Multiple determinants controlling activation of yeast replication origins late in S phase

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Analysis of a 131-kb segment of the left arm of yeast chromosome XIV beginning 157 kb from the telomere reveals four highly active origins of replication that initiate replication late in S phase. Previous work has shown that telomeres act as determinants for late origin activation. However, at least two of the chromosome XIV origins maintain their late activation time when located on large circular plasmids, indicating that late replication is independent of telomeres. Analysis of the replication time of plasmid derivatives containing varying amounts of chromosome XIV DNA show that a minimum of three chromosomal elements, distinct from each tested origin, contribute to late activation time. These late determinants are functionally equivalent, because duplication of one set of contributing sequences can compensate for the removal of another set. Furthermore, insertion of an origin that is normally early activated into this domain results in a shift to late activation, suggesting that the chromosome XIV origins are not unique in their ability to respond to the late determinants.

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The process of DNA replication is intricately controlled and integrated within the eukaryotic cell cycle. For example, the onset of DNA replication is delayed in response to damage of the DNA template (Siede et al. 1993), mitosis does not ensue until replication has been completed (Weinert and Hartwell 1988, 1989), and replication of the genome is restricted to a single round during each S phase (Blow and Laskey 1988). Additional controls are exerted over the time at which chromosomal regions initiate replication. This temporal pattern can be visualized in mammalian cells by incorporating BrdU into DNA at discrete times during S phase and examining the chromosomes at mitosis (e.g., Drouin et al. 1990). BrdU incorporation reveals interspersed early and late-replicating regions. The average DNA content of these regions is estimated at 2500–3000 kb, an amount sufficient to contain 10–50 origins of replication (Drouin et al. 1990). Because most neighboring origins have been found by DNA fiber autoradiography to initiate replication fairly synchronously (Hand 1975), the origins lo-

cated within a given temporal band are likely to initiate replication at similar times.

The replication time of a chromosomal region is not invariant, however. In a mammalian female, the inactive X chromosome replicates late in S phase, but the active homolog replicates early (Lyon 1972). Furthermore, sub-chromosomal regions show differences in replication time that correlate with gene activity (Hatton et al. 1988). For example, the human β -globin gene locus is transcriptionally silent and late replicating in nonerythroid cell types but early replicating when genes within the cluster are expressed (Dhar et al. 1988; Epner et al. 1988). Although it is clear that the temporal pattern of replication is flexible, the causal relationship between replication time and transcriptional activity is not understood. Essentially, no information is available about the mechanistic basis for variations in replication time along a mammalian chromosome.

Chromosomes of the yeast *Saccharomyces cerevisiae* have many advantages for studying the basis of temporal patterns of replication. Sequences that function as origins of replication have been identified by their ability to allow maintenance of plasmid DNA (Stinchcomb et al. 1979). Some (but not all) of these autonomous replication sequences (ARSs) have been shown, through the use of two-dimensional (2D) agarose gel electrophoresis techniques, to function as chromosomal origins of replication (Huberman et al. 1988; Ferguson et al. 1991b).

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Because replication initiates at defined origins in yeast and cells can be synchronized prior to S phase, the activation time of specific origins can be determined accurately (Ferguson et al. 1991b; Brewer et al. 1993).

Like mammalian chromosomes, yeast chromosomes exhibit a reproducible temporal pattern of replication. In general, yeast telomeres replicate late in S phase and centromeres replicate in the first half of S phase (McCarroll and Fangman 1988). Late replication at telomeres arises in at least two ways. First, DNA sequences can be passively replicated late in S phase by a replication fork from an early activated origin. The left telomere of chromosome III lies ~40 kb from the nearest active origin of replication, *ARS305* (Huberman et al. 1988; Dubey et al. 1991; Newlon et al. 1991). Although *ARS305* is activated early in S phase, slow replication fork progression through the subtelomeric region delays telomere replication until the second half of S phase (Reynolds et al. 1989). Second, late replication can arise through activation of an origin late in S phase. The right end of chromosome V contains an efficient origin (*ARS501*) located 31 kb from the telomere (Ferguson et al. 1991b). In this case, late replication of the adjacent telomere is achieved through late activation of *ARS501* and probably of an ARS in the subtelomeric Y' sequence (Ferguson et al. 1991b). In the case of *ARS501*, the presence of the telomere appears to be sufficient for late activation. Placement of *ARS501* on a circular plasmid changes its time of activation to early in S phase; subsequent linearization of the plasmid and addition of telomeres restore late activation (Ferguson and Fangman 1992). Thus, the telomere can cause late activation of nearby origins.

We are only beginning to explore the complexity of the replication program in yeast. The initiation times of origins along the left arm of the small chromosome III have been characterized (Reynolds et al. 1989). All of these origins are activated in the first half of the S phase. Little is known, however, about the temporal pattern of replication along the arms of larger chromosomes. We report here the discovery and characterization of a region on chromosome XIV, located 157 kb from the closest telomere, that contains four replication origins, all of which initiate replication at mid- to late S phase. The late activation time of at least two of these origins is maintained when the origins and flanking sequences are located on circular plasmids, indicating that late activation of these origins is independent of the telomere. Furthermore, multiple sequences contained in the DNA flanking two of these origins influence replication initiation time.

