at 65° in 5× SSC, 0.1% SDS, 1× Denhardt's solution, 100 μg/ml sonicated calf thymus DNA. Blots were washed at 65° in 1× SSC, 0.1% SDS. Low-stringency hybridizations of the libraries were performed at 55° in 5× SSC, 1× Denhardt's solution. Filters were washed in 5× SSC at 55°.

Yeast transformation: *S. cerevisiae* cells were transformed with plasmid DNA using the lithium acetate procedure described by Elble (1992). *S. monacensis* cells were transformed by electroporation. Cells were grown in YM medium (Difco) to a density of $\sim 3.0 \times 10^7$/ml. Cells were prepared for electroporation as described by Becker and Guarente (1991). Approximately 100 ng of plasmid DNA and 2 μg of sonicated calf thymus DNA (Sigma, St. Louis) were added to 50 μl of cell suspension. Electroporation was carried out at 1.43 kV, 200 ohms, and 25 μF in a 0.2-cm cuvette. After electroporation, cells were resuspended in 1 ml of 1 m sorbitol and incubated in a roller drum for 3–5 hr prior to spreading on YM plates containing 50 μg/ml G418 (Geneticin, Life Technologies, Inc.). Plates were incubated at 23°. For Ars^+ plasmids, 500–2000 transformants were obtained; pRS333 gave no transformants.

Subcloning and sequencing analysis: The ARS-containing clones isolated from the *S. carlsbergensis* chromosome III libraries were subcloned in pRS306, using either convenient restriction sites or limited Exonuclease III digestion (Henikoff 1987) to generate smaller clones. For each ARS element, the smallest subclone was sequenced (cycle sequencing kit, Life Technologies, Inc.). If the smallest ARS-containing subclone did not contain an open reading frame (ORF) to anchor it to the *S. cerevisiae* chromosome III sequence, the ends of larger clones were sequenced to detect open reading frames. Sequencing primers were provided by the New Jersey Medical School Molecular Resources Facility and Life Technologies, Inc. Sequence analysis was performed using the Wisconsin package (Genetics Computer Group, Inc.) provided by UMDNJ Academic Computing Services.

Analysis of replication intermediates by 2-D gel electrophoresis: All the ARS elements isolated from the *S. carlsbergensis* chromosome III were examined for their chromosomal replication origin activity by the 2-D gel electrophoresis method of Brewer and Fangman (1987). *S. monacensis* cells were harvested at a density of 2–4 × 10^7 cells/ml. DNA was prepared as described by Theis and Newlon (1994). The fragments labeled to detect replication intermediates were the 1.5-kb *Eco*RI from 4EH2 (*ARS304*^{carl}), the 1.3-kb *Dra*I fragment of D/D1.3 (*ARS305*^{carl}), the 3.9-kb *Xba*I-*Bam*HI from Xb/B3.9 (*ARS306*^{carl}), the 2.2-kb *Hin*dIII from H/H2.2 (*ARS307*^{carl}), the 3.0-kb *Bam*HI-*Eco*RI from 19EG1 (*ARS308*^{carl}), the 3.4-kb *Eco*RI-*Xho*I from −14 (*ARS309*^{carl}), and the 1.0-kb *Bam*HI from pB5B1(*ARS310*^{carl}).

RESULTS

Identification of *S. carlsbergensis* ARS elements: To identify ARS elements from the *S. carlsbergensis* chromosome III, a *Bam*HI library enriched for fragments of the gel-purified chromosome was constructed in the integrating shuttle vector pRS306 (Sikorski and Hieter 1989) as described in materials and methods. DNA from pools of 40–48 clones was screened for plasmids that transformed *S. cerevisiae* at high frequency, yielding unstable transformants. Using this strategy, 35 independent ARS-containing plasmids were identified. The chromosomal locations of these ARS elements were determined by Southern blot analysis (as described in materials and methods). Four different *S. carlsbergensis* chromosome III ARS elements were identified. Two were from the diverged region of the chromosome, one hybridizing at or near *ARS305* and the second at or near *ARS310* (Figure 2). The other two corresponded to *ARS315* and *ARS316* (A. Dershowitz, A. Poloumienko, J. Shah and C. S. Newlon, unpublished re-

