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Time of Replication of ARS Elements along Yeast Chromosome III

ANN E. REYNOLDS,[1]* ROBERT M. McCARROLL,[1]† CAROL S. NEWLON,[2] AND WALTON L. FANGMAN[1]

Department of Genetics, SK-50, University of Washington, Seattle, Washington 98195,[1] and Department of Microbiology and Molecular Genetics, UMDNJ-New Jersey Medical School, 185 South Orange Avenue, Newark, New Jersey 07103[2]

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The replication of putative replication origins (ARS elements) was examined for 200 kilobases of chromosome III of *Saccharomyces cerevisiae*. By using synchronous cultures and transfers from dense to light isotope medium, the temporal pattern of mitotic DNA replication of eight fragments that contain ARSs was determined. ARS elements near the telomeres replicated late in S phase, while internal ARS elements replicated in the first half of S phase. The results suggest that some ARS elements in the chromosome may be inactive as replication origins. The actively expressed mating type locus, *MAT*, replicated early in S phase, while the silent cassettes, *HML* and *HMR*, replicated late. Unexpectedly, chromosome III sequences were found to replicate late in G1 at the arrest induced by the temperature-sensitive *cdc7* allele.

Replication of eucaryotic chromosomes is achieved by initiation at multiple sites along the DNA. It was first observed over 30 years ago that different regions of a chromosome replicate at different times (33). Analysis of S phase in several organisms subsequently indicated that replication proceeds according to a temporal program. In *Saccharomyces cerevisiae* (11, 23), in *Physarum* spp. (3, 24), and in mammalian cells (for examples, see reference 16), specific DNA sequences have been shown to replicate at particular times in S phase. Cytological analyses indicated that a mammalian chromosome is divided into interspersed regions of early and late replication (10, 19), with a single region containing about 20 replication origins (19). The existence of these regions suggests that clusters of adjacent origins are coordinately activated, and indeed fiber autoradiography experiments have shown that clusters of at least three adjacent origins are activated at about the same time (14).

Yeast chromosomes, with an average size of about 850 kilobases (kb) (7), are considerably smaller than mammalian chromosomes. However, the spacing of origins on yeast chromosomes is similar to the spacing on mammalian chromosomes (reviewed in reference 26). While most pairs of adjacent origins are activated within a few minutes of each other (27, 30), the activation of a small fraction of adjacent origins is staggered by as much as half the length of S phase. These may represent origins located in adjacent early- and late-replicating regions of the same chromosome (30). An analysis of specific yeast chromosomal sequences has shown that centromeres replicate in the first half of S phase while telomeres replicate late (23). Therefore, both early- and late-replicating DNA can exist on a yeast chromosome, and there is likely to be at least one transition from early to late replication on each arm.

In this paper, we report an analysis of the time of replication along a 200-kb region of yeast chromosome III. This region, representing approximately 60% of the chromosome and extending from the telomere on the left arm to the mating type locus (*MAT*) on the right arm, was cloned and mapped, and the positions of ARS elements were determined (see Fig. 2a and reference 28). Since several of these ARS elements have recently been shown to function as replication origins in vivo (21; Greenfeder and Newlon, unpublished), temporal analysis of replication in this region can provide information about the control of the time of activation of a large cluster of adjacent origins.

* Corresponding author.

† Present address: Department of Molecular Genetics and Cell Biology, University of Chicago, 1103 E. 57th Street, Chicago, IL 60637.

MATERIALS AND METHODS

Cell synchronization and density transfer. Cultures of *S. cerevisiae* RM14-3A (*MATa cdc7-1 bar1 his6 trp1-289 ura3-52 leu2-3,112*) were synchronized and samples were processed as described before (23). For the experiments described in this paper, DNA from each time point was digested with the enzyme *Eco*RI.

