MOLECULAR AND CELLULAR BIOLOGY, Oct. 1991, p. 5346–5355
0270-7306/91/105346-10$02.00/0

Vol. 11, No. 10

Evidence Suggesting that the *ARS* Elements Associated with Silencers of the Yeast Mating-Type Locus *HML* Do Not Function as Chromosomal DNA Replication Origins

DHARANI D. DUBEY,[1]† LESLIE R. DAVIS,[1] SCOTT A. GREENFEDER,[2] LOKE Y. ONG,[2] JIGUANG ZHU,[1] JAMES R. BROACH,[3] CAROL S. NEWLON,[2] AND JOEL A. HUBERMAN[1]*

Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, New York 14263[1]; Department of Microbiology and Molecular Genetics, UMDNJ-New Jersey Medical School, Newark, New Jersey 07103[2]; and Department of Biology, Princeton University, Princeton, New Jersey 08544[3]

Received 7 May 1991/Accepted 29 July 1991

The silent mating-type loci of *Saccharomyces cerevisiae*, *HML* and *HMR*, are flanked by transcriptional silencers that have *ARS* activity (i.e., they function as replication origins when in plasmids). To test whether these *ARS* elements are chromosomal origins, we mapped origins near *HML* (close to the left telomere of chromosome III). Our results indicate that the *HML*-associated *ARS* elements either do not function as chromosomal replication origins or do so at a frequency below our detection level, suggesting that replication from a silencer-associated origin in each S phase is not essential for the maintenance of transcriptional repression at *HML*. Our results also imply that the ability of a DNA fragment to function as an *ARS* element in a plasmid does not ensure its ability to function as an efficient chromosomal replication origin. Telomere proximity is not responsible for inactivating these *ARS* elements, because they are not detectably functional as chromosomal origins even in genetically modified strains in which they are far from the telomere.

The complex process of chromosomal DNA replication in eukaryotes is poorly understood, partly because origins of DNA replication are not well characterized. In *Saccharomyces cerevisiae*, autonomously replicating sequences (*ARS* elements) promote efficient plasmid replication and thus permit plasmids containing them to transform yeast cells at high frequency without integration into the genome. *ARS* elements can be classified as strong or weak, depending on the mitotic stabilities of plasmids containing them. Strong *ARS* elements promote high mitotic stabilities, and weak *ARS* elements promote lower mitotic stabilities. The properties of *ARS* elements suggest that they may serve as replication origins (7, 47). Indeed, two-dimensional (2D) gel electrophoretic replicon mapping techniques have permitted the direct demonstration that *ARS* elements are active as replication origins in the yeast 2μm plasmid and in an *ARS1*-containing plasmid (4, 24). Results obtained by these techniques have also revealed that four strong *ARS* elements on yeast chromosome III—*ARS305* (A6C [25]), *ARS306* (C1G [12, 52]), *ARS307* (C2G1 [19]), and *ARS309* (J11D [19]; see references 40 and 40a for the locations of these *ARS* elements on chromosome III)—and a weak *ARS* in tandemly repeated ribosomal DNA (31) are all active as chromosomal replication origins. In ribosomal DNA, however, only 5 to 30% of available *ARS* elements are active as origins in each cell cycle (31). Whether all the *ARS* elements in nonrepeated chromosomal DNA are active as replication origins was, until recently, unknown. The experiments described in this paper reveal that some *ARS* elements in unique DNA do not detectably function as origins in their normal chromosomal environment.

In *S. cerevisiae*, there are two mating types, **a** and α. Information specifying mating types is present at three locations on chromosome III, the *HML*, *MAT*, and *HMR* loci (Fig. 1). Under normal conditions, only the information at the *MAT* locus (either **a** or α) is expressed. Haploid yeast cells can switch mating type by replacing the *MAT* information with a copy of either the information present at *HML* (usually α) or the information present at *HMR* (usually **a**) (reviewed by Herskowitz [22]). The *HML* and *HMR* loci are located near the left and right telomeres of chromosome III, and information present at *HML* and *HMR* is usually repressed. Products of four *trans*-acting *SIR* genes are required for this repression (26, 43). In addition, both *HML* and *HMR* are flanked by *cis*-acting "silencer" sequences, E and I, which are necessary for their *SIR*-mediated repression. These silencers have strong *ARS* activity when in plasmids, and they share sequence properties (1, 3, 15, 33). An additional *ARS* element is located near the *HML* I silencer within a 1.4-kbp *Hin*dIII-*Bam*HI fragment (Fig. 1, lower panel) (40, 40a).

In a comprehensive study of the *HMR* E silencer, Brand et al. (3) identified three functional domains: A, E, and B. Domain A contains an 11-bp match to the *ARS* consensus sequence. Domains E and B have binding sites for the RAP1 and ABF1 proteins, respectively, and are required for *SIR*-dependent plasmid stability (3, 29). Since all four silencers (*HML* E, *HML* I, *HMR* E, and *HMR* I) have strong *ARS* activity, and a round of replication is required to establish repression at *HML* and *HMR* (36), DNA replication initiated at these loci has been assumed to play an important role in their function as transcriptional repressors (3, 15, 36).

In higher eukaryotes, the processes of DNA replication and transcription are correlated so that most actively transcribed genes are replicated early in S phase (17, 21). In addition, transcriptional elements are closely associated with replication origins in several viral systems (reviewed by DePamphilis [11]). While transcription near *oriC* seems to activate replication in *Escherichia coli* (2), transcription into *ARS1* has an adverse effect on its activity in *S. cerevisiae*

* Corresponding author.
† Present address: Department of Zoology, Kutir Mahavidyalaya, Chakkey, Jaunpur-222146, U.P., India.

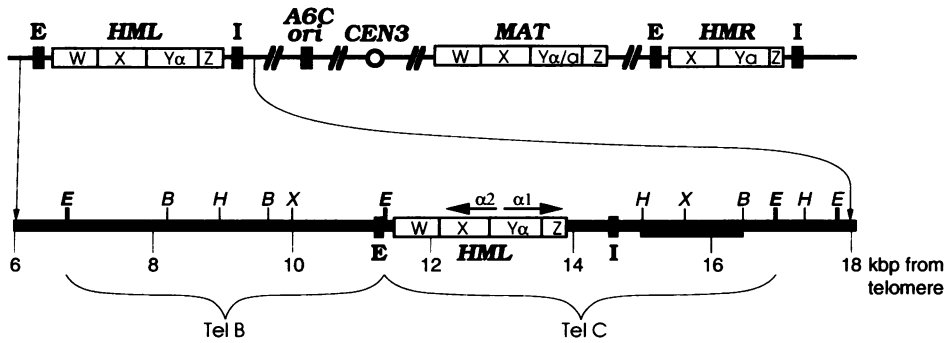

FIG. 1. Relationship of the yeast *HML* silent mating-type region to other relevant loci on chromosome III. Upper portion: diagram of chromosome III showing the *HML*, *MAT*, and *HMR* mating-type loci, the A6C origin, and the centromere. E and I silencers flank the *HML* and *HMR* loci. The blocks labeled W, X, Yα or Ya, and Z indicate regions of identical sequence at *HML*, *MAT*, and *HMR*. Lower portion: enlargement of the *HML* region showing restriction sites (E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; X, *Xho*I), distances from the telomere, directions of transcription at *HML* (normally repressed), and the boundaries of the two *Eco*RI fragments, TelB and TelC. The diagonally slashed *Hin*dIII-*Bam*HI restriction fragment (about 16 kbp from the telomere) and the E and I silencers each contain an *ARS* element (see text).

(44). It would be interesting to see whether there is a relationship between transcription and replication at *HML*, where *ARS* elements (possible replication origins) are integral parts of transcriptional repressors. In addition, it has recently been demonstrated that proximity to yeast telomeres can repress the transcription of genes (18). Therefore, it is also of interest to determine whether proximity to a telomere can affect replication origin function.

To answer these questions, we have used 2D gel electrophoresis to test the chromosomal origin function of the *ARS* elements that flank *HML*. Our results suggest that none of these *ARS* elements functions detectably in vivo as a chromosomal replication origin regardless of the transcriptional status of *HML*. In addition, their proximity to a telomere does not appear to be responsible for their inability to function as origins. Finally, our results also suggest that this region of chromosome III is replicated by a fork moving toward the telomere from the distant *ARS305* origin (A6C origin [25]).