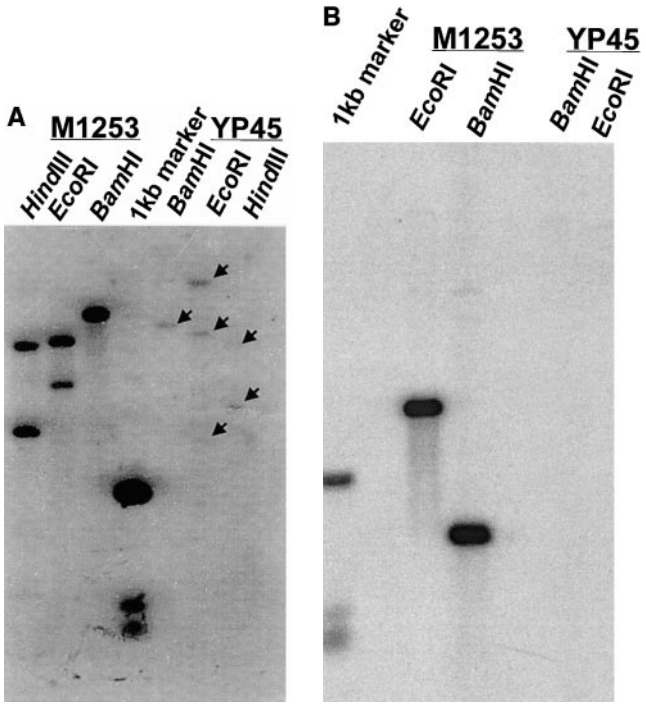


Figure 2.—Genomic Southern hybridization. Southern blots of genomic DNA from strains M1253 and YP45 digested with *Bam*HI, *Eco*RI, or *Hin*dIII were hybridized with the *S. carlsbergensis* ARS-containing fragments (A) 1B3a and (B) pB5B1 at high stringency. The divergence of the *S. carlsbergensis* sequences is indicated by the fact that the pB5B1 probe fails to hybridize with *S. cerevisiae* sequences and that the 1B3a probe hybridizes only weakly (arrows, Figure 2A).

sults), which are located in the *S. cerevisiae*-like part of the mosaic chromosome, and were not analyzed further.