Results

Identification and characterization of a domain of late-activated origins on chromosome XIV

We discovered a 17-kb region of the left arm of chromosome XIV that contains an ARS (*ARS1412*) as indicated by its ability to support autonomous plasmid replication. Subsequent mapping localized the ARS within a 1.2-kb

*Pst*I fragment that lies 196 kb from the left telomere and 432 kb from the centromere of chromosome XIV. Replication time of *ARS1412* was determined by synchronizing cells at the G1/S-phase boundary after growth in dense isotopes of carbon and nitrogen and measuring the kinetics with which a 6.4-kb *Eco*RI restriction fragment containing the ARS changed in density after transfer to isotopically light media (McCarroll and Fangman 1988). The time at which half of the cycling cells in the population have replicated a specific restriction fragment is expressed as T_{rep} . The replication kinetics of three previously studied *Eco*RI restriction fragments were used as standards (Fig. 1): a fragment from chromosome III containing *ARS305*, one of the earliest replicating origins known (T_{rep} = 16.4 min); a fragment adjacent to *ARS1* on chromosome IV (T_{rep} = 19.2 min); and R11, a fragment near *ARS501* that is one of the latest replicating sequences known (T_{rep} = 32.4 min). The *Eco*RI fragment containing *ARS1412* replicated late in S phase with a T_{rep} of 28.8 min (Fig. 1B).

To determine the extent of the late replicating domain near *ARS1412*, we cloned 131 kb of surrounding chromosome XIV sequences (Fig. 2). This overlapping series

Figure 1. Time of replication of chromosome XIV ARSs. In the map at top, the relative chromosomal positions of *ARS1411*, *ARS1412*, *ARS1413*, and *ARS1414* are indicated by symbols that correspond to the symbols in the replication graphs below. These data are derived from a single culture. For simplicity, the data points for *ARS305*, *ARS1*, and R11 replication have been omitted. The replication curves for *ARS305* and R11 are shown as dotted lines, while the replication curve for *ARS1* is shown as a broken line. The time at which half of the cycling cells have replicated an ARS fragment (T_{rep} ; illustrated in B) is calculated for each chromosome XIV fragment. (A) Replication kinetics of a 5.0-kb *Eco*RI fragment that contains *ARS1411*. (B) Replication kinetics of a 6.4-kb *Eco*RI fragment that contains *ARS1412*. (C) Replication kinetics of a 5.2-kb *Eco*RI fragment that contains *ARS1413*. (D) Replication kinetics of a 6.2-kb *Eco*RI fragment that contains *ARS1414*.

Figure 2. Summary of chromosome walk on chromosome XIV. The wide bar represents 131 kb of chromosome XIV beginning 157 kb from the telomere and extending toward the centromere. *Eco*RI restriction sites are shown (||) above the bar; *Hind*III sites are below the bar. The first *Hind*III site (*) corresponds to position 159,841 bp, and the last *Eco*RI site (**) corresponds to position 288,209 bp of the chromosome XIV DNA sequence (*Saccharomyces* Genome Database). The location of the *KEX2* and *RAP1* genes are included for reference. The minimal sequence that has been shown to contain ARS activity is indicated for each ARS by a solid black line within the chromosome bar. Plasmid inserts isolated from the chromosome walk or by other techniques as described in Materials and methods are shown below the map. Plasmid inserts capable of providing high-efficiency transformation are indicated by a wide black bar over the ARS region. A triangle signifies that the indicated ARS fragment was deleted from the corresponding plasmid. All such deletions resulted in the loss of ARS activity.

of plasmid inserts represents 16.7% of the chromosome. The region begins 157 kb from the telomere and includes a number of known yeast genes such as *KEX2* and *RAP1* (Fig. 2). The map derived from the chromosome walk agrees well with the map predicted from the recently completed genome sequence (Fig. 2; *Saccharomyces* Genome Database).

The chromosome walk utilized libraries of yeast sequences cloned into a CEN vector lacking ARS sequences (YIp5-5; Ferguson et al. 1991b). Therefore, the inserts were tested directly for ARS activity by assaying for high-efficiency transformation in yeast (Stinchcomb

et al. 1979). Seven plasmids showed ARS activity (Fig. 3), and subcloning revealed four fragments of ≤ 2 kb with ARS activity (including *ARS1412*) in this 131-kb region. When each of these small fragments or a portion thereof was deleted from the corresponding plasmids, high-frequency yeast transformation was lost. Therefore, a minimum of four ARS elements appear to be located within this 131-kb region; the possible presence of more than one ARS within the ~ 2 -kb ARS fragments cannot be eliminated by the data. These ARSs have been named (from telomere to centromere) *ARS1411*, *ARS1412*, *ARS1413*, and *ARS1414*.

Figure 3. Reproducibility of replication times of *ARS305*, *ARS1*, and R11. T_{rep} s for the early chromosomal markers *ARS305* and *ARS1* and the late marker R11 were plotted from 14 separate experiments. The horizontal lines indicate the extent of the *ARS305*-R11 interval. Wide, vertical bars indicate T_{rep} s of *ARS1*. The replication index for *ARS1* corresponds to the fraction of the *ARS305*-R11 interval elapsed at the T_{rep} for *ARS1*. Mean and standard deviations of T_{rep} s and *ARS1* replication indices are given at the top.