DNA probes. Fragments used as probes in the quantitative hybridizations were isolated from *Bam*HI clones that were derived from the 200 kb of chromosome III extending from *MAT* to the left telomere (28). Each subfragment used as a probe detects a single *Eco*RI fragment in yeast genomic DNA. We have designated these *Eco*RI fragments with the same nomenclature used for the original *Bam*HI fragment (e.g., C1G fragment refers to the 8.6-kb *Eco*RI fragment found within the 23-kb C1G *Bam*HI fragment). The fragments were analyzed, and their sizes are listed in Table 1. Hybridization to Southern blots of *Bam*HI, *Eco*RI, and *Hin*dIII digests of genomic DNA were carried out to verify that each probe detected only the appropriate single-copy sequence. DNA was labeled with [α-^{32}P]dATP by random primer extension (12). Hybridization and quantitation were carried out as described before (23).

RESULTS

Replication times along chromosome III. The temporal pattern of replication of chromosome III sequences was determined by measuring the kinetics of replication of DNA fragments after transferring synchronous cultures from dense to light isotope medium (11, 23). Cultures of *MATa cdc7*(Ts) cells were grown in medium containing [^{13}C]glucose and [^{15}N]ammonia to uniformly label the DNA and then treated with α-factor to arrest the cells in G1. Cells were then removed from α-factor and dense medium by filtration and suspended in fresh medium containing [^{12}C]glucose and [^{14}N]ammonia at the restrictive temperature for *cdc7*. The medium also contained pronase to de-

TABLE 1. Timed fragments and ARS elements[a]

Fragment	Fragment length (kb)	Distance from left telomere[b] (kb)	ARS(s)	T_{REP} (min) Expt 1	T_{REP} (min) Expt 2
3LT	4.4	4.7	None	37	39
HML	5.3	14.2	HMLI, E5F4	33	30
J10A	3.8	20.2	None	27	23
A6C	5.0	40.0	A6C	18	17
C1G	8.7	77.3	C1G	18	14
C2G	3.4	100.6	C2G1	23	23
J11D	13.1	116.3	J11D	30	26
H9G	13.8	153.9	H9G	23	21
MAT	6.6	181.0	E5F3	25	24
HMR	5.6	~350[c]	HMRE, HMREI	36	37
ARS1-R	3.0	Unlinked (chromosome IV)	None	25	21
10Z	3.8	Unlinked (chromosome V)	None	38	38

[a] *Eco*RI fragments detected by quantitative hybridizations are listed. ARS1-R is the fragment adjacent to the 1.45-kb *Eco*RI fragment which contains ARS1.

[b] Distance from left telomere was measured from the chromosome terminus to the center of the listed restriction fragment.

[c] The distance from the left telomere to *HMR* was estimated from genetic and physical distances in the interval between *MAT* and *HMR*.

grade residual α-factor. Under these conditions, cells release from the α-factor block and arrest again at the *cdc7* block (G1/S boundary). A synchronous S phase was initiated by returning the culture to the permissive temperature, and cell samples were collected throughout the S phase. DNA was prepared from each sample, digested with *Eco*RI endonuclease, and centrifuged in CsCl gradients to separate the newly replicated, hybrid-density DNA from the unreplicated, fully dense DNA. The kinetics of appearance of particular DNA sequences in newly replicated DNA were examined by hybridizing fractions from each gradient with radiolabeled cloned DNA fragments. The fraction of cells that had replicated that sequence (F_{REP}) was calculated by using the formula $F_{REP} = 1/2(F_{HL})/(F_{HH} + 1/2F_{HL})$, where HH is fully dense unreplicated DNA and HL is replicated hybrid-density DNA.

We determined the time of replication of two fragments representing the most distal cloned fragments on each arm of chromosome III (3LT and *HMR*) and seven fragments that contain ARS elements which are located at 20- to 30-kb intervals in the contiguous 200-kb cloned region (see Fig. 2a). Fragment 3LT does not contain an ARS element, but it lies about 3.2 kb centromere-proximal from an X sequence, a class of moderately repetitive elements that contain ARS elements and are found adjacent to many telomeres (6, 8). The kinetics of replication of the left- and right-arm fragments from one experiment are shown in Fig. 1, along with standards representing early (ARS1-R, chromosome IV) and late (10Z, chromosome V) S-phase replication (23). The results show that sequences on chromosome III replicated at different times during S phase, with the earliest two internal fragments, A6C and C1G, replicating before ARS1-R and the telomere-adjacent fragments, *HMR* and 3LT, replicating at about the same time as 10Z.