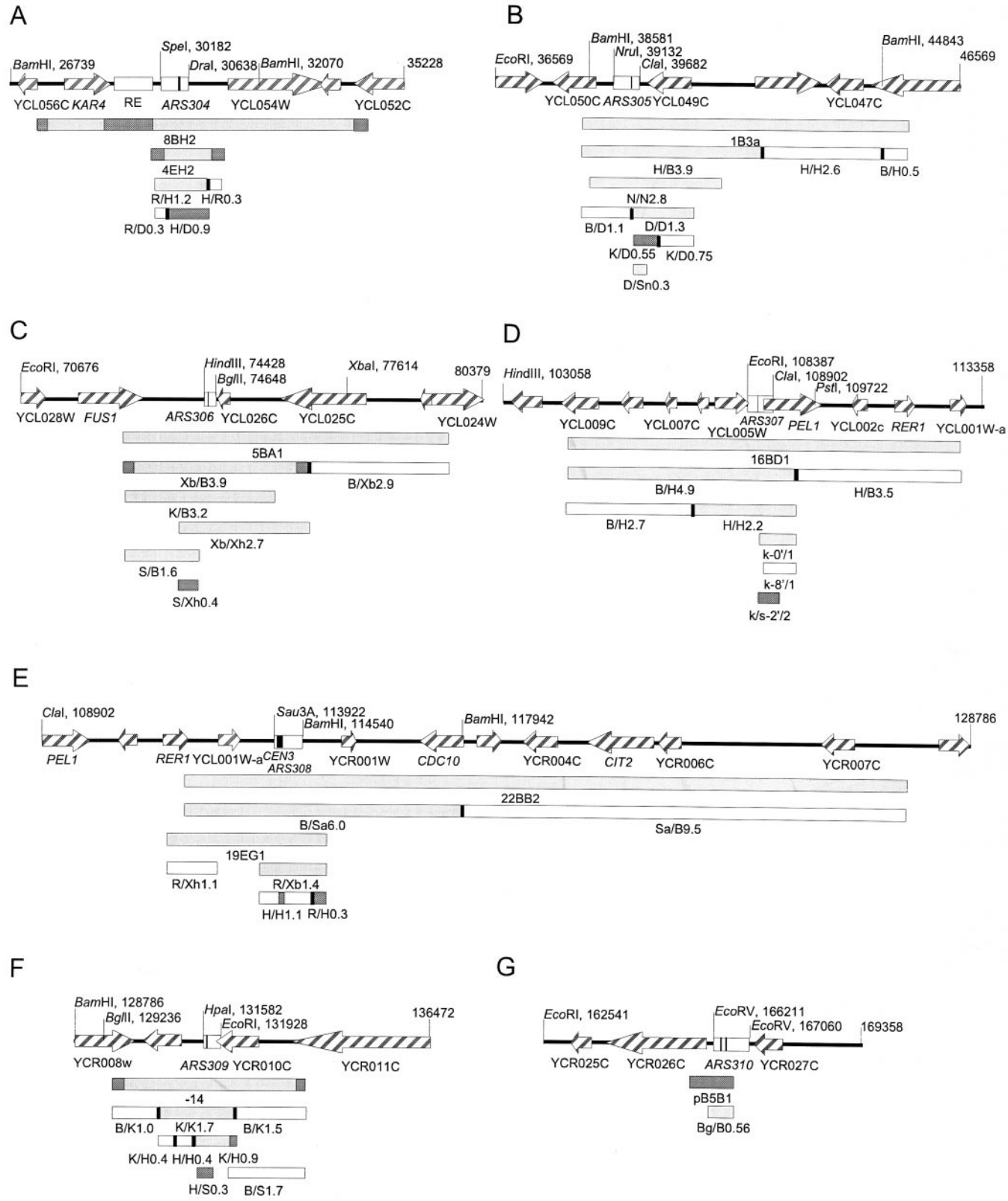
This preliminary screening suggested that the chromosomal locations of ARS elements are conserved between the two homeologous chromosomes III, enabling further screening for *S. carlsbergensis* ARS elements using specific *S. cerevisiae* chromosome III ARS-containing fragments as probes to hybridize at low stringency to the *S. carlsbergensis* chromosome III libraries. Plasmids were identified that hybridized to *ARS304*, *ARS306*, *ARS307*, *ARS308*, and *ARS309*. Each of these clones transformed *S. cerevisiae* at high frequency, yielding transformants that carried unstable plasmids. These results indicated that each of these plasmids carries one or more ARS elements. To distinguish the *S. cerevisiae* ARS elements from their *S. carlsbergensis* homeologues, we employ the superscript "*carl*"; *e.g.*, the *ARS304* homeologue is *ARS304*^{carl}.

To further define the locations of the new ARS elements, the *S. carlsbergensis* inserts in these plasmids were subcloned and tested for ARS activity (Figure 3); a single ARS element was present in each of the initial plasmids. Analysis of the DNA sequences of the subclones revealed that the *S. carlsbergensis* ARS elements are located within intergenic regions corresponding to their *S. cerevisiae* counterparts. For example, the *ARS305*^{carl} is downstream of an ORF that is 60% identical to the C-terminal 112 aa of YCL049C, just as *ARS305* is downstream of YCL049C (Figure 3B). *ARS307*^{carl} is between ORFs homologous to *PEL1* (76% identity over 78 amino acids) and YCL005W (82% identity over 34 aa), just as *ARS307* is located between *PEL1* and YCL005W. *ARS310*^{carl} is upstream of an ORF 68% identical to the predicted N-terminal 106 aa of the YCR026C ORF (Figure 3G), placing it in the intergenic region that corresponds to *ARS310*.