MATERIALS AND METHODS

Strains and cell growth. The yeast strains used in this study are listed in Table 1. 4910-3-3 cells (from L. Hartwell, University of Washington) and the other cells used were grown at 23 and 30°C, respectively, in YPD medium to a final density of $\approx 1.5 \times 10^7$ cells per ml.

Strain Y903 was constructed by transforming strain DMY53 (*MATa ura3-52 leu2-3,112 ade2-1 lys1-1 his5-2 can1-100* [33]) to uracil prototrophy with *Hpa*I-digested pDM33 DNA. Plasmid pDM33 consists of plasmid YIp19 (plasmid pUC19 containing the 1.1-kbp *Hin*dIII fragment spanning *URA3* inserted at the *Hin*dIII site) carrying a 1.1-kbp *Xba*I fragment encompassing the *HML* E site and the adjacent 0.5 kbp of the *HML* W region. Digestion of plasmid pDM33 with *Hpa*I prior to the transformation of *S. cerevisiae* directs circular integration of the plasmid into the W region of either *HML* or *MAT*. Accordingly, Ura^+ transformants were examined by Southern analysis for the location of the integration site, and one such transformant in which the plasmid had been inserted at *MAT* was retained as strain Y903. A map of this strain's altered *MAT* locus is provided in Fig. 8.

Strain Y574 (referred to in this paper as BJ2169*sir4*$^-$) was constructed by transforming strain BJ2169 to leucine prototrophy with a linearized *SIR4* gene (35) into whose *Bgl*II site was inserted the *LEU2* gene. BJ2169 is a direct descendant of S288C (27).

Strain Y554 (referred to in this paper as S150-2B*sir4*$^-$) was constructed as previously described (35).

DNA isolation and purification. DNA from logarithmically growing cells was isolated and purified as described previously (24). For strains XJ24-24A, K45, and K191, the initial solution of nuclear DNA was extracted three times with

TABLE 1. Genotypes of yeast strains

Name	Genotype	Source	Background strain	Reference
A364A	*MATa ade1 ade2 ura1 tyr1 his7 lys2 gal1*	L. Hartwell	A364A	20
4910-3-3	*MATa his7 ura1 cdc7-4 bar1*	L. Hartwell	A364A	41
XJ24-24A	*MATa ade6 arg4-17 trp1-1 aro7-1*	C. Newlon	S288C[a]	46
BJ2169	*MATa leu2 trp1 ura3-52 prb1-1122 prc1-407 pep4-3*	E. Jones	S288C	28
BJ2169*sir4*$^-$ (Y574)	BJ2169 *sir4::LEU2*	A. Rose and J. Broach	S288C	This paper
S150-2B	*MATa leu2-3,112 ura3-52 trp1-289 his3Δ gal2*	J. Broach	S288C	35
S150-2B*sir4*$^-$ (Y554)	*S150-2B sir4Δ-2::HIS3*	A. Rose and J. Broach	S288C	This paper
Y903	E^+ *MATα ade2-1 lys1-1 his5-2 leu2-3,112 can1-100*	D. Mahoney and J. Broach	S288C	This paper
K45	*MATα HMLα HMRa sir1 ho trp1-1 thr1 arg4-17 lys1-1 ade8-10 his2 ura1*	A. Klar	S288C	30
K191	Like K45, but with large circular chromosome III derived by *HML-HMR* fusion	A. Klar	S288C	30

[a] Other strains, in addition to S288C, may have contributed to the background of XJ24-24A (45).

phenol-chloroform-isoamyl alcohol (25:24:1) instead of being purified in a CsCl gradient.

BND-cellulose fractionation. Samples of isolated, purified DNA (100 to 500 μg) were digested to completion with the restriction enzymes indicated in the figure legends. In most experiments, replication-fork-containing fragments were then enriched with 250 to 500 μl of packed BND (benzoylated, naphthoylated DEAE)-cellulose as described previously (24, 25). Where noted in the figure legends, such enrichment was omitted.

2D gel electrophoresis. Both the neutral/neutral (4) and neutral/alkaline (38) techniques were used as previously described (24, 25).

Transfer to nylon membranes and hybridization. Alkaline capillary transfer, probe preparation, and hybridization were carried out as described previously (24, 25). The probes used are described in the text and figure legends.

Transfer to nitrocellulose membranes and hybridization. Where noted in the figure legends, DNA was transferred to nitrocellulose membranes as previously described (13). Hybridizations were carried out in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium pyrophosphate–5× Denhardt's solution–0.5% sodium dodecyl sulfate (SDS)–100 μg of sonicated, denatured calf thymus DNA per ml at 65°C overnight. Membranes were washed in three to four changes of 1× SSC–0.1% SDS at 65°C. The probes used are described in the text and were labeled by using a Multi Prime kit (Amersham) according to the manufacturer's instructions.

RESULTS

2D gel replicon mapping techniques. For the studies described in this paper, we used both the neutral/alkaline (24, 38) and neutral/neutral (4) 2D gel electrophoresis techniques. The advantages of using both techniques have been discussed elsewhere (32, 49). In both techniques, restriction fragments from replicating DNA are separated according to the extent of replication in the first (neutral) dimension of electrophoresis. During the alkaline second dimension of the neutral/alkaline technique, nascent strands are separated from full-size parental strands and fractionated according to size. The combination of first and second dimensions leads to the separation of nascent strands from other strands so that the sizes of nascent strands able to hybridize with any probe sequence can be measured. By sequential hybridization with probes from different portions of the restriction fragment of interest, the direction(s) of replication through any restriction fragment can be determined, and the locations of origins and termination sites can also be determined (38). The location of the origin in the yeast 2μm plasmid was measured with an error of ±100 bp (24), and the errors for chromosomal origins have been only slightly larger (ca. ±250 bp [25, 31]).

In the neutral/neutral technique, second-dimension electrophoresis is carried out by using elevated agarose and ethidium bromide concentrations at high voltage and low temperature. These conditions maximize the dependence of second-dimension mobility on DNA structure: nonlinear structures migrate more slowly than linear structures, and different types of nonlinear structures migrate at characteristically different rates (4). The consequence is that the family of replicating molecules generated when a replication fork traverses a restriction fragment from one end to the other ("simple Y's") produces an arc in the final 2D gel that can easily be distinguished from the arc produced by the replicating forms of a restriction fragment containing an internal origin near its center ("bubbles" [4]).

Replication forks move leftward through the *HML*-containing *Bam*HI fragment. A map of the left end of chromosome III, which contains *HML*, is shown in the lower portion of Fig. 1. Clones containing this region have been isolated from chromosome III in at least four different yeast strains and analyzed for *ARS* function on plasmids. The results from all of these analyses are in agreement, not only with regard to the location of *ARS* elements but also concerning details of the restriction map of this region. The strains used for these analyses were DC56 (7, 23), XJ24-24A (source of the ring chromosome in strain XG1#24 [40, 46]), A364A (48), and DMY1 (34). The complete ancestries of strains DC56 and XJ24-24A cannot easily be determined because of uncertainties about the backgrounds of many of the strains (contributed by numerous laboratories) which went into their construction (45), but the genomes of both strains contain large contributions from the commonly used strain S288C (37). DMY1 is an immediate descendent of S150-2B, which is employed in this study (Table 1). Both DMY1 and S150-2B are derived from S288C. A364A is another commonly used strain (20) that appears to be related to S288C (37). It is important to note that the strains used in this study for analysis of origin function (Table 1) are the same as or closely related to the strains used for *ARS* analyses.

The two *Eco*RI fragments referred to as TelB and TelC in Fig. 1 were derived from strain A364A and were cloned into the yeast shuttle vector YIp5 (47) by Synn and Newlon (48). The locations of *ARS* elements in clones derived from both A364A and XJ24-24A were determined. In addition to the previously identified *ARS* elements coinciding with the E and I silencers, a new *ARS* element was detected in the indicated *Hin*dIII-*Bam*HI fragment (Fig. 1) (40, 40a).