Progress of the yeast genome sequencing project allows analysis of these potential origins at the sequence level. Yeast origins of replication require at least one match to an 11-bp ARS consensus sequence (ACS; Broach et al. 1983; Deshpande and Newlon 1992; Rivier and Rine 1992), the binding site for a complex of six proteins known as the origin recognition complex (Bell and Stillman 1992; Diffley and Cocker 1992). All four ARS regions contain matches at 10 of 11 positions of the ACS (Broach et al. 1983). The *ARS1411*, *ARS1412*, and *ARS1413* regions (Fig. 2, black bars) contain two of these 10 out of 11 matches each, and the *ARS1414* region contains one 10 out of 11 match and one perfect match to the consensus. All of the ARS regions contain multiple (8–14) 9 out of 11 matches to the ACS. Yeast origin activity requires an additional 100–150 bp of sequence, although a comparison of these sequences at different origins does not identify any additional consensus sequences. Rather, yeast origins are modular (Marahrens and Stillman 1994; Rao et al. 1994; Theis and Newlon 1994) and contain a number of important and essential elements including transcription factor binding sites (Walker et al. 1990; Marahrens and Stillman 1992; Newlon and Theis 1993) and easily unwound regions (Natale et al. 1992, 1993; Huang and Kowalski 1993). No common transcription factor binding sites [e.g., *ABF1*; Halfter et al. 1989] were found by computer search among the four ARSs. The helical stability (free energy difference between the duplex and single-strand states; kcal/mole) of each of the ARS regions was determined using a computer program (Natale et al. 1992). Each ARS element contains at least one easily unwound region near one or both ACS matches (data not shown). The minimal helical stability observed for each ARS ranges from 70 to 84 kcal/mole and encompasses the value observed previously for helical stability at *ARS305* (Huang and Kowalski 1993). Although the number of properties that can be compared at this level of analysis is small, these chromosome XIV ARSs do not appear to share sequence motifs that distinguish them from other well-characterized regions.

The replication times of *EcoRI* fragments containing each of the newly identified ARSs were determined by synchronous density transfer. As shown in Figure 1, A–D, all of the regions replicate late in S phase with T_{rep} s of 24.0–28.8 min. To determine the reliability of these measurements, the four ARSs were assayed in two independent timing experiments. The variation in T_{rep} s of the chromosome XIV origins was <2 min between experiments, and the order of origin activation times was nearly identical [*ARS1413* replicated slightly later than *ARS1412* in one experiment; data not shown]. To explore further the extent of variation between experiments, we assessed the variability of T_{rep} s among experiments by analyzing the three reference fragments in 14 independent cultures that differed in growth rate and plasmid content. The T_{rep} values for each of these three *EcoRI* fragments showed s.d.s of 1.2 min or less (Fig. 3). For comparisons among experiments, we express timing as a replication index. Here, we define the replication

index of *ARS305* as 0.0 and *R11* as 1.0. The time of replication of an intermediate fragment can then be expressed as a fraction of the *ARS305*–*R11* interval. Such a normalized replication index is quite reproducible: The replication indices for *ARS1* in the 14 experiments (also shown in Fig. 3) yield a mean value of 0.27 ± 0.05 . *ARS1411*, *ARS1412*, *ARS1413*, and *ARS1414* have replication indices of 0.66, 0.78, 0.75, and 0.48, respectively. Assuming a s.d. of ± 0.05 for all replication indices, we conclude that *ARS1411*, *ARS1412*, and *ARS1413* are all activated at approximately the same late time in S phase but *ARS1414* is activated earlier, at mid-S phase.

The regions containing each of the ARSs may function as late-activated chromosomal origins or may be passively replicated by forks traveling from distant origins. To distinguish between these possibilities, the efficiency of activation in the chromosome was determined by 2D agarose gel electrophoresis for each of the four ARSs (Fig. 4A–D). For an origin located asymmetrically in a restriction fragment, the intermediates that begin as bubble structures are converted to simple-Y forms as the replication fork reaches the end of the restriction fragment. These replicating structures can be distinguished by 2D agarose gel electrophoresis (Brewer and Fangman 1987; Friedman and Brewer 1995). Fragments containing each of the chromosome XIV ARSs display this discontinuous pattern (Fig. 4A–D). Very few Y-shaped intermediates containing short replicated branches were detected, suggesting that the origins are utilized in most or all cell cycles. Analysis of the direction of replication fork movement (Friedman and Brewer 1995) in sequences immediately adjacent to each origin confirmed that the origins are utilized in >85% of cell cycles (data not shown).

Late activation of ARS1412 and ARS1413 is telomere independent and not intrinsic to the ARS

Previous experiments have shown that proximity to the telomere can influence origin activation time (Ferguson and Fangman 1992). However, the group of chromosome XIV ARSs is located far from the telomere, suggesting that the late activation time of these origins might be telomere independent. To test this hypothesis, the replication times of large circular plasmids containing *ARS1412* (p12; 24.1 kb) and *ARS1413* (p13; 20.3 kb) were measured. (Fig. 5, top) The chromosome locations of the plasmid inserts are shown in Figure 2. Both plasmids replicated at mid to late S phase (Fig. 5A,B) with replication indices of 0.72 (p12) and 0.57 (p13), only slightly earlier than the chromosomal activation times of *ARS1412* (0.78) and *ARS1413* (0.75). The late replication of p12 and p13 is striking in comparison with the behavior of plasmid pR151 (22.8 kb) that contains *ARS501* and flanking sequences from chromosome V (Ferguson and Fangman 1992). Although *ARS501* replicates late in S phase in its native location on chromosome V, it replicates at the same time as *ARS1* (replication index of ~ 0.27) when located on the circular plasmid (Ferguson and Fangman 1992). On the basis of the results with p12