The time of replication of a fragment can be expressed quantitatively as T_{REP}, the time after release from *cdc7* arrest at which half the cells have replicated that DNA (23). Since most chromosome III fragments undergo some replication during *cdc7* arrest (an unexpected result considered further below), we defined T_{REP} for chromosome III sequences as the time at which half the replication that follows release from the *cdc7* block has occurred. The T_{REP} values obtained for chromosome III fragments in two independent density transfer experiments are summarized in Table 1. The interval between the T_{REP} of the earliest-replicating fragment and the latest-replicating fragment in the two experiments was similar (20 and 25 min) and was close to the length of the interval previously reported from a survey of randomly selected fragments (23). The T_{REP} values for experiment 1 were generally later than those seen in experiment 2, but the relative times during S phase at which chromosome III sequences replicated in the two experiments were the same. The T_{REP} for all the fragments analyzed is shown in Fig. 2 relative to their chromosomal map positions. Most of the fragments, including the mating type locus, *MAT*, replicated relatively early, with T_{REP} values that clustered within the first half of the S phase. The fragments 3LT, *HML*, and *HMR*, which are located near the two telomeres, replicated late in S phase. The largest untimed region, between A6C and C1G, was about 37 kb.

The 8.6-kb *Eco*RI fragment C1G, which was not thought to contain an ARS element, is approximately equidistant from two known ARS elements, A6C and C2G1 (28). We predicted that this fragment would have a later T_{REP} than either the A6C or C2G fragments if the ARS elements in these two fragments were behaving as origins of replication. To our surprise, C1G replicated earlier than either of these fragments. This result caused us to reevaluate ARS activity within this region. It had previously been shown in two separate analyses that the 23-kb *Bam*HI C1G fragment, which includes the C1G *Eco*RI fragment we analyzed and the *HIS4* locus, had no ARS activity when cloned into vectors which would reach high copy number when an ARS element is added (18, 28). However, we subcloned the 8.6-kb *Eco*RI fragment (28) from the original 23-kb *Bam*HI fragment and found that it had ARS activity, based on the standard criteria of high-frequency transformation and maintenance of the DNA as an extrachromosomal element. The identification of ARS activity within this region provides an explanation for the early time of replication. One plausible explanation for the failure to detect ARS activity in the large *Bam*HI fragment is that the fragment contains a sequence that is deleterious at high copy number. Since the 8.6-kb *Eco*RI fragment had ARS activity in both low-copy centromere-containing vectors and high-copy YIp vectors (data not shown), the putative deleterious sequence must reside in some other part of the *Bam*HI fragment.

Previous analysis had indicated that the telomere-adjacent sequence 3LT replicates late in S phase (23). We were interested in extending the analysis of sequences adjacent to the left telomere to determine the size of the late-replicating region. We analyzed the replication of an additional DNA fragment, J10A, which is immediately centromere-proximal to *HML* (data from experiment 2 are shown in Fig. 3). The kinetics of replication for 3LT, *HML*, J10A, and A6C revealed a progressive transition through this chromosomal region to earlier times of replication (Fig. 1A and 3 and Table 1). It is clear from these data (summarized in Fig. 2b) that there is not a large region of contiguous chromosome III DNA that is replicating at a similar late time in S phase. Sequences in the region adjacent to the telomere may be replicated by the late activation of origins in this region or by a replication fork moving distal from the *ARS* A6C toward the telomere (see Discussion).