The DNA sequences of the remaining four *S. carlsbergensis* ARS elements failed to reveal the presence of ORFs. Therefore, the ends of larger clones were sequenced to find homologies to ORFs that would allow alignment with *S. cerevisiae* DNA sequences. The ends of the *ARS306*^{carl} clone, Xb/B3.9, are homologous to *FUS1* (83% identity over 216 bp) and YCL025C (77% identity over 232 bp), placing *ARS306*^{carl} in a location similar to *ARS306* (Figure 3C). Similarly, *ARS309*^{carl} is in a position analogous to *ARS309* (Figure 3F), and *ARS304*^{carl} is in the same position as *ARS304*. *CEN3*^{carl} (located within clone H/H1.1) is 63% identical to *CEN3* over 158 bp (T. H. Anderson and T. Nilsson-Tillgren, personal communication). While *ARS308* is closely associated with *CEN3* (Greenfeder and Newlon 1992a), *ARS308*^{carl} is found in the adjacent fragment (Figure 3E).

Analysis of chromosomal replication origin activity: Only about two-thirds of the ARS elements on *S. cerevisiae* chromosome III function detectably as replication origins in their natural locations (Newlon *et al.* 1993). Therefore, we examined the replication intermediates of chromosomal DNA fragments carrying the *S. carlsbergensis* ARS elements using the 2-D gel electrophoresis

Figure 3.—Subcloning of *S. carlsbergensis* ARS elements. The top line of each panel represents the physical map of *S. cerevisiae* chromosome III. On the map line, arrows represent open reading frames, while boxes denote the smallest ARS-containing restriction fragments, the recombination enhancer (RE; Wu and Haber 1995), and the centromere (*CEN3*); names are written underneath the line. The position of the essential ARS consensus sequence (ACS) of each ARS element is indicated by a bar or bars within the box; similarly, the bar within the *CEN3* box denotes CDEI, CDEII, and CDEIII. Below the map lines, each box represents an *S. carlsbergensis* restriction fragment. The shaded boxes represent Ars$^+$ fragments, while the open boxes represent Ars$^-$ fragments. The dark shading denotes the regions which have been sequenced. The dark bars between the boxes represent the boundaries of the adjacent subclones. The capital letters used in the name of the subclones show the restriction sites used for cloning: B, *Bam*HI; Bg, *Bgl*II; D, *Dra*I; H, *Hin*dIII; K, *Kpn*I; N, *Nco*I; R, *Eco*RI; S, *Spe*I; Sa, *Sac*I; Sn,*Sna*BI; Xb, *Xba*I; and Xh, *Xho*I. All the maps are drawn to the same scale. For simplicity, only the elevant restriction sites are shown; in most cases, there are additional sites for the enzymes that are not shown. Numbering is based on the chromosome III sequence (Munich Information Center for Protein Sequences, 02.09.96 release) (A) *ARS304*^{carl}. Clones 8BH2 and 4EH2 were identified in the *Bam*HI



and *Eco*RI libraries, respectively, using the indicated 5.3 kb *Bam*HI fragment as a probe. (B) *ARS305*^{carl}. Clone 1B3a was identified in the *Bam*HI library using the indicated 6.2-kb *Bam*HI fragment. (C) *ARS306*^{carl}. 5BA1 was obtained from the *Bam*HI library using the 3.2-kb *Hin*dIII/*Xba*I fragment as a probe. (D) *ARS307*^{carl}. 16BD1 was obtained by probing the *Bam*HI library with the 1.3-kb *Eco*RI/*Pst*I fragment. (E) *ARS308*^{carl}. 22BB2 and 19EG1 were pulled out of the *Bam*HI and *Eco*RI libraries, respectively, by probing with the 3.4 kb *Bam*HI fragment. (F) *ARS309*^{carl}. Clone 14 was obtained from the *Bgl*II library probed with the 2.7-kb *Bgl*II/*Eco*RI fragment. (G) *ARS310*^{carl}. pB5B1 was an Ars^{+} clone obtained from the *Bam*HI library.