As shown in Fig. 1, a 6.8-kbp *Bam*HI fragment contains *HML*, the E and I silencers, and the new *ARS* element. Replication of this fragment by initiation at any one of its three *ARS* elements would lead to predictable patterns after neutral/alkaline electrophoresis, as diagrammed in Fig. 2.

In drawing Fig. 2, we have used a new convention for naming *ARS* elements (8) in which each *ARS* element is assigned a three- or four-digit number. The first one or two digits correspond to the number of the chromosome on which the element is located, and the last two digits identify the element uniquely. Since most or all of the *ARS* elements at the left end of yeast chromosome III have been identified and mapped, they have been numbered serially from left to right.

The upper part of Fig. 2 is a magnified drawing of the 6.8-kbp *HML*-containing *Bam*HI fragment (also shown in Fig. 1) illustrating the two subfragments used as hybridization probes for neutral/alkaline 2D analysis. The lower part of Fig. 2 diagrams the autoradiographic signals predicted if each of the *ARS* elements in this *Bam*HI fragment were to serve as the sole bidirectional origin responsible for replicating the entire fragment. In each cartoon, the large round spot indicates the signal from the nonreplicating intact fragment, the horizontal line extending to the left from this spot indicates the signal from parental strands, the vertical line below this spot indicates the signal from occasional randomly nicked strands in nonreplicating DNA, and the diagonal line indicates the signal from nascent strands. In each cartoon, the minimum detectable nascent strand length is determined by the distance from the origin to the probe. See reference 24 for further explanation. Note that identical results are predicted for origin function by *ARS303* and for

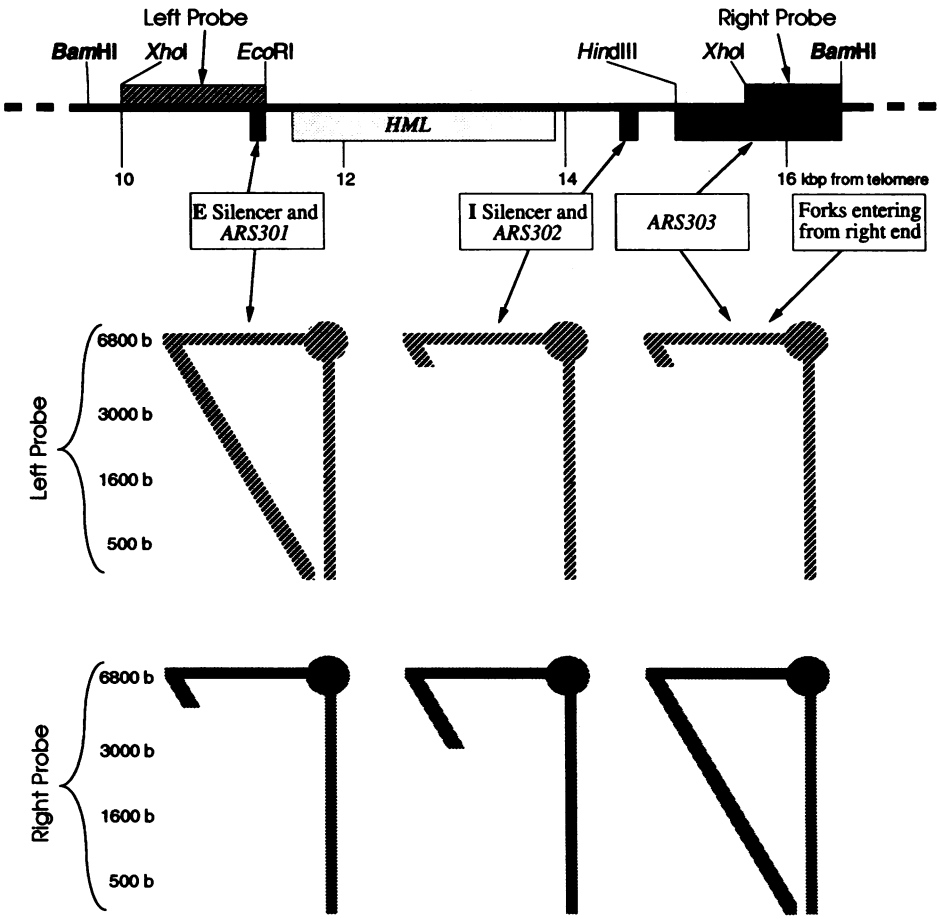

FIG. 2. Neutral/alkaline 2D gel electrophoresis results predicted, assuming origin activity of the *HML*-flanking *ARS* elements. The upper portion is an enlargement of the 6.8-kbp *HML*-containing *Bam*HI fragment shown in Fig. 1. The *ARS* elements are numbered as described in the text. For use as hybridization probes, the indicated *Xho*I-*Eco*RI and *Xho*I-*Bam*HI fragments were purified by electrophoresis in low-gelling-temperature agarose and labeled to high specific activity with [^{32}P]dTTP as described previously (24). The lower portion presents, in cartoon form, the results expected if each of the indicated *ARS* elements were to serve as the sole origin responsible for replicating the entire fragment. The upper (diagonally striped) diagram in each pair represents the pattern expected after hybridization with the left probe; the lower (shaded) diagram shows the results predicted with the right probe. See text for details. This cartoon was drawn assuming first-dimension electrophoresis from left to right.

replication forks entering this *Bam*HI fragment from the right end and moving all the way through to the left end, with no initiations anywhere within the fragment.

When *Bam*HI-digested DNA from the yeast strain 4910-3-3 (derived from A364A; Table 1) was subjected to neutral/alkaline 2D gel electrophoresis and then hybridized sequentially with the two probe fragments indicated in Fig. 2, the results shown in Fig. 3 were obtained: the right probe detected nascent strands of all sizes, while the left probe detected only the very longest nascent strands (which were not resolved from the 6.8-kbp parental strands). These results show clearly that neither *ARS301* nor *ARS302* (the silencer-associated *ARS* elements) functions as the sole replication origin responsible for replicating this region. Although the results are consistent with the view that *ARS303* functions as the sole origin or that *ARS302* and *ARS303* function in combination, they are equally consistent with the possibility that *ARS301*, *ARS302*, and *ARS303* are all nonfunctional and that this *Bam*HI fragment is replicated by an external origin located to its right (Fig. 2). The results of other experiments, some of which will be presented below, favor the latter possibility.

To further test the possibility that the *HML* region is replicated by an external origin, we applied the neutral/neutral 2D technique to DNA isolated from both strain 4910-3-3 (Fig. 4A and B) and strain XJ24-24A (Fig. 4C and D). Both the 5.4-kbp *HML*-containing *Eco*RI fragment (TelC in Fig. 1) and the 6.8-kbp *HML*-containing *Bam*HI fragment (Fig. 1 and 2) carry *ARS302* and *ARS303*. The *Bam*HI fragment also contains *ARS301*. The arcs of replicating molecules identified by probes recognizing these fragments in neutral/neutral gels of both *Eco*RI- and *Bam*HI-digested DNA (Fig. 4A to C) rise upwards and leftwards from the major spot of nonreplicating DNA and then curve downwards and end at the line formed by double-stranded linear fragments. This is the type of arc predicted for restriction fragments that are replicated as simple Y's (4) by forks passing from one end to the other. There is no evidence of the type of arc that would be produced by a restriction fragment containing an internal origin or bubble, i.e., an arc that rises more steeply away from the nonreplicating monomer fragments and that does not return to the line of linear fragments (4) (see example in Fig. 4D).