Chromosome III replicates during *cdc7* arrest. An unex-

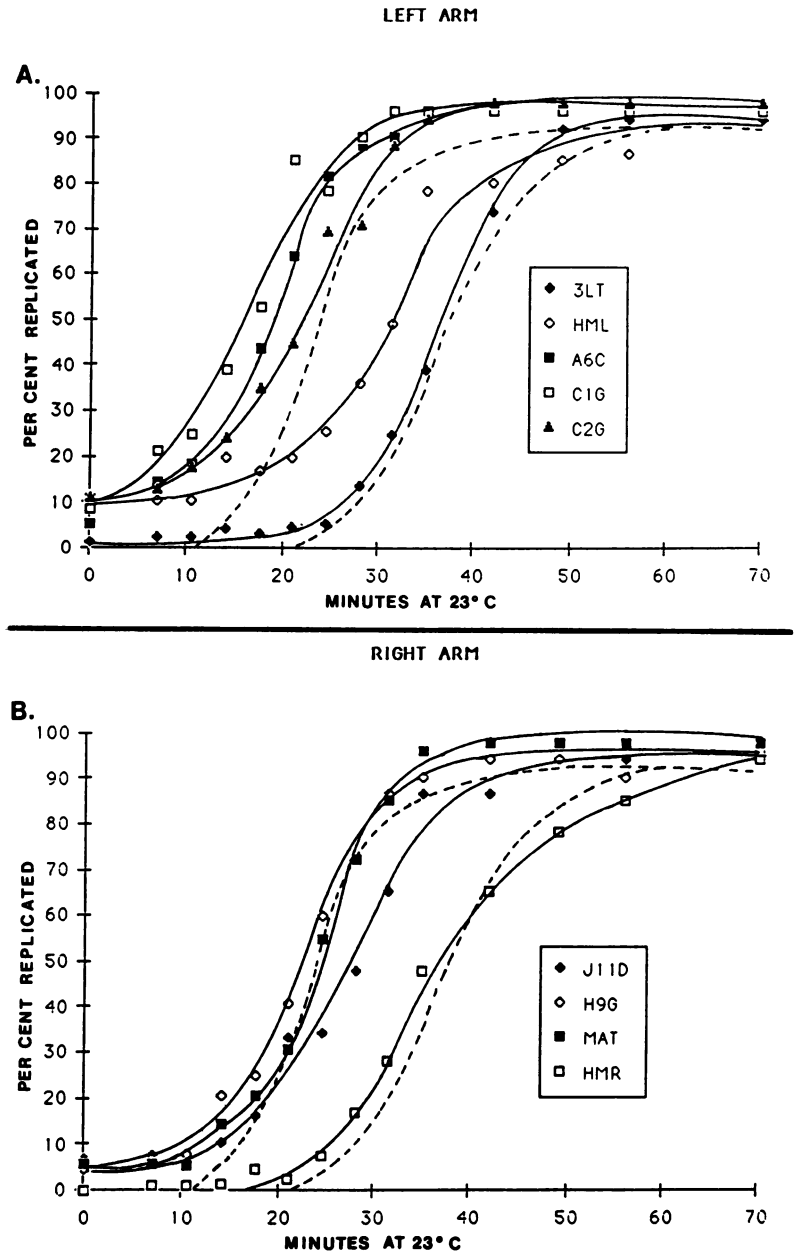

FIG. 1. Kinetics of replication of chromosome III fragments. The data are from experiment 1. The early- and late-replicating standards ARS1-R and 10Z (23) are indicated by dashed lines. (A) Fragments from the left arm. (B) Fragments from the right arm.

pected finding in these experiments was that for each of the chromosome III fragments except *HMR*, some replication had occurred during the G1 (*cdc7*) arrest (see 0-min values in Fig. 1 and 3). This "escape replication" was surprising because previous studies had indicated that both completion of the α-factor-sensitive step and the function of the *CDC7* gene product were required for the initiation of mitotic DNA synthesis (17). An intriguing aspect of escape replication is that it has so far been observed only for chromosome III. We have examined more than 30 genomic sequences located on 11 other chromosomes, including seven sequences on chromosome I and 13 sequences on chromosome V, and none of these exhibited escape replication (see reference 23 for examples). The extent of escape replication which had occurred by the time the cultures were shifted to 23°C varied in different experiments as well as with the fragment analyzed (Fig. 1 and 3). The chromosome III escape replication was specific to *cdc7*-induced arrest, since it did not occur when chromosome III replication was analyzed in cells arrested with α-factor alone (data not shown). In addition, the order of replication of chromosome III fragments appeared to be unaffected by escape replication during *cdc7* arrest, since, in cells synchronized only with α-factor, fragments J10A, *HML*, C1G, A6C, ARS1-R, and 10Z all replicated in the same order as in the α-factor, *cdc7* double-arrest synchronization experiments (unpublished observations).