technique developed by Brewer and Fangman (1987). If the fragment being examined lacks a replication origin, it is replicated by a fork originating outside the fragment, and the replication intermediates are Y-shaped and migrate in the pattern labeled "Y arc" in Figure 4. If the fragment contains a replication origin, replication intermediates are bubble shaped and migrate more slowly than Y-shaped intermediates in the second dimension, falling along the arc labeled "bubble" in Figure 4. If the origin is asymmetrically located within the fragment, a composite pattern is produced in which early replication intermediates are bubble shaped and late intermediates are Y-shaped. The detection of bubble-shaped intermediates indicates that the fragment contains an active replication origin. If a particular origin is active in every cell in the culture, then no small Y-shaped intermediates should be detected. If the origin is active in only a fraction of the cells, then some of the time the fragment is replicated by a fork entering the fragment from outside, resulting in the presence of Y-shaped intermediates of all sizes.

We first examined the origin activity of the *S. carlsbergensis* ARS elements in the chromosome III substitution strain M1253. Bubble-shaped replication intermediates were detected in the 2-D gels for *ARS305*^{carl}, *ARS306*^{carl}, *ARS307*^{carl}, *ARS309*^{carl}, and *ARS310*^{carl} (Figure 4, panels 305, 306, 307, 309, and 310), indicating that these ARS elements function as chromosomal replication origins during normal cell growth. In addition to the bubble arc, a complete Y arc of uniform intensity was present on the 2-D gels for *ARS307*^{carl}, *ARS309*^{carl}, and *ARS310*^{carl}, suggesting that these origins are active in only a fraction of the population. In contrast, the 2-D gels for *ARS305*^{carl} and *ARS306*^{carl} showed an intense signal on the late part of the Y arc with little signal from small Y-shaped intermediates, suggesting that these two origins are active in most or all of the cells in the population (Figure 4, compare panels 305 and 306 to panels 307, 309, and 310). *ARS304*^{carl} and *ARS308*^{carl} did not function detectably as chromosomal replication origins because only Y-shaped intermediates were detected (Figure 4, panels 304 and 308). A conspicuous feature of the 2-D analysis of *ARS308*^{carl} is the presence of an intense spot of hybridization along the Y arc, mapping to the position of the centromere (Figure 4, panel 308), and reflecting a replication-fork pause site similar to the pause site coincident with *CEN3* (Greenfeder and Newlon 1992a).

Analyses of *S. monacensis*: The preceding analyses were performed in an *S. cerevisiae* background. We were concerned that the replication of the *S. carlsbergensis* chromosome by *S. cerevisiae* proteins might make use of different replicator sequences than are used in the species from which the chromosome was derived. Indeed, studies of *Schizosaccharomyces pombe* and *Kluyveromyces lactis* have shown that the DNA sequences required for ARS activity differ from those required in *S. cerevisiae* (Maundrell *et al.* 1985, 1988; Fabiani *et al.* 1996). The obvious choice for these experiments is the species that contributed the *S. carlsbergensis* set of chromosomes to