Figure 4. 2D gel analysis of chromosome XIV origins. The relative positions of *ARS1411*, *ARS1412*, *ARS1413*, and *ARS1414* are indicated on the large map. Small maps above each autoradiograph depict the restriction fragment probed in the 2D gel and the region of the fragment that contains the ARS [wide black bar]. (A) 2D gel of DNA digested with *NheI* and *PstI* and probed to detect a 3.8-kb *ARS1411* fragment. The spot below the end of the bubble arc is background hybridization and does not appear on other 2D gels of this origin. (B) 2D gel of DNA digested with *MscI* and probed to detect a 2.6-kb *ARS1412* fragment. (C) 2D gel of DNA digested with *XbaI* and probed to detect a 2.7-kb *ARS1413* fragment. (D) 2D gel of DNA digested with *EcoRV* and probed to detect a 4.0-kb *ARS1414* fragment. DNA analyzed in these 2D gels was harvested from several independent cultures of yeast strain RM14-3a.

and p13, we conclude that the late activation of *ARS1412* and *ARS1413* is independent of a functional telomere.

These results indicate that either origins *ARS1412* and *ARS1413* are inherently activated late or there are sequences flanking each of these origins that cause late activation. To distinguish between these two possibilities, we subcloned the ARSs from p12 and p13 to remove much of the adjacent chromosomal sequences. A 2.9-kb *EcoRI*–*BamHI* fragment containing *ARS1412* and a 1.46-kb *MluI*–*XmnI* fragment containing *ARS1413* were cloned into the *BamHI* site of YIp5-5 to create plasmids p12ARS and p13ARS, respectively (Fig. 5, top). These plasmids replicated very early in S phase (Fig. 5C,D), with replication indices of 0.03 and 0.0, respectively. We conclude that late activation is not an intrinsic property of *ARS1412* and *ARS1413* but rather is conferred by chromosomal sequences adjacent to these ARSs.

Many of the conclusions in this paper are dependent on comparisons of plasmid replication times between different cultures. As demonstrated above, the T_{rep} s and replication indices of chromosomal fragments are highly reproducible. To assess the reproducibility of replication times for extrachromosomal DNA, the replication index of a plasmid containing *ARS1413* (p13ΔRight; see below) was determined in two experiments on independently isolated yeast transformants. The variability in replication index of the plasmid in the two experiments (0.32 vs. 0.30) was comparable to that observed for chromosomal sequences (Fig. 3). Thus, the estimates of reproducibility for chromosomal sequences appear to be valid for plasmid sequences as well.

Mapping the sequences that contribute to late origin activation

We searched the flanks of *ARS1412* and *ARS1413* for sequences that might be responsible for late origin activation. Because a 450-bp tract of telomere sequences

($[C_{1-3}A]_n$) located ~8 kb from an ARS in a circular plasmid caused a slight delay in activation (Ferguson and Fangman 1992), sequences of the complete inserts of plasmids p12 and p13 were scanned by computer (see Materials and methods) for tracts of $[C_{1-3}A]_n$. The sequences near *ARS1* and from the insert of pR151 (~15 kb each) also were scanned, as early replicating controls. All possible 6-bp segments from a 118-bp cloned tract of chromosomal telomeric $[C_{1-3}A]_n$ sequences (Louis and Haber 1992) were used as a probe. The longest continuous matches found were 9 bp. No differences in the length or density of matches distinguished the inserts of the late-replicating plasmids from the early replicating controls.

To map the determinants of late origin activation, derivatives of plasmids p12 and p13 were created in which different sections of material adjacent to the ARSs were deleted and the effect on time of replication was determined (Fig. 6). p12ΔRight is deleted for an 8.3-kb *SfiI*–*BamHI* fragment on the centromere side of *ARS1412*. Removal of a 2.1-kb *KpnI*–*SfiI* fragment from the center of the insert generated plasmid p12ΔCenter, and deletion of a 5.5-kb *Sau3A*–*BamHI* fragment on the telomere side of *ARS1412* created p12ΔLeft. Surprisingly, none of the three flank deletions resulted in early origin activation (Fig. 6A). Plasmids p12ΔRight, p12ΔCenter, and p12ΔLeft had replication indices of 0.59, 0.46, and 0.53, respectively. The replication indices for these plasmids appear to be different from that of p12 (replication index of 0.72). Furthermore, the p12ΔCenter deletion may have a larger effect on activation time than the p12ΔRight deletion. The simple interpretation of these results is that each of the segments remaining in the deletion plasmids makes a contribution to late activation of the *ARS1412* origin. The number of elements required by the data could be as few as two if cleavage disrupts an element at the *SfiI* site used to create p12ΔRight and p12ΔCenter. However, because these two plasmids appear to have different replication indices, the latter in-