The escape replication suggests that chromosome III replication might be independent of *CDC7* action. If so, then continued incubation of a culture at the nonpermissive temperature for the *cdc7* mutation should result in the

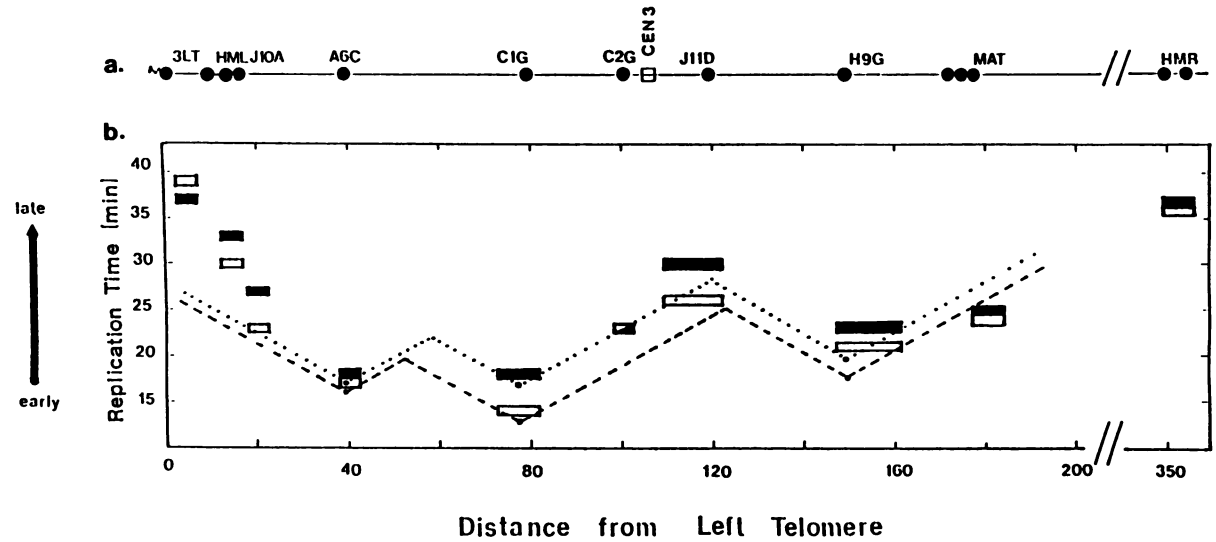

FIG. 2. (a) Map of chromosome III. Approximately 200 kb of cloned chromosome III DNA, from the left telomere to the *MAT* locus, is represented to scale. The distance from the left telomere to the *HMR* fragment was approximated from genetic and physical distance relationships in the interval between *MAT* and *HMR*. The fragments whose times of replication were determined in this work are indicated above the line. The designation of fragment names is described in Materials and Methods. Solid circles represent strong ARS elements. Two additional weak ARS elements, D10B and C2G2 (28), are not shown in this figure. Centromere, open box; left telomere, zigzag line. (b) T_{REP} values for experiments 1 and 2. The T_{REP} values are indicated by boxes which correspond in size to the length of the *Eco*RI fragment analyzed. Solid boxes, Experiment 1; open boxes, experiment 2. The broken lines represent the timing pattern expected if replication begins at only three ARS elements, ARSs A6C, C1G, and H9G, and proceeds at a fork rate of 3.6 kb/min. The time at which activation of an origin occurs (indicated as a small dot) was extrapolated from the location of the ARS within the fragment and the T_{REP} for the entire fragment. Dotted lines, Experiment 1; dashed lines, experiment 2.