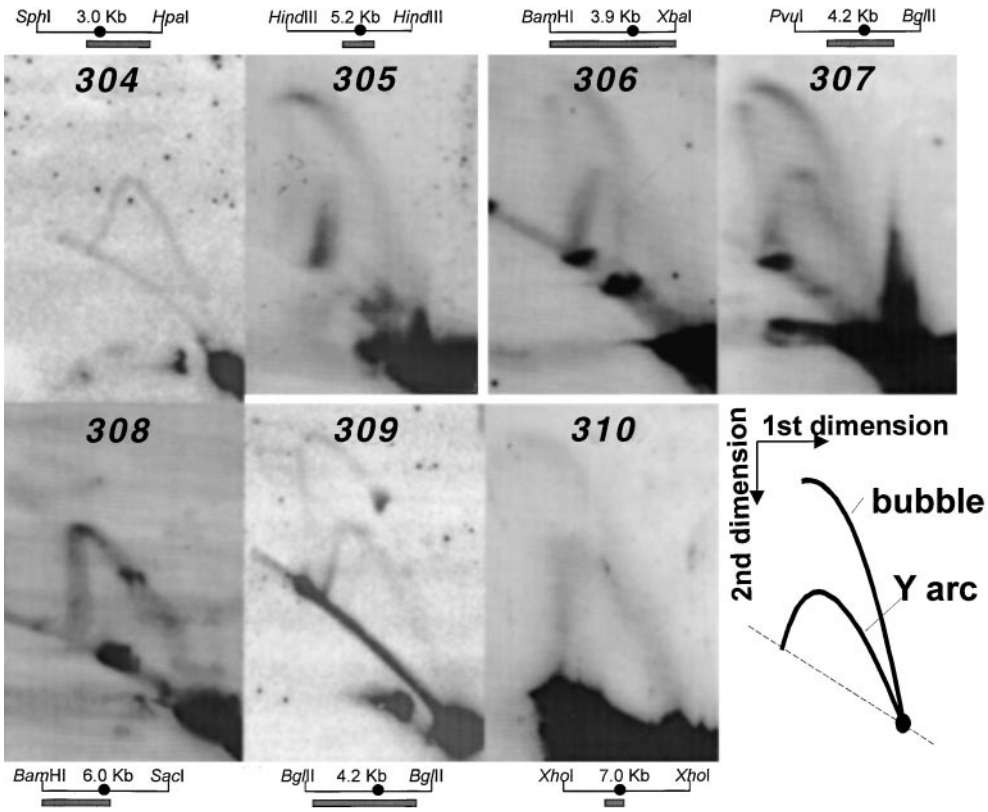


Figure 4.—2-D gel analysis in the chromosome III substitution strain. Each of the *S. carlsbergensis* ARS elements was analyzed for origin function using DNA prepared from M1253. For each ARS element, the restriction fragment examined is indicated. The position of the ARS within the fragment is denoted by the dot, and the fragment used as a probe is indicated by the box below the line. The lower right panel is a diagram of the migration patterns of bubble- and Y-shaped replication intermediates. The presence of bubble-shaped intermediates indicates that a fragment contains a replication origin.