The absence of bubble arcs in Fig. 4A and C is not a

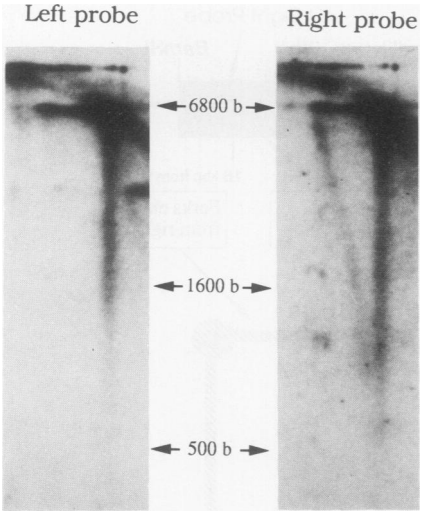

FIG. 3. Replication forks enter the *HML*-containing *Bam*HI fragment from the right or initiate at *ARS303*. The probes indicated in Fig. 2 were incubated under hybridization conditions with a membrane containing *Bam*HI-digested DNA from cell strain 4910-3-3 that had been enriched for replicating molecules and then subjected to neutral/alkaline 2D gel electrophoresis. Comparison of these results with those predicted in Fig. 2 shows that the detected *Bam*HI fragment must be replicated via initiation at *ARS303* or by replication forks entering from the right end. b, bases.

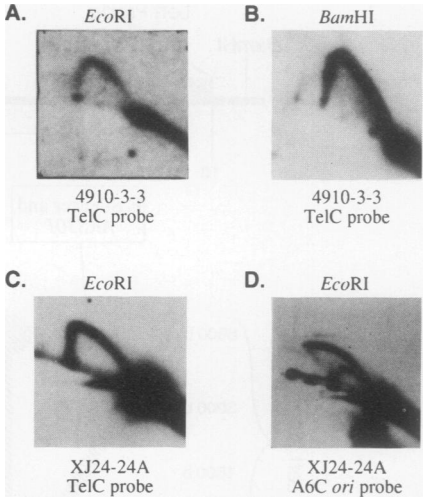

FIG. 4. The *HML*-containing *Eco*RI and *Bam*HI fragments replicate predominantly as simple Y's. DNA from growing 4910-3-3 (A and B) or XJ24-24A (C and D) cells was digested to completion with *Eco*RI or *Bam*HI, enriched for replicating fragments (A and B only), subjected to neutral/neutral 2D gel electrophoresis, and transferred to nylon (A and B) or nitrocellulose (C and D) membranes. The membranes were incubated under hybridization conditions with the right probe from Fig. 2 (A and B), the adjacent 0.7-kbp *Hin*dIII-*Xho*I fragment (C), or the 1.9-kbp *Bam*HI-*Pst*I fragment encompassing the A6C origin (D) (25). In panel D, both the large spot below the dense signal from the monomeric nonreplicating fragment and the small spot and streak just above and parallel to the faint line of linear molecules are spurious background and are nonreproducible.

consequence of damage suffered by the DNA during isolation. When these membranes were rehybridized with probes able to detect the known yeast chromosomal replication origin at *ARS305* (the A6C origin [25]), clear bubble arcs were obtained (strain 4910-3-3 [25]; strain XJ24-24A in Fig. 4D). In each case, fainter arcs, resembling those generated by simple Y's, were also obtained. Such faint Y-like arcs are consistently observed when the 5.8-kbp *Eco*RI fragment containing the A6C origin is analyzed by the neutral/neutral 2D technique and may be due to occasional nicks at replication forks which convert bubbles to Y-like structures (32). Because the autoradiogram in Fig. 4D was photographed at reduced (64%) magnification compared with the autoradiogram in Fig. 4C, the Y-like arc in Fig. 4D appears smaller than the simple Y arc in Fig. 4C. In the original autoradiograms, however, the two arcs are nearly superimposable, as would be expected since the two restriction fragments are about the same size (the Y-like arc does not rise quite as high as the simple Y arc).

Quantitations of weak bubble arc or nascent strand signals detected in previous investigations (19, 31) suggest that weak arcs representing as little as 5% of the total signal from replicating structures can be detected. Arcs containing about 15% of the total signal are routinely detected in our experiments. Therefore we conclude, conservatively, that if DNA replication were initiating in the investigated region in more than 15% of the cells in the experimental cultures, we would detect evidence of a bubble arc in Fig. 4B (if *ARS301* were active) or in Fig. 4A to C (if *ARS302* were active).

The evidence suggesting that *ARS303* is not an efficient origin is less complete because it is not yet known where *ARS303* is located within the *Hin*dIII-*Bam*HI fragment (Fig. 1 and 2). A bubble arc is usually difficult to detect unless the origin is located within the central portion of the fragment, i.e., more than about 20% of the fragment length from the end (32, 50). If *ARS303* is located between the *Hin*dIII and *Xho*I sites, then it is greater than 20% of the fragment length from the right end of the fragment and would be expected to produce a detectable bubble arc in Fig. 4A and C if active as an origin. No bubble arc is detected. Similarly, if there were a functional origin between the *Xho*I and *Bam*HI sites, examination of the *Hin*dIII fragment to the right of *HML* (Fig. 1) would be expected to reveal a bubble arc. However, when *Hin*dIII digests of DNA from strain XJ24-24A or 4910-3-3 were probed with the right probe shown in Fig. 2, only simple Y arcs were revealed (data not shown for XJ24-24A; Fig. 6, left panel, for 4910-3-3). Thus, these results confirm that neither *ARS301*, *ARS302*, nor *ARS303* functions detectably as a chromosomal replication origin.

The overall conclusion that replication forks move from right to left through the *HML* region and that there are no functional origins in this region is further confirmed by neutral/alkaline analyses of the TelB and TelC *Eco*RI fragments and of the J10A *Bam*HI fragment (the 10.9-kbp *Bam*HI fragment whose left terminus is the rightmost *Bam*HI site shown in Fig. 1 and 2; see references 40 and 40a for the location of this fragment relative to others on chromosome III) and by neutral/neutral analyses of the TelB and J10A fragments (10).

The *HML*-flanking *ARS* elements are inactive as origins even when the *HML* locus is transcribed. To test whether the inability of the *HML*-flanking *ARS* elements to function as chromosomal origins was related to repression of transcription from *HML* in the 4910-3-3 cell strain, two pairs of isogenic strains were constructed that differed from each other only in one of the *trans*-acting genes required for the repression of *HML* (the *SIR* genes; see the introduction). In both cases, the *SIR4* gene was disrupted by the insertion of

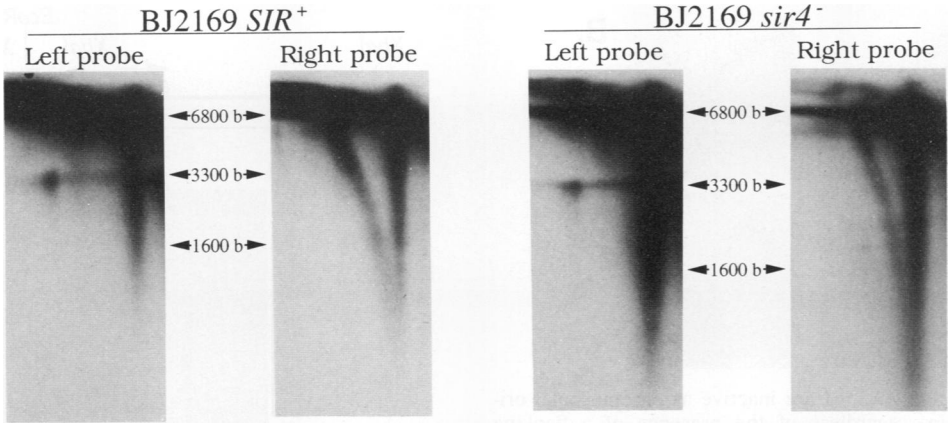

FIG. 5. The *HML*-containing *Bam*HI fragment is replicated from right to left whether or not transcription at *HML* is repressed. This experiment was carried out identically to that in Fig. 3, except that DNA was isolated from BJ2169 cells with repressed (*SIR*$^+$) or derepressed (*sir4*$^-$) *HML* loci. The left-probe autoradiograms were exposed for a much longer time than the right-probe autoradiograms to increase the probability of detecting signals from short nascent strands. Minor back-smearing in the first dimension in this experiment led to the presence of a horizontal line at 3,300 bases, detected by the left probe. This signal is due to back-smearing from the spot of nonreplicating DNA of a 3,300-base fragment that has partial sequence homology with the left probe.

a selectable marker (see Materials and Methods and genotypes in Table 1). That disruption of the *SIR4* gene led to the expression of *HML* was confirmed for both strains by mating tests and by Northern (RNA) analyses (data not shown).