replication of chromosome III fragments by the entire population of cells. To test this possibility, samples were taken 2, 3, and 4 h after the shift to the nonpermissive temperature (*cdc7* block). The 2-h sample represents the time at which a culture is normally shifted to 23°C to begin S phase. The fragments listed in Table 1 were analyzed; representative examples are shown in Fig. 4. All the chromosome III fragments, but not ARS1-R or 10Z, replicated to some extent at the restrictive temperature. Most of the fragments were replicated to an apparent maximum of 40 to 50% by 4 h. Since no fragment on chromosome III was replicated by all the cells in the population when held at the restrictive temperature, we conclude that chromosome III replication is not totally independent of *CDC7* function in the population.

DISCUSSION

This study provides the first systematic analysis of the time of replication along a large section of a chromosome in which several putative replication origins (ARS elements) have been mapped. The pattern of replication from the left telomere to *MAT* is that of a short, telomere-adjacent region of late replication and a central portion in which replication initiates during the first half of S phase. A fragment from near the right telomere, *HMR*, also replicates late, suggesting that a region of late replication exists at the right end of the chromosome. The centromere-adjacent fragments (C2G and J11D) replicate slightly later than surrounding fragments containing ARS elements, indicating that the centromere region is not the earliest-replicating region of the chromosome. The time during S phase at which the centromere region of chromosome III replicates is very similar to that found for centromeres of other chromosomes (23).

The region between A6C and *MAT* contains six ARS elements. The strength of ARS elements, as determined by the rate of loss of plasmids containing these sequences, varies greatly depending on the context of the ARS element (1, 26). It is difficult, therefore, to predict the behavior of the ARS elements in their chromosomal context from plasmid loss data. The kinetic data are compatible with the possibility that only three of these, ARSs A6C, C1G, and H9G, are active as origins. The time of replication of the ARS fragments C2G, J11D, and *MAT* can be accounted for by assuming bidirectional replication from ARSs A6C, C1G, and H9G at the replication fork rate of 3.6 kb/min (measured previously for these culture conditions [30]). The expected kinetics are illustrated by the lines in Fig. 2b. If the ARS elements C2G1 and J11D are active as origins, then either replication forks are slower in this region or a barrier to the movement of forks emanating from ARSs C1G and H9G must exist.

Alternatively, the initiation of replication at ARSs C2G1, J11D, and E5F3 (in the *MAT* fragment) may actually be heterogeneous in the population of cells. In some cells all ARSs may be activated early, and in other cells individual ARSs may fail to be activated and instead rely on replication from neighboring ARSs. The actual times of replication measured for each ARS would then reflect the weighted average of these two events. In support of this hypothesis, the replication intermediates observed on two-dimensional gels for ARSs A6C, C2G1, and J11D are a mixture of bubbles and simple Y's (J. Huberman, personal communication; Greenfeder and Newlon, unpublished). The simplest interpretation of these results is that in some cells these ARSs are active as origins (forming bubbles) and in other cells the sequences are passively replicated by replication forks initiating elsewhere (forming simple Y's). The proportion of bubble structures from C2G1 and J11D is low (Greenfeder