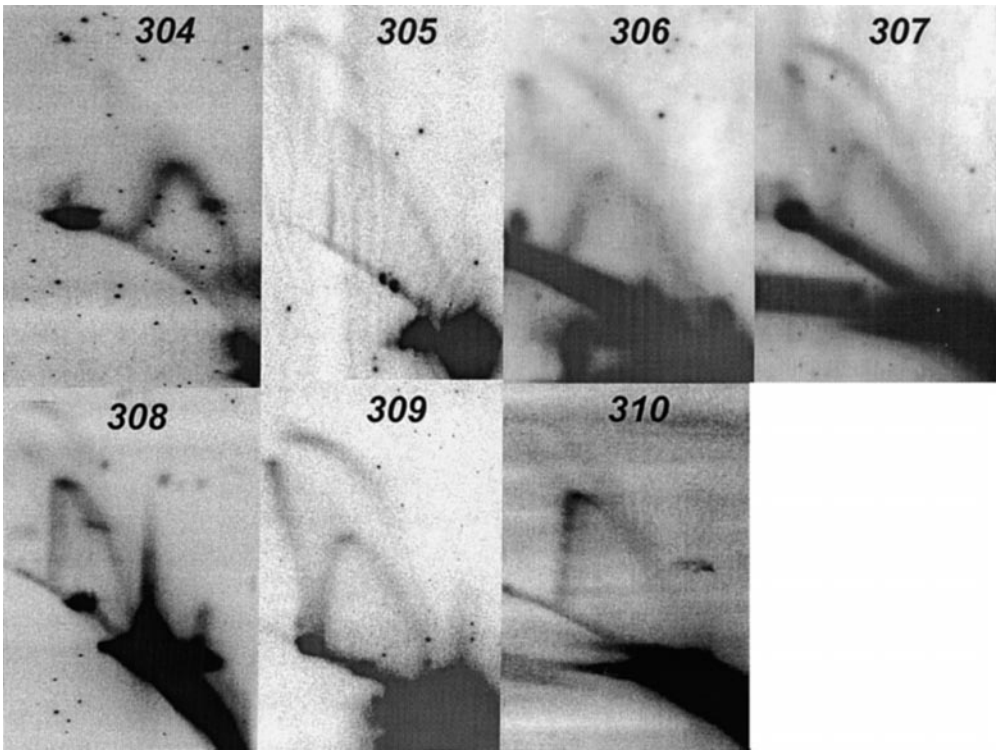


Figure 5.—2-D gel analysis in *S. monacensis*. Origin function was examined using DNA isolated from *S. monacensis* cells. Restriction digests and probes are the same as shown in Figure 4.

the brewing strain, which seems to be *S. monacensis* (Hansen and Kielland-Brandt 1994).

Using primers designed from our sequences of *ARS305*^{carl} and *ARS306*^{carl}, we amplified by PCR the homologues from *S. monacensis*. The sequences of the PCR products were identical to the sequences obtained from our clones (Theis *et al.* 1999, and data not shown), supporting the idea that the *S. carlsbergensis* chromosomes are derived from *S. monacensis*. On the basis of this observation, we decided to analyze the function of the *S. carlsbergensis* ARS elements in *S. monacensis*. Because *S. monacensis* is a prototroph, we constructed a plasmid that carries *CEN3*^{carl} and KanMX, encoding G418 resistance (see materials and methods). Three ARS elements were inserted into this plasmid and examined for ARS activity: *ARS305*^{carl}-fragment D/Sn0.3, *ARS307*^{carl}-fragment k/s-2′/2, and *ARS310*^{carl}-fragment Y. All three of these constructs yielded G418-resistant colonies, while the vector pRS333 did not. The plasmids were unstable in the *S. monacensis* transformants, and, in the case of the *ARS310*^{carl} transformants, Southern blot analysis showed that the plasmid had not integrated into one of the *S. monacensis* chromosomes (data not shown). We conclude that these three ARS elements are able to function in both *S. monacensis* and *S. cerevisiae*.

We examined replication origin activity in *S. monacensis* (Figure 5). *ARS304*^{carl} was inactive while *ARS307*^{carl} and *ARS309*^{carl} were active, though not in every cell cycle as shown by the Y arc of uniform intensity (compare panels in Figure 4 with those in Figure 5). *ARS305*^{carl} and *ARS306*^{carl} appeared to function less efficiently as chromosomal replication origins in *S. monacensis*, as indicated by the uniform intensity of the Y arc detected in Figure 5. *ARS308*^{carl} was weakly active as an origin in *S. monacensis*, as indicated by the faint bubble arc, while it was inactive in the substitution strain (Figure 5, panel 308).

DISCUSSION

We have isolated nine ARS elements from the *S. carlsbergensis* chromosome III. The first approach was to screen a plasmid library enriched for clones from the *S. carlsbergensis* chromosome. The advantage of this approach is that it requires no assumptions about the positions of ARS elements on the chromosome. Of the 35 independent Ars$^+$ clones obtained in the screen of the *Bam*HI library, 18 were derived from chromosome III and represented four different ARS elements. Two of these were derived from the *S. cerevisiae* region of the chromosome and were not studied further. *ARS305*^{carl} was isolated twice, and *ARS310*^{carl} was isolated four times. While this screen was certainly not saturated, it was relatively inefficient. Moreover, it showed that ARS elements are located in analogous positions on the two chromosomes. To supplement the data from the initial screen, five more ARS elements were identified by hybridization to the *S. carlsbergensis* libraries at reduced stringency using probes containing *S. cerevisiae* ARS elements.

The analysis of subclones indicated that each of the clones obtained from the *S. carlsbergensis* libraries con-

tained a single ARS element. These seven ARS elements were located between homologous ORFs on the two chromosomes, despite the fact that the large clones often contained many intergenic regions. Assuming the organization of ORFs is identical on the two chromosomes, only 7 of the 27 intergenic regions present on the seven *S. carlsbergensis* clones contain ARS elements. The precise correspondence of the *S. carlsbergensis* ARS elements to the only ARS elements in a 150-kb segment of chromosome III (nucleotides 30,000–180,000; Newlon *et al.* 1991) makes it unlikely that the use of the directed strategy missed one or more ARS elements in the corresponding region of the *S. carlsbergensis* chromosome.