*Bam*HI-digested DNA from these strains was analyzed by the neutral/alkaline technique as in Fig. 2 and 3. Identical results were obtained in one experiment with S150-2B and in two experiments with BJ2169. The autoradiograms from one of the BJ2169 experiments are shown in Fig. 5. These autoradiograms are not as clear as those of Fig. 3 because of minor artifactual back-smearing (trailing due to weak adsorption of the migrating DNA molecules to the agarose gel matrix) in the first dimension. This back-smearing produced an extended horizontal streak from a 3,300-base fragment detected by the left probe. Nevertheless, it is clear that, regardless of whether the *HML* locus is transcribed (Fig. 5, right panels) or not transcribed (left panels), only the right probe detects a diagonal signal due to nascent strands of all sizes (Fig. 5), just as observed with strain 4910-3-3 (Fig. 3). Thus, neither the *HML* E- nor I-associated *ARS* element functions as the sole replication origin in this region, even in cells actively transcribing the *HML* locus.

This conclusion was further tested and confirmed by neutral/alkaline analyses of the TelC *Eco*RI fragment and by neutral/neutral analyses of the *HML*-containing *Bam*HI fragment and of the TelC *Eco*RI fragment in these strains as well as in strain K45 (Table 1), which, due to a defect in the *SIR1* gene, also permits transcription from *HML* (most data not shown; data for strain K45 shown in Fig. 7B). As in Fig. 4A and C, the absence of detectable bubble arcs in the neutral/neutral *Eco*RI analyses suggested that *ARS303* is not functional in any of these strains if it is located in the left portion of its *Hin*dIII-*Bam*HI fragment.

To examine the possibility that *ARS303* is present and functional in the right portion of its *Hin*dIII-*Bam*HI fragment, neutral/neutral analyses of *Hin*dIII-digested BJ2169 and K45 DNA were carried out (Fig. 6, center and right panels, and Fig. 7D). Again, no hints of bubble arcs were detected. Thus, like *ARS301* and *ARS302*, *ARS303* is not detectably functional as a chromosomal replication origin even when transcription is permitted at *HML*.

Removal of *ARS301*, *ARS302*, and *ARS303* from near the telomere does not convert these *ARS* elements to active chromosomal origins. The three *HML*-flanking *ARS* elements shown to be not detectably active as chromosomal origins in the experiments described above are all located within 17 kbp of the left telomere of chromosome III, whereas the most telomere-proximal *ARS* element known to function as a chromosomal origin, *ARS305* (formerly the A6C *ARS* [25, 40, 40a]), is located about 40 kbp from the telomere. To test whether the inability of these *ARS* elements to function as chromosomal origins is due to their proximity to a telomere, we performed two experiments.

First, we used a strain carrying a ring derivative of chromosome III, resulting from an intrachromosomal recombination between *HML* and *HMR*. The ring chromosome lacks telomeres but does contain *ARS302* and *ARS303*. This strain, K191, was isolated from K45 by selecting for cells capable of mating and has been shown to carry a ring chromosome (30, 39). The recombination event restored the ability of the *sir1* parent to mate by deleting the *MAT***a** information at *HMR* and replacing it with a hybrid *HML-HMR* cassette carrying *MAT*α information. Figures 7A and C show that in K191, which lacks chromosome III telomeres

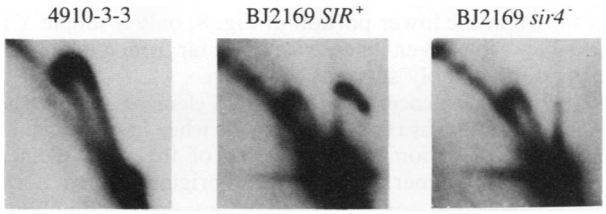

FIG. 6. The *ARS303*-containing *Hin*dIII fragment is replicated predominantly as a simple Y regardless of the transcriptional status of *HML*. DNA from growing 4910-3-3 or BJ2169 cells (*SIR*$^+$ or *sir4*$^-$) was digested to completion with *Hin*dIII, enriched for replicating fragments, subjected to neutral/neutral 2D gel electrophoresis, and transferred to nylon membranes. The membranes were incubated under hybridization conditions with the right probe from Fig. 2. The elongated spot above the dense signal from the nonreplicating monomeric fragment in the center panel is spurious background and is nonreproducible.

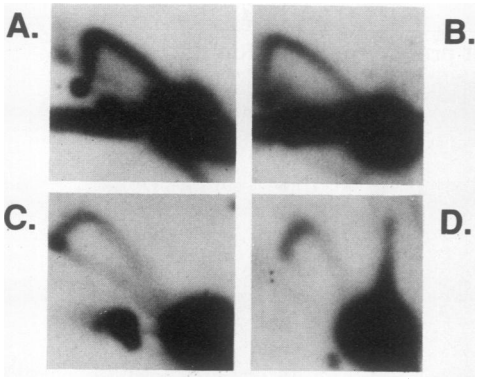

FIG. 7. *ARS302* and *ARS303* are inactive as chromosomal origins in a *sir1* strain, regardless of the presence of a flanking telomere. DNA from growing cells of strain K45 (B and D) or K191 (A and C) was digested to completion with *Eco*RI (A and B) or *Hin*dIII (C and D), subjected to neutral/neutral 2D gel electrophoresis (without prior enrichment for replicating DNA), and transferred to nitrocellulose membranes. The membranes were incubated under hybridization conditions with the right probe from Fig. 2. Subsequently, the membranes bearing *Eco*RI-digested DNA were rehybridized with the same probe used in Fig. 4D, and, as in Fig. 4D, strong bubble arcs were obtained (data not shown).

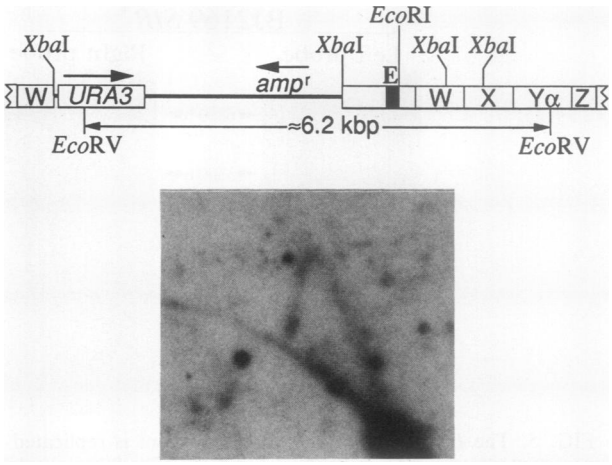

FIG. 8. The E-silencer-associated *ARS* element, *ARS301*, does not function as an origin even when transposed to the *MAT* region in the interior of chromosome III. The upper portion shows a map of the modified *MAT* region in strain Y903. As a consequence of the manipulations described in Materials and Methods, the *MAT* sequences to the left of W in Y903 have been rendered identical to the corresponding sequences at *HML* for an additional 0.6 kbp, a region that encompasses the entire E silencer. The thin line represents DNA derived from pUC19 (the orientation of β-lactamase transcription is indicated). Note that Y903 contains a second copy of the E silencer at its normal *HML*-flanking position. The lower portion of the figure shows the results of probing *Eco*RV-digested, replication-fork-enriched, neutral/neutral-2D-gel-electrophoresed Y903 DNA with pBR322 DNA. The pBR322 probe detects only the indicated E- and *MAT*-containing *Eco*RV fragment. The fragment containing the *HML*-flanking copy of the E silencer is not detected by this probe.

and expresses the hybrid *HML-HMR* locus, both the *Eco*RI fragment carrying the hybrid cassette and the *Hin*dIII fragment to its right are replicated as simple Y's. Therefore, neither *ARS302* nor *ARS303* is detectably active as a chromosomal replication origin even when removed from a telomere.