Figure 5. Plasmids and time of replication assays. (Top) Plasmids are drawn to scale. Thin black lines represent vector sequences; open lines represent chromosome XIV inserts. Thick hatched lines represent a 1.1-kb *EcoRI* fragment containing *CEN5*. Thick, stippled lines indicate ARS fragments found within the inserts. (Bottom) Time of replication assays for four plasmids are shown. Plasmid names and replication indices (R.I.) are indicated. Data for the plasmids are indicated in the graphs by solid lines and filled circles. Control, chromosomal markers are indicated by broken lines; earliest to latest: *ARS305*, *ARS1*, *R11*. The density shift experiments shown here were obtained from different cultures than those shown in Fig. 1. (A) Replication kinetics of p12 containing a 17.4-kb insert with *ARS1412*; $T_{rep} = 28.3$ min. (B) Replication kinetics of p13 containing a 13.6-kb insert with *ARS1413*; $T_{rep} = 25.7$ min. (C) Replication kinetics of p12ARS containing the 2.9-kb ARS fragment from p12; $T_{rep} = 20.2$ min. (D) Replication kinetics of p13ARS containing the 1.4-kb ARS fragment from p13; $T_{rep} = 16.0$ min.

interpretation seems unlikely. Thus, elements affecting the time of origin activation may be present in three (or more) different regions of the p12 insert.

Similar experiments were performed with the sequences adjacent to *ARS1413*. Beginning with plasmid p13, three deletion plasmids were constructed. Each deletion removes flanking regions but leaves the 1.46-kb *XmnI*–*MluI* *ARS1413* region intact. p13 Δ Left is a 6.2-kb deletion of the telomere-flanking region; p13 Δ Right is a 5.9-kb deletion of the centromere-flanking region (Fig. 6B). Both p13 Δ Left and p13 Δ Right (with replication indices of 0.19 and 0.32, respectively) replicate considerably earlier than intact p13 (Fig. 6B). Although the shift to earlier replication is especially pronounced with p13 Δ Left, neither deletion plasmid replicated as early as p13ARS. The sequences that influence *ARS1413* activa-

tion time appear to behave in an additive manner. The replication indices of p13 Δ Left and p13 Δ Right sum to a value (0.51) that is very similar to the replication index of the original plasmid (p13; 0.57). A third deletion plasmid (p13 Δ Left1/2) was constructed that contains the centromere-flanking region and ~ 2.5 kb of the telomere-flanking region immediately adjacent to the ARS. This plasmid has a replication index of 0.43 (Fig. 6B), intermediate between that of p13 Δ Left (0.19) and p13 (0.57). Thus, the telomere-flanking region may contain at least two separable sequences that contribute to the activation time of *ARS1413*.

p13 ($T_{rep} = 25.7$ min) replicates several minutes earlier than the corresponding chromosomal origin ($T_{rep} = 28.4$ min), suggesting that some sequences that contribute to late replication may have been excluded from the plasmid. As a preliminary test of this possibility, we determined the replication time of plasmid p113-6b, which was isolated from the chromosome walk (see Fig. 2). This plasmid contains the same telomere-proximal junction as p13 Δ Left1/2, but contains 13.5 kb of DNA to the centromere side of *ARS1413*. Therefore, any difference in the replication index between p113-6b and p13 Δ Left1/2 should be attributable to the additional 7.5 kb of centromere-flanking sequence present on the insert of p113-6b (see Fig. 6B, map). p113-6b has a replication index of 0.57 (Fig. 6B), identical to that of p13, and somewhat later than that of p13 Δ Left1/2 (0.43; Fig. 6B). These results suggest that sequences capable of influencing activation time of *ARS1413* lie >6 kb from the origin. These additional, distal sequences may contribute to *ARS1413* activation time in the chromosome and thus may help account for the observation that plasmid p13 replicates earlier than the chromosomal *ARS1413*.

The possibility that sequences in the two flanks of *ARS1413* in plasmid p13 might have some functional equivalency was tested by duplicating the right flank present in p13 Δ Left. The new plasmid, p13RightDup, contains the *XmnI*–*MluI* *ARS1413* fragment flanked on one side by the normal centromere-proximal sequences and on the other side by a mirror-image duplication of this region (Fig. 6B). p13RightDup replicated even later than the original p13 plasmid, with a replication index of 0.76 (Fig. 6B). This result indicates that the right flank can replace the left flank in delaying origin activation. It also suggests that two sets of right flank sequences can interact synergistically with each other, because a single right flank (on p13 Δ Left) gives a replication index of 0.19 but two right flanks provide the fourfold greater replication index of 0.76.

The deletion derivatives of plasmid p13 appear to show a correlation between increasing plasmid size and later replication in S phase. Although the deletion derivatives of p12 with *ARS1412* clearly do not show this correlation, we tested the possibility that time of activation of the *ARS1413* origin might simply be related to plasmid size. *ARS1413* was placed on a plasmid containing a large insert from chromosome V. pR151 (22.8 kb) contains *ARS501* and was previously shown to replicate relatively early in S phase, at the same time as *ARS1*

effects of the telomere on the activation time of the HO ARS (Raghuraman et al. 1994) and the 2 μ plasmid ARS (Wellinger et al. 1993) have been observed in small linear plasmids.