We addressed the ability of these ARS to function as chromosomal replication origins by examining replication intermediates in both the chromosome *III* substitution strain and *S. monacensis*. In *S. cerevisiae*, *ARS305*, *ARS306*, *ARS307*, *ARS309*, and *ARS310* are all strong origins, *i.e.*, active in most cells (Deshpande and Newlon 1992; Greenfeder and Newlon 1992b; Zhu *et al.* 1992; Newlon *et al.* 1993; Theis and Newlon 1997). The *S. carlsbergensis* counterparts of these ARS elements are also active as chromosomal replication origins, although their levels of activity appear to differ. For example, $ARS305^{carl}$ and $ARS306^{carl}$ appear to be active in most *S. cerevisiae* cells, as indicated by the absence of small Y-shaped intermediates (Figure 4), yet these origins appear to be less efficiently used in *S. monacensis*, as suggested by the uniform intensity of the Y arc present below the bubble arc (Figure 5). $ARS309^{carl}$ and $ARS310^{carl}$ appear to be partially active in both species, as indicated by the similar intensities of both bubble- and Y-shaped intermediates (Figures 4 and 5). $ARS304^{carl}$ is not active in either case, nor is *ARS304* an active origin in *S. cerevisiae* (Dubey *et al.* 1991; Newlon *et al.* 1993). $ARS308^{carl}$ does not appear to be used as an origin in the chromosome III substitution strain, but is weakly active in *S. monacensis*, just as *ARS308* is a weak origin in *S. cerevisiae* (Greenfeder and Newlon 1992b). Therefore, the ability of these ARS elements to function as chromosomal replication origins is also conserved.

While the analysis of *S. monacensis* suggests that these ARS elements are likely to function in the yeast that contributed the *S. carlsbergensis* genome to the alloploid brewing strain, our further analysis of the *S. monacensis* type strain suggests that these results must be interpreted with some caution. We have found that *S. monacensis* is also an alloploid, and it appears to carry *S. cerevisiae*-like chromosomes I and III (J. F. Theis and C. S. Newlon, unpublished results).

It was somewhat surprising that all five of the efficient origins of replication in the region of *S. cerevisiae* chromosome *III* to the left of *MAT*, *ARS305*, *ARS306*, *ARS307*, *ARS309*, and *ARS310* were conserved on the *S. carlsbergensis* chromosome because many of the origins can be deleted from the *S. cerevisiae* chromosome without affecting its stability (Dershowitz and Newlon 1993; Newlon *et al.* 1993). Even more surprising was the conservation of *ARS304*, which is not active as a chromosomal replication origin. While it is possible that *ARS304* is active under conditions that we have not tested, we consider it unlikely because our analysis was done on cultures grown in rich medium at optimal temperatures, a situation in which the rapid growth rate might be expected to require the use of all available origins. Moreover, we have examined origin function under a very different set of conditions, premeiotic S phase, and have found that *ARS304* is not active during meiosis either (Collins and Newlon 1994). Alternatively, *ARS304* might be used for some purpose other than replication. ORC binding sites are an important component of the silencers at *HML* and *HMR* (reviewed by Laurenson and Rine 1992), and the function of the ORC in silencing is distinct from its role in replication (Fox *et al.* 1995, 1997; Dillin and Rine 1997). Perhaps ORC binding at *ARS304* is being used for some other purpose.

Our finding that the ARS elements on chromosome III of *S. cerevisiae* and *S. carlsbergensis* are in conserved locations provides strong evidence that these pairs of ARS elements are homologues. Comparisons of different ARSs with one another have failed to identify any common elements beyond the ARS consensus sequence (Broach *et al.* 1983). This may indicate that, while ORC binding may be a common feature of all ARS elements, the accessory elements may be different. By comparing homologous ARS elements from different species, we hope to identify ARS-specific functional elements.

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