Second, to test whether the inability of *ARS301* to function as an origin is due to its proximity to a telomere, we took advantage of a yeast strain, Y903 (see Materials and Methods and Table 1), in which the E silencer with its associated *ARS301* had been moved to the left of the *MAT* locus in the interior of the chromosome. The translocated E silencer in Y903 was shown to effectively repress transcription from the *MAT* locus. A map of the modified *MAT* locus is provided in the top portion of Fig. 8. In this map, the thin horizontal line represents DNA from the plasmid pUC19, while the thicker boxes represent yeast-derived DNA. The E silencer and its associated *ARS* element are located 34% of fragment length from the right end of a 6.2-kbp *Eco*RV restriction fragment. If the *ARS* element is active as an origin in its new chromosomal location, then neutral/neutral analysis of *Eco*RV-digested replicating DNA should reveal a partial bubble arc. As shown in the lower portion of Fig. 8, only a simple Y arc is visible. Thus, even when *ARS301* is far from a telomere, it does not detectably serve as an origin.

The E- and I-silencer-associated *ARS* elements (*ARS301* and *ARS302*) function as replication origins when in plasmids. One possible explanation for the failure of the *ARS* elements studied in this paper to function as origins in their normal chromosomal environment is that they do not function as origins even when located in plasmids. *ARS* elements promote efficient replication of plasmids containing them, and, in theory, there are several ways to improve replication efficiency. For example, an *ARS* element might simply provide an entry site for proteins that would initiate replication at some other position on the plasmid.

To test this possibility, we have used both 2D gel electrophoretic techniques to map replication origins in the plasmids constructed earlier (48), consisting of the TelB and TelC *Eco*RI restriction fragments (Fig. 1) cloned into the yeast shuttle vector YIp5. Results obtained for the TelB clone (pTelB) are shown in Fig. 9. DNA from yeast cells containing pTelB was digested with *Eco*RI, which cuts pTelB into two linear fragments: the TelB fragment (Fig. 1) and the YIp5 vector. The *Eco*RI fragments were subjected to both neutral/alkaline and neutral/neutral 2D gel electrophoresis, blotted to membranes, and then probed with the short fragments indicated in Fig. 9. The neutral/alkaline gel electrophoretic data presented in the lower portion of Fig. 9 show that replication within both the TelB fragment and the YIp5 fragment proceeds from right to left, indicating that the origin responsible for replicating these fragments must be located near their right-hand junction (at or near *ARS301*). The neutral/neutral gel electrophoretic data in the upper portion of Fig. 9 show that both the TelB fragment and the YIp5 fragment are replicated predominantly as simple Y's, from one end to the other. However, the weaker, upturned, smeared signal at the far end (left end) of the YIp5 arc suggests (on the basis of similarity to results obtained by other laboratories [5, 6, 16]) that a second fork frequently enters this fragment after the first fork has nearly traversed it, leading to termination near the far end of the fragment. Termination near the left end of the YIp5 fragment is anticipated if bidirectional replication initiates at *ARS301* and if both forks move at the same rate.

Similar data (not shown) reveal that in the TelC plasmid (which contains both *ARS302* and *ARS303*), *ARS302* serves as origin most or all of the time. We have not yet tested a plasmid containing only *ARS303*, but the results obtained so far show that, at least for *ARS301* and *ARS302*, failure to

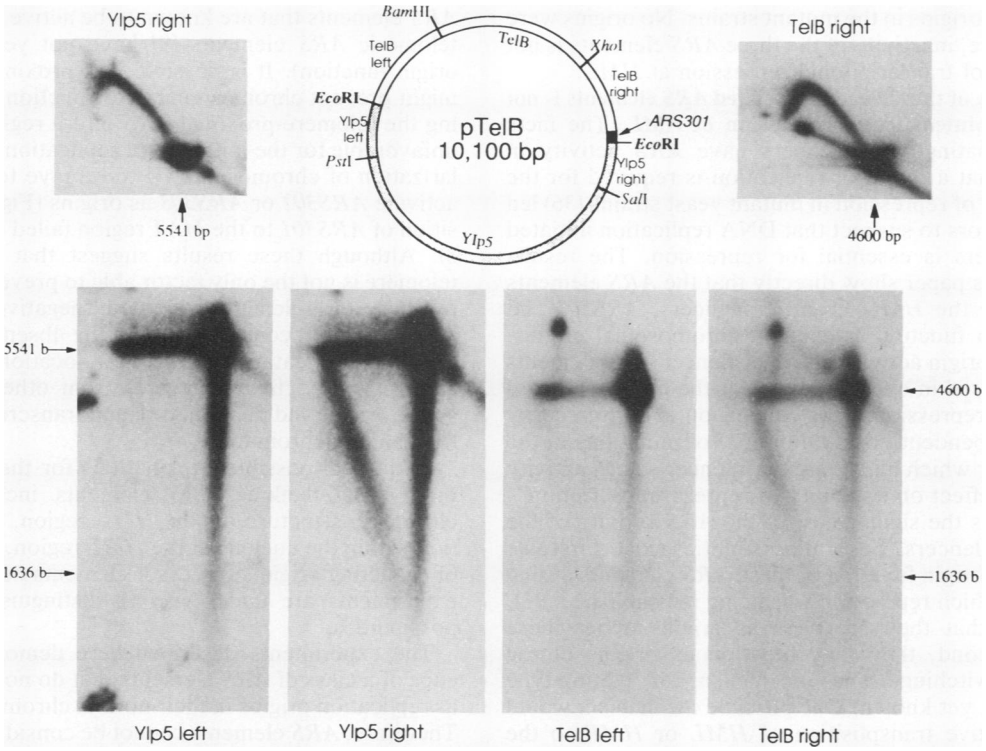

FIG. 9. *ARS301* functions as a replication origin when in a plasmid. The plasmid, pTelB, was constructed (48) by cloning the TelB *Eco*RI fragment from chromosome III, which contains *ARS301* (Fig. 1), into the *Eco*RI site of the vector YIp5 (pBR322 containing the yeast *URA3* gene [47]). pTelB was then introduced into BJ2169 yeast cells by selecting for uracil prototrophy. Southern blotting of undigested and *Apa*I-digested DNA from transformed cells confirmed that the introduced plasmid was not integrated into the chromosomal DNA of the transformed cells. Total DNA was isolated from the transformed yeast cells, digested with *Eco*RI, enriched for replication intermediates, subjected to 2D gel electrophoresis, blotted to a nylon membrane, and then hybridized with the probes indicated in the figure. Top left and top right: neutral/neutral electrophoresis; the membrane was hybridized first with the "YIp5 right" probe, which detects the YIp5 *Eco*RI fragment (top left), and then with the "TelB right" probe, which detects the TelB *Eco*RI fragment (top right). Bottom panels: neutral/alkaline electrophoresis; the membrane was hybridized sequentially with the indicated probes. The TelB right and YIp5 right probes detect short nascent strands, while the TelB left and YIp5 left probes detect only the longest nascent strands, suggesting that replication forks move from right to left through both restriction fragments and that the origin must be at or near their right-hand junction in pTelB.

function as an origin in plasmids is not the reason for failure to function as an origin in the chromosome.

DISCUSSION

Replication forks move from right to left through the *HML* region. The results described above show that the *HML*-containing 7 kbp of chromosome III diagrammed in Fig. 1 and 2 (the *HML* region) are replicated as a simple Y from right to left. Additional measurements (10) reveal that replication forks initiated at the A6C origin (25) move leftwards for 23 kbp to the right-hand boundary of the *HML* region and continue leftwards past the *HML* region to at least 2 kbp from the telomere, probably all the way to the telomere. Taken together, these results suggest that the A6C origin is responsible for replicating most or all of the leftmost 40 kbp of chromosome III.

These observations are consistent with replication timing studies which show that the centromere and other internal regions of yeast chromosome III replicate early whereas telomeres replicate late and that a gradient of replication timing exists between *ARS305* (formerly the A6C *ARS*), which replicates early, and the left telomere, which replicates late (42).