Late replication is not ARS specific

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Figure 7. Replication kinetics of an ectopic copy of *ARS1* in a strain lacking *ARS1413* activity. (Top) Restriction map of *EcoRI* sites (▲) in the region surrounding *ARS1413*. *ARS1* is inserted ~10 kb telomere proximal to *ARS1413*. *ARS1413* contains a mutation in the essential ACS as described in Materials and methods. The scale is indicated. (Bottom) Replication kinetics of an ~12.5-kb fragment containing *ARS1* (●). The probe utilized does not detect *ARS1* in its native location. The data points for *ARS305*, *ARS1*, and *R11* replication have been omitted; the replication curves for *ARS305* and *R11* are shown as short broken lines, whereas the replication curve for *ARS1* (on chromosome IV) is shown as a long broken line. The probe utilized does not hybridize to the ectopic copy of *ARS1* on chromosome XIV.

tion index of 0.66 [Fig. 7]. This initiation time is significantly later than the time of initiation of the endogenous copy of *ARS1* on chromosome IV in the same cells [replication index of 0.27; Fig. 7]. We conclude that the initiation time of *ARS1* is affected by the late determinants on chromosome XIV. Furthermore, this result suggests that the late determinants may be widely spaced throughout this domain of chromosome XIV.

Discussion

The large size of the early and late-replicating bands in mammalian chromosomes suggests that multiple origins must be located within these regions and, consequently, that groups of adjacent origins must be activated coordinately at different times during S phase (Hand 1978). We have shown here that a 131-kb region of yeast chromosome XIV replicates at mid- to late S phase. In contrast to the large mammalian chromosome bands, a region this small could be replicated by a single fork traversing it during the period of S phase. However, our 2D gel results demonstrate that there are four origins of replication located within this domain and that all four origins are used in all (or nearly all) cell cycles. Because these origins are extremely efficient, the late time of

replication determined for restriction fragments containing the origins reflects their late activation time.

Although the telomere can determine late activation time of origins, we considered it unlikely that the telomere causes late replication of the chromosome XIV origins because the distance over which the telomere can influence replication time is limited (Ferguson and Fangman 1992). Sequences located >60 kb from the right telomere of chromosome V normally replicate early in S phase. However, a chromosomal truncation that places the telomere ~30 kb from these early replicating sequences results in a shift to late replication (Ferguson and Fangman 1992). Thus, on chromosome V, the effect of the telomere on replication time can extend 30 kb but not more than 60 kb. However, it is clear that the relationship between replication time and the distance of an origin from the telomere is not simple. *ARS305*, which is located only 40 kb from the nearest telomere, is one of the earliest replicating origins known (Reynolds et al. 1989).

To test the telomere dependence of *ARS1412* and *ARS1413* late replication, the activation times of these origins were determined on circular plasmids. Large genomic fragments containing either *ARS1412* or *ARS1413* replicated rather late in S phase in this context, only several minutes earlier than the chromosomal origins. In contrast, *ARS501*, which is a telomere-adjacent, late-activated chromosomal origin (Ferguson and Fangman 1992), is activated significantly earlier on a plasmid than *ARS1412* or *ARS1413*. These results confirm our suspicion that late replication of *ARS1412* and *ARS1413* is telomere independent and suggest that a second type of late determinant is contained in the insert sequences of p12 and p13. Analysis of the replication time of plasmids containing minimal origin fragments shows that the late determinants are located in sequences that flank the two origins.

If the determinant that delays origin activation consists of repetitive sequence elements or if *ARS1412* and *ARS1413* flanks contain the same determining sequences, these sequences might be detected by computer analysis. Many matches could be found by searching, for example, for exact 11-bp matches [see Materials and methods]. However, no matches could be demonstrated within or between the inserts of the late replicating plasmids that were not found at equivalent frequency in sequences known to be permissive for earlier activation of origins. In addition, no patterns of sequence composition (such as GC content or telomeric $[C_{1-3}A]_n$) could be found that clearly distinguish the p12 and p13 inserts from early replicating sequences.

The results with derivatives of plasmid p12 suggest that a minimum of three separate elements contribute to the late activation of *ARS1412*. Based on the end points of the deletion constructs, these elements must be dispersed over a distance of at least 3.6 kb. Our data do not eliminate the possibility that many more than three elements are involved. The slightly different effects of the p12ΔRight and p12ΔCenter deletions (Fig. 6A) would be consistent with the possibility that different deleted re-

regions contain different numbers of contributing elements. For example, a high density of such elements in the small 2.1-kb *KpnI-SfiI* fragment might explain why the p12 Δ Center deletion results in the largest shift to an earlier replication time and why the effect of the large p12 Δ Right deletion is more modest.

ARS1413 initiation time is also affected by multiple elements contained in the flanking regions. Deletion of either 6-kb flanking region of p13 results in a shift to an intermediate replication time, suggesting that additive late determinants reside on both sides of *ARS1413*. Furthermore, plasmid p13 Δ Left1/2 has a replication index (0.43) that is intermediate between that of p13 Δ Left (0.19) and intact p13 (0.57), suggesting that the telomere flanking 6 kb might be comprised of at least two separable late determinants. Finally, the fact that p113-6b replicates later than p13 Δ Left1/2 suggests that at least one additional determinant lies centromere proximal to the p13 insert. Thus, as many as four (or more) elements may contribute to late replication of *ARS1413*.