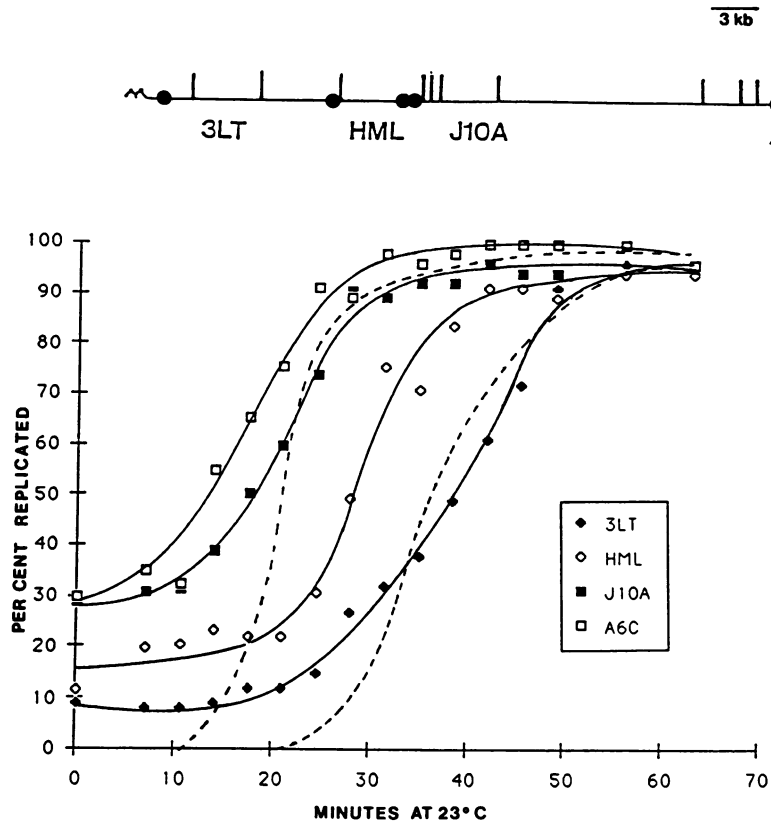

FIG. 3. Kinetics of replication of the terminal 40 kb of chromosome III left arm. A map of the region is shown above the graph. *Eco*RI sites are designated by vertical lines; ARS elements and left telomere are indicated as in Fig. 2. Kinetic curves were derived from the data of experiment 2. Early- (ARS1-R) and late- (10Z) replicating fragments are shown by dashed lines.

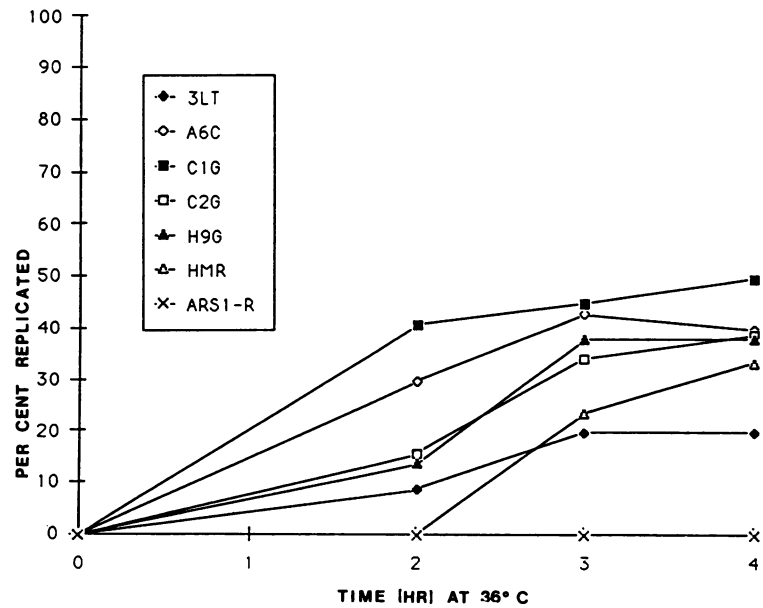

FIG. 4. Escape replication of chromosome III sequences at *cdc7* arrest. The data, indicating the amount of replication during the 4-h incubation at 36°C, were derived from experiment 2. The zero-time point represents the time at which α-factor-arrested cultures were filtered and suspended in isotopically normal medium at 36°C.

and Newlon, unpublished), suggesting that they are used less frequently as origins than A6C.

The transition in time of replication from early to late in S phase at the left end of chromosome III was of particular interest because no detailed information has been obtained for such a region in any organism. The simplest explanation of the data is that the timing transition in this case reflects the movement of a single replication fork from an early-replicating origin to the end of the chromosome. However, the replication of this region cannot be explained by the earlier-measured fork rate (3.6 kb/min [30]). The calculated fork rate (distance between the fragments divided by the difference between T_{REP} values) in the region from J10A to 3LT averaged less than 1.3 kb/min. A reduction in fork rate may be caused by specific DNA sequences, particular proteins bound to DNA, altered chromatin configuration, or highly active transcription units which impede the movement of the replication complex. An alternative explanation of the timing data for this region of chromosome III is the presence of one or more active origins in the late-replicating region that are activated near the time that a fork approaches from the interior region of the chromosome. ARS elements have been identified on both sides of the *HML* locus, and at least one of these ARSs (*HML* E) is involved in transcriptional repression of mating type information at the locus (13). We are assessing whether these ARSs are functional origins by using two-dimensional gel electrophoresis (4, 20).