What is surprising about these results is the implication that the three *ARS* elements located in the *HML* region do not detectably function as chromosomal replication origins. Efficient origin activity of *ARS301* and *ARS302* is clearly ruled out by both neutral/alkaline (Fig. 3 and 5) and neutral/neutral (Fig. 4) analyses. Although both analyses employ 2D electrophoresis, the rationales of the two analyses are different and independent. Consequently, when the identical conclusion is suggested by the two analyses, that conclusion is likely to be correct. Although the precise location of *ARS303* is not known, our results strongly suggest that this *ARS* element, too, does not normally serve as a chromosomal origin. Neutral/alkaline analyses provide no evidence for rightward fork movement in any of the restriction fragments containing *ARS303* or within 23 kbp to the right of it (Fig. 3 and 5) (10), and neutral/neutral analyses of restriction fragments containing *ARS303* (Fig. 4, 6, and 7) show no evidence of bubble arcs.

We considered the possibility that the repression of potential origin function for the three *ARS* elements in the *HML* region might be related to the normal repression of transcriptional activity in that region. We tested strains with altered *SIR1* or *SIR4* genes (genes whose products are essential for the establishment and/or maintenance of transcriptional repression at *HML*), demonstrated that the mutations had abolished such repression, and then searched for

HML-proximal origins in the mutant strains. No origins were found. Therefore, inactivity of the three *ARS* elements is not a consequence of transcriptional repression at *HML*.

Origin activity of the silencer-associated *ARS* elements is not required for maintenance of repression of *HML*. The facts that all four mating-type silencers have *ARS* activity in plasmids and that a round of replication is required for the reestablishment of repression in mutant yeast strains (36) led many investigators to suspect that DNA replication initiated at these silencers is essential for repression. The results presented in this paper show directly that the *ARS* elements associated with the *HML* E and I silencers, *ARS301* and *ARS302*, do not function as efficient chromosomal origins. Therefore, the origin activity of these silencer *ARS* elements cannot be required in every S phase for the maintenance of transcriptional repression. This conclusion is reinforced by the recent independent observation (34) of mutations in the *HML* E silencer which inactivate the silencer's *ARS* activity but have little effect on its ability to repress transcription.

What, then, is the significance of the *ARS* activity of the *HML* E and I silencers? Several possibilities exist. First, we did not test the origin function of these *ARS* elements during an S phase in which repression was being reestablished (36). It is possible that they are used as origins under these conditions. Second, they may function as origins during mating-type switching. The mechanism of mating-type switching is not yet known. One possible mechanism would involve replicative transposition of *HML* or *HMR* to the *MAT* locus. If replication is a part of the mating-type switching mechanism, the silencer-associated *ARS* elements may serve as origins for such replication. Third, silencers may exhibit *ARS* activity in plasmids only coincidentally. This plasmid *ARS* activity may be completely irrelevant to the normal function of silencers in chromosomes.

Absence of correlation between transcription and replication at *HML*. Despite multiple examples of correlations between replication and transcription in other cases (see the introduction), this study provided no evidence for such a correlation at *HML*. Instead, the data presented in this paper and additional data (10) show that replication forks move through the chromosomal copy of *HML* from right to left regardless of whether *HML* is being transcribed or not. In addition, earlier studies (15) demonstrated that silencers are competent to repress transcription from *HML* even when they are present on plasmids and thus, presumably, are functioning as replication origins. It is interesting, however, that repression of *HML* in these earlier plasmid studies (15) was not as complete as it is when the *HML* locus is present in its normal chromosomal environment (33), raising the possibility that the origin function of the *HML* silencers may interfere somewhat with their ability to repress transcription.

Possible explanations for the inability of the *HML*-flanking *ARS* elements to function as chromosomal origins. We have considered several possible explanations for the surprising failure of the *HML*-flanking *ARS* elements to detectably function as origins in their normal chromosomal environment. For example, abundant transcription into the vicinity of an *ARS* element has been found to abolish its activity (44). However, Northern analyses reveal slight (51) or undetectable (14) transcription near *ARS301* and *ARS302*. Moderate transcription is detectable near *ARS303* (14, 51). Consequently, this potential explanation is excluded for *ARS301* and *ARS302* but not for *ARS303*.

One interesting feature common to all three inactive *ARS* elements is that they are closer to a telomere than are all *ARS* elements that are known to be active as origins (known telomeric *ARS* elements [9] have not yet been tested for origin function). It is possible that proximity to a telomere might prevent chromosomal *ARS* function, perhaps by forcing the telomere-proximal *ARS* into a region of the nucleus unfavorable for the initiation of replication. However, circularization of chromosome III to remove telomeres failed to activate *ARS302* or *ARS303* as origins (Fig. 7), and transposition of *ARS301* to the *MAT* region failed to activate it (Fig. 8). Although these results suggest that closeness to the telomere is not the only factor able to prevent origin function for these *ARS* elements, they are "negative" results and are not completely conclusive given the absence of understanding of other features of the new locations for these *ARS* elements, e.g., their distances from other active chromosomal origins and the structure and transcriptional activity of the flanking chromatin.

Additional possible explanations for the inactivity of the three *HML*-flanking *ARS* elements include unfavorable chromatin structure for the *HML* region, unfavorable position within the nucleus of the *HML* region, and the existence of *cis*-acting origin suppressor elements near *HML*. Further experiments are under way to distinguish between these possibilities.

The experiments described here demonstrate the existence of a class of *ARS* elements that do not detectably serve as replication origins in their normal chromosomal locations. Therefore, *ARS* elements cannot be considered equivalent to chromosomal replication origins; each *ARS* element must be tested for origin function in its normal chromosomal location. All chromosomal replication origins characterized in yeast strains to date colocalize, within experimental error, with *ARS* elements, but only a few examples of replication origins are known. It will be necessary to identify and characterize numerous additional yeast replication origins before one can have confidence in the validity of the generalization that all yeast origins are *ARS* elements.

ACKNOWLEDGMENTS

We are grateful to Leland Hartwell for yeast strain 4910-3-3, to Elizabeth Jones for strain BJ2169, and to Jeffrey Strathern for help with strain genealogy.

This work was supported by NSF grant DCB-8616046 and ACS grant MV506 to J.A.H., by NIH grant GM-35679 to C.S.N., and by NIH grant GM-34545 to J.B. D.D.D. was a Fogarty International Fellow of the NIH. S.A.G. and L.Y.O. were partially supported by stipends from the UMDNJ Foundation.

REFERENCES

1. **Abraham, J., K. A. Nasmyth, J. N. Strathern, A. J. S. Klar, and J. B. Hicks.** 1984. Regulation of mating-type information in yeast: negative control requiring sequences both 5′ and 3′ to the regulated region. J. Mol. Biol. **176**:307–331.
2. **Baker, T. A., and A. Kornberg.** 1988. Transcriptional activation of initiation of replication from the E. coli chromosomal origin: an RNA-DNA hybrid near *oriC*. Cell **55**:113–123.
3. **Brand, A. H., G. Micklem, and K. Nasmyth.** 1987. A yeast silencer contains sequences that can promote autonomous plasmid replication and transcriptional activation. Cell **51**:709–717.
4. **Brewer, B. J., and W. L. Fangman.** 1987. The localization of replication origins on ARS plasmids in S. cerevisiae. Cell **51**:463–471.
5. **Brewer, B. J., and W. L. Fangman.** 1988. A replication fork barrier at the 3′ end of yeast ribosomal RNA genes. Cell **55**:637–643.
6. **Brewer, B. J., E. P. Sena, and W. L. Fangman.** 1988. Analysis of replication intermediates by two-dimensional agarose gel electrophoresis, p. 229–234. *In* T. Kelly and B. Stillman (ed.),