The late elements on chromosome XIV can affect an origin at a distance. This phenomenon can be seen by comparing the replication indices of plasmids p13 Δ Left1/2 and p113-6b. The insert in p13 Δ Left1/2 contains 6 kb of sequences centromere-proximal to *ARS1413* and ~2.5 kb of telomere-proximal sequences. This plasmid has a replication index of 0.43. Plasmid p113-6b replicates somewhat later, with a replication index of 0.57, suggesting that the insert of this plasmid contains additional late determinants. The insert of p113-6b contains all insert sequences from p13 Δ Left1/2 but extends ~7.5 kb farther toward the centromere. Therefore, late determinants within the additional 7.5 kb appear to influence *ARS1413* replication time, despite the fact that these sequences lie >6 kb from the ARS. Finally, orientation with respect to the ARS element itself may also influence the strength of a late determinant. Orientation might explain the dramatic delay of *ARS1413* activation by the mirror-image duplication of the right flank; a late element in the duplicated flank would be inverted compared with its orientation in the original insert. Additional plasmid constructs are being made to test these possibilities.

The mechanism for initiation delay is unknown. In one simple model, multiple, dispersed elements interact with the origin, making independent contributions to its late activation (Fig. 8). Removal of one or more of the elements results in a shift to an earlier activation time. As diagrammed, these late activation elements may represent binding sites for proteins that interact directly with replication origins and impede a step in initiation. Alternatively, they may function by nucleating a specific chromatin structure that is inhospitable for early initiation, a mechanism that has been proposed for the telomere's effect on the time of activation of nearby origins (Ferguson et al. 1991a) and on transcriptional silencing (Gottschling et al. 1990). Another possibility is that the late elements target chromosome regions to a specific subcompartment of the nucleus where the origins are acted upon late in S phase. This subcompartment could

Figure 8. Model of the interaction of late activation determinants with an origin. The top diagram shows three elements in sequences flanking the origin. (Thick solid bar) Origin; (open box) late element. Each element can contribute to the delay in time of activation of the origin during S phase. The remaining diagrams show the origin with fewer late determinants in the flanks. The fewer the number of determinants, the earlier the origin is activated. These diagrams may represent the plasmid constructs described in this paper or replication origins in different regions of the genome that become active at different times.

be the nuclear periphery, where late-replicating yeast telomeres are located (Klein et al. 1992).

The mechanism of late origin activation appears to be independent of the origin itself. *ARS1412* and *ARS1413* revert to extremely early activation times when located on plasmids lacking flanking chromosome XIV sequences. Insertion of *ARS1* at an ectopic location on chromosome XIV results in a shift of its activation time to late in S phase. These results suggest not only that late activation is independent of the origin itself, but that the chromosome XIV origins are not unique in their ability to respond to the late determinants.

Further analysis of the *ARS1412* and *ARS1413* regions will determine if there is a small number of elements that contribute to activation of these origins late in the S phase or if there are many contributing sequence motifs distributed throughout this chromosome region. Whatever the outcome, it may be possible to use cloned late elements to assess the functional significance of temporal regulation of chromosome replication. For example, late elements and responding origins can be substituted for early activated origins in different regions of the yeast genome. It will be of particular interest to test whether the early replication of a centromere is required for the construction of a chromosome that is capable of proper segregation (Ferguson et al. 1991a) and if gene expression in early replicating regions of the genome is altered by the late activation of nearby origins. An understanding of the function of late elements in yeast may provide a model system for investigating how origins in the late-replicating bands of human chromosomes are controlled.

Materials and methods

Chromosome walk

Two libraries were utilized in the chromosome walk. Construction of a library containing partial *Sau3AI* fragments has been described [Ferguson et al. 1991b]. A second library was constructed with fragments resulting from a complete *Bam*HI digest of yeast DNA [Ferguson 1990]. Both libraries were made with the vector YIp5-5 [Ferguson et al. 1991] that contains *URA3* and *CEN5* but lacks an ARS sequence. It should be noted that this work predated the release of the completed yeast genome sequence.

The chromosome walk was conducted using probes from plasmid inserts and from overlapping λ clones of chromosome XIV [Olson et al. 1986] obtained from the American Type Culture Collection. DNA fragments from plasmids to be used as probes were separated by gel electrophoresis and eluted from the agarose using an IBI Unidirectional Electrocluter. The λ DNA was isolated as described [Helms et al. 1985] and used as a probe without digestion. DNA was labeled by the hexanucleotide priming method [Feinberg and Vogelstein 1983]. Colonies containing the desired plasmids were identified by colony hybridization using circular Hybond-N filters [Amersham]. Briefly, filters were placed on plates for 5 min, the inverted filters were placed on Whatman paper soaked in 0.5 M NaOH, 1 M NaCl, for 7 min, transferred twice to Whatman paper soaked in 0.5 M Tris, 3 M NaCl for 3 min each, and washed briefly in 2 $\times$ SCP [Brewer and Fangman 1987], UV cross-linked in a UV Stratalinker 1800 (Stratagene), and hybridized as described previously [Brewer and Fangman 1987].

Overlap of plasmid inserts from the chromosome walk was determined by restriction enzyme mapping. Plasmid inserts were mapped by partial digestion and indirect end labeling. The *Eco*RI-*Hind*III restriction map was confirmed through single and double digestion of the plasmids. Multiple probes throughout the chromosome walk were hybridized to pulsed-field gel-separated chromosome DNAs [Carle and Olson 1985] to confirm that all probes derived from chromosome XIV.