Three of the ARS fragments examined contain the loci involved in mating type determination (*MAT*) and mating type switching (*HML* and *HMR*). It has been suggested that the *MAT* locus may replicate late in S phase, after a switch of mating type has been completed, to account for the observation that cells switch mating type in pairs (9). Our results show that *MAT* replicates in the first half of S phase. Since this strain is defective in the HO endonuclease, which introduces the double-strand break at the *MAT* locus, we cannot rule out the possibility that the cut made at *MAT* during a mating type switch might influence the time of replication of the *MAT* locus. The mating cassettes *HML* and *HMR*, which are transcriptionally repressed by factors acting in *trans* at sites located on both sides of the cassettes (2, 5, 32), replicate in the second half of S phase. Interestingly, many tissue-specific mammalian genes replicate late in S phase when they are inactive (16).

The observation that chromosome III replicates during the cell cycle arrest induced by a temperature-sensitive allele of CDC7 was surprising. DNA sequence analysis of the *CDC7* gene has indicated that the gene product has homology with protein kinases (31), and it has been suggested that the *CDC7* protein is part of a multiprotein replication complex (22). In radiolabeling experiments, *cdc7*(Ts) mutants behave like classic "slow-stop" mutants, apparently completing a round of DNA replication but not initiating a new one at the restrictive temperature (15). An electron microscopic analysis of DNA isolated from a *cdc7*(Ts) strain arrested at the restrictive temperature revealed that only 4% of the DNA molecules examined contained replication structures (29), consistent with the major defect being in the initiation of DNA replication. Our results are not at variance with these earlier observations since only chromosome III, which constitutes approximately 3% of the genome, appears to replicate at the restrictive temperature, and it replicates in only about one-half of the cells.

The unique escape replication observed for chromosome III would be explained if it contains a replication origin with an especially high affinity for an initiation factor(s) that may be available in a limited amount during *cdc7* arrest. This unusual origin might then successfully compete for such factors in a subset of cells, for example, in mother cells which retain more cytoplasm at cell division. Perhaps a unique origin is important for the process of mating type switching which occurs on chromosome III. Alternatively, the escape replication may be due to other features of chromosome III, such as its chromatin folding. It is possible that an interaction between *MAT* and *HML*, predicted to occur by the pattern of mating type switching in homothallic strains (reviewed in reference 25), perturbs the normal organization of chromosome III and somehow facilitates initiation at origins within the perturbed region. While the strain used in this work has a mutation in the HO gene and therefore does not switch mating type, synapsis between the *MAT***a** locus and *HML* during G1 may still occur and affect the activation of origins on the chromosome. Regardless of the mechanism of escape replication, it does not appear to alter the temporal pattern of chromosome III replication during the S phase following *cdc7* arrest. The pattern of replication seen in cells synchronized only with α-factor is the same as that observed in cells synchronized by treatment with α-factor and *cdc7* (unpublished observations).

Our analysis of chromosome III replication demonstrates that, as with mammalian chromosomes, different regions along a chromosome replicate at different times. The earliest-replicating sequences are centrally located, while the latest-replicating sequences are adjacent to the telomeres. We are currently testing the possibility that proximity to a telomere influences time of replication. Our data are consistent with the 200-kb region from *MAT* to the left telomere being replicated predominantly by initiation at three ARS elements early in S phase. These origins initiate replication at very similar times, suggesting coordinate control of activation. Further analysis will be necessary to determine whether ARS elements located in the later-replicating regions are nonfunctional or whether they initiate late in S phase.

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