Eukaryotic DNA replication. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
7. **Broach, J. R., Y. Li, J. Feldman, M. Jayaram, J. Abraham, K. A. Nasmyth, and J. B. Hicks.** 1982. Localization and sequence analysis of yeast origins of DNA replication. Cold Spring Harbor Symp. Quant. Biol. **47**:1165–1173.
8. **Campbell, J. L., and C. S. Newlon.** 1991. Chromosomal DNA replication, p. 41–146. *In* J. R. Broach, E. W. Jones, and J. R. Pringle (ed.), The molecular biology of the yeast Saccharomyces, vol. 1, Genome, dynamics, protein synthesis, and energetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **Chan, C. S. M., and B.-K. Tye.** 1983. Organization of DNA sequences and replication origins at yeast telomeres. Cell **33**: 563–573.
10. **Davis, L. R., D. D. Dubey, and J. A. Huberman.** Unpublished data.
11. **DePamphilis, M. L.** 1988. Transcriptional elements as components of eukaryotic origins of DNA replication. Cell **52**:635–638.
12. **Deshpande, A., and C. S. Newlon.** Unpublished data.
13. **Devenish, R. J., and C. S. Newlon.** 1982. Isolation and characterization of yeast chromosome III by a method applicable to other circular DNAs. Gene **18**:277–288.
14. **Dubey, D. D., and J. A. Huberman.** Unpublished data.
15. **Feldman, J. B., J. B. Hicks, and J. R. Broach.** 1984. Identification of sites required for repression of a silent mating type locus in yeast. J. Mol. Biol. **178**:815–834.
16. **Gahn, T. A., and C. L. Schildkraut.** 1989. The Epstein-Barr virus origin of plasmid replication, *oriP*, contains both the initiation and termination sites of DNA replication. Cell **58**:527–535.
17. **Goldman, M. A., G. P. Holmquist, M. C. Gray, L. A. Caston, and A. Nag.** 1984. Replication timing of genes and middle repetitive sequences. Science **224**:686–692.
18. **Gottschling, D. E., O. M. Aparicio, B. L. Billington, and V. A. Zakian.** 1990. Position effect at S. cerevisiae telomeres: reversible repression of Pol II transcription. Cell **63**:751–762.
19. **Greenfeder, S. A., and C. S. Newlon.** Unpublished data.
20. **Hartwell, L. H., R. K. Mortimer, J. Culotti, and M. Culotti.** 1973. Genetic control of the cell division cycle in yeast. V. Genetic analysis of *cdc* mutants. Genetics **74**:267–286.
21. **Hatton, K. S., V. Dhar, E. H. Brown, M. A. Iqbal, S. Stuart, V. T. Didamo, and C. L. Schildkraut.** 1988. Replication program of active and inactive multigene families in mammalian cells. Mol. Cell. Biol. **8**:2149–2158.
22. **Herskowitz, I.** 1989. A regulatory hierarchy for cell specialization in yeast. Nature (London) **342**:749–757.
23. **Hicks, J., J. N. Strathern, and A. J. S. Klar.** 1979. Transposable mating type genes in *Saccharomyces cerevisiae*. Nature (London) **282**:478–483.
24. **Huberman, J. A., L. D. Spotila, K. A. Nawotka, S. M. El-Assouli, and L. R. Davis.** 1987. The in vivo replication origin of the yeast 2μm plasmid. Cell **51**:473–481.
25. **Huberman, J. A., J. Zhu, L. R. Davis, and C. S. Newlon.** 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.
26. **Ivy, J. M., A. J. S. Klar, and J. B. Hicks.** 1986. Cloning and characterization of four *SIR* genes of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **6**:688–702.
27. **Jones, E. (Carnegie Mellon University).** 1991. Personal communication.
28. **Jones, E. W.** 1990. Vacuolar proteases in yeast *Saccharomyces cerevisiae*. Methods Enzymol. **185**:372–386.
29. **Kimmerly, W. J., and J. Rine.** 1987. Replication and segregation of plasmids containing *cis*-acting regulatory sites of silent mating-type genes in *Saccharomyces cerevisiae* are controlled by the *SIR* genes. Mol. Cell. Biol. **7**:4225–4237.
30. **Klar, A. J. S., J. N. Strathern, J. B. Hicks, and D. Prudente.** 1983. Efficient production of a ring derivative of chromosome III by the mating-type switching mechanism in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **3**:803–810.
31. **Linskens, M. H. K., and J. A. Huberman.** 1988. Organization of replication of ribosomal DNA in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8**:4927–4935.
32. **Linskens, M. H. K., and J. A. Huberman.** 1990. Ambiguities in results obtained with 2D gel replicon mapping techniques. Nucleic Acids Res. **18**:647–652.
33. **Mahoney, D. J., and J. R. Broach.** 1989. The *HML* mating-type cassette of *Saccharomyces cerevisiae* is regulated by two separate but functionally equivalent silencers. Mol. Cell. Biol. **9**:4621–4630.
34. **Mahoney, D. J., R. Marquardt, G.-J. Shei, A. B. Rose, and J. R. Broach.** 1991. Mutations in the *HML* E silencer of *Saccharomyces cerevisiae* yield metastable inheritance of transcriptional repression. Genes Dev. **5**:605–615.
35. **Marshall, M., D. Mahoney, A. Rose, J. B. Hicks, and J. R. Broach.** 1987. Functional domains of *SIR4*, a gene required for position effect regulation in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7**:4441–4452.
36. **Miller, A. M., and K. A. Nasmyth.** 1984. Role of DNA replication in the repression of silent mating type loci in yeast. Nature (London) **312**:247–251.
37. **Mortimer, R. K., and J. R. Johnston.** 1986. Genealogy of principal strains of the Yeast Genetic Stock Center. Genetics **113**:35–43.
38. **Nawotka, K. A., and J. A. Huberman.** 1988. Two-dimensional gel electrophoretic method for mapping DNA replicons. Mol. Cell. Biol. **8**:1408–1413.
39. **Newlon, C. S.** Unpublished data.
40. **Newlon, C. S., R. P. Green, K. J. Hardeman, K. E. Kim, L. R. Lipchitz, T. G. Palzkill, S. Synn, and S. T. Woody.** 1986. Structure and organization of yeast chromosome III, p. 211–223. *In* J. Hicks (ed.), Yeast cell biology. Alan R. Liss, Inc., New York.
40a. **Newlon, C. S., L. R. Lipchitz, I. Collins, A. Deshpande, R. J. Devenish, R. P. Green, H. L. Klein, T. G. Palzkill, R. Ren, S. Synn, and S. T. Woody.** Analysis of a circular derivative of *Saccharomyces cerevisiae* chromosome III: a physical map and identification and location of *ARS* elements. Genetics, in press.
41. **Potashkin, J. A., and J. A. Huberman.** 1986. Characterization of DNA sequences associated with residual nuclei of *Saccharomyces cerevisiae*. Exp. Cell Res. **165**:29–40.
42. **Reynolds, A. E., R. M. McCarroll, C. S. Newlon, and W. L. Fangman.** 1989. Time of replication of ARS elements along yeast chromosome III. Mol. Cell. Biol. **9**:4488–4494.
43. **Rine, J., and I. Herskowitz.** 1987. Four genes responsible for a position effect on expression from *HML* and *HMR* in *Saccharomyces cerevisiae*. Genetics **116**:9–22.
44. **Snyder, M., R. J. Sapolsky, and R. W. Davis.** 1988. Transcription interferes with elements important for chromosome maintenance in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8**:2184–2194.
45. **Strathern, J. (Frederick Cancer Research Facility).** 1991. Personal communication.
46. **Strathern, J. N., C. S. Newlon, I. Herskowitz, and J. B. Hicks.** 1979. Isolation of a circular derivative of yeast chromosome III: implications for the mechanism of mating type interconversion. Cell **18**:309–319.
47. **Struhl, K., D. T. Stinchcomb, S. Scherer, and R. W. Davis.** 1979. High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proc. Natl. Acad. Sci. USA **76**:1035–1039.
48. **Synn, S., and C. S. Newlon.** Unpublished data.
49. **Umek, R. M., M. H. K. Linskens, D. Kowalski, and J. A. Huberman.** 1989. New beginnings in studies of eukaryotic DNA replication origins. Biochim. Biophys. Acta **1007**:1–14.
50. **Yang, L., and M. Botchan.** 1990. Replication of bovine papillomavirus type 1 DNA initiates within an E2-responsive enhancer element. J. Virol. **64**:5903–5911.
51. **Yoshikawa, A., and K. Isono.** 1990. Chromosome III of *Saccharomyces cerevisiae*: an ordered clone bank, a detailed restriction map, and analysis of transcripts suggest the presence of 160 genes. Yeast **6**:383–401.
52. **Zhu, J., C. S. Newlon, and J. A. Huberman.** Unpublished data.