Two sections of the chromosome walk could not be isolated from the libraries in use. One gap was covered by plasmid YIp5-RAP1 (Fig. 2) in the following manner: Fragments from the centromere-proximal end of p124-10 and the telomere-proximal end of p13 were used as probes on Southern blots of digested genomic DNA [Southern 1975]. A 5.7-kb *Pvu*II fragment was detected by both probes. *Pvu*II fragments of ~5.7 kb were isolated by electroelution from genomic DNA separated in an agarose gel. These fragments were cloned into the *Bam*HI site of YIp5-5 after blunting of the *Bam*HI site with Klenow polymerase [Boehringer Mannheim]. Colony hybridization (as described above) was used to detect plasmids that had incorporated the desired *Pvu*II fragment.

A small region located between the inserts of plasmids p110A-5a and p12 (Fig. 2) had not been recovered from plasmid, cosmid, or λ vector libraries propagated in *Escherichia coli*. A gap repair approach in yeast was used to clone the region and test for ARS sequences [Iwasaki et al. 1991]. Two versions of a plasmid were constructed that contained small segments of sequence from either side of the cloning gap, separated by a unique *Sac*I site to allow linearization. One version of the plasmid (pGAP) contained the *URA3*, *CEN5* vector YIp5-5; the other (pGAP-ARS) was identical except for the addition of a 422-bp fragment containing *ARS1*.

The plasmids were linearized at the *Sac*I site and transformed into yeast selecting for *Ura*⁺ colonies. Eight of 14 transformants with linearized pGAP-ARS had repaired the plasmid using

chromosomal sequences. Supercoiled, gap-repaired plasmid was isolated by CsCl-ethidium bromide equilibrium gradient centrifugation and was restriction-mapped extensively by Southern blotting. The recovered genomic DNA of 18.1 kb had a restriction map that was identical to the map of the chromosomal region determined by genomic Southern blotting. Furthermore, as expected from the initial failure to recover these sequences from *E. coli*, the gap-repaired plasmid failed to yield stable *E. coli* transformants. These results confirm that the gap-repaired plasmid contains the chromosomal sequences between the inserts of p110A-5a and p12.

The *Sac*I linearized form of pGAP gave rise to *Ura*⁺ transformants that grew very slowly. Slow growth might arise from plasmid integration into the chromosome, because the resulting dicentric chromosomes would be unstable [Haber et al. 1984]. All nine *Ura*⁺ colonies analyzed by Southern hybridization contained chromosomal integrations of plasmid sequences. Because transformation with linearized pGAP would be expected to yield plasmids that can be maintained extrachromosomally if and only if there were an ARS within the region covered by the gap repair, it is concluded that the gap region does not contain an ARS.

Identification of ARS fragments

ARS elements were identified by the ability of plasmids from the chromosome walk to transform yeast strain RM14-3a (*MATa cdc7-1 bar1 ura3-52 trp1-289 leu2-3.112 his6*) at high efficiency [Stinchcomb et al. 1979]. The region containing the ARS was narrowed to 2 kb or less for each ARS by subcloning fragments of the original plasmids into the polylinker of yeast-integrating plasmid pRS306 [Sikorski and Hieter 1989] or the *Bam*HI site of YIp5-5 and testing the resulting plasmids for ARS activity as described above. The minimal fragments identified are *ARS1411*, *Clal*-*Nhe*I (2.04 kb); *ARS1412*, *Pst*I (1.23 kb); *ARS1413*, *Mlu*I-*Xmn*I (1.44 kb); and *ARS1414*, *Bam*HI-*Stu*I (1.31 kb). *ARS1412* was known previously as the *KEX2* ARS [Brewer et al. 1993].

ARS1413 mutagenesis and construction of *ARS1* integration strain

The putative ACS in *ARS1413* was altered by oligonucleotide-directed mutagenesis [Deng and Nickoloff 1992] of plasmid pRS306-ARS. A 10 out of 11 match to the ACS was altered by site-directed mutagenesis from 5'-ATTGTATTTA-3' to 5'-ATTGTATACA-3'. The oligonucleotide used was 5'-ggtcacccaactGtatacaattgtttc-3'. The 10 out of 11 match to the ACS is italicized, altered nucleotides are capitalized, and an *Acc*I site introduced by the mutations is underlined. As a selection for oligonucleotide incorporation, a unique *Hind*III site was altered to a *Stu*I site in the polylinker of pRS306-ARS using the following oligonucleotide: 5'-ggaattcgatatcaGgcCtatcgataccg-3'. The altered nucleotides are capitalized, and the *Stu*I site is underlined. Plasmids were initially screened for the presence of the engineered *Stu*I site and were subsequently screened with *Acc*I for the ACS mutation. All plasmids that had the *Stu*I site had also incorporated the second oligonucleotide. The mutagenized plasmid is designated pRS306-ARS ACS2 and was unable to transform yeast at high efficiency.

For integration of *ARS1* on chromosome XIV, a plasmid (pRS306-CS) was constructed that contains a 1.67-kb *Clal*-*Sac*I fragment from chromosome XIV inserted at the *Clal* and *Sac*I sites of the polylinker in the yeast integrating plasmid pRS306 [Sikorski and Hieter 1989]. This insert was derived from plasmid YIp5-RAP1 (see Fig. 2) and contains a single *Bam*HI site

that was used as the insertion site for *ARS*. The *ARS1* insertion construct [pRS306-CS *ARS1*] was created by cleaving pRS306-CS with *Bam*HI and ligating to a 1.45-kb *Eco*RI fragment containing *ARS1* and *TRP1*. Both fragments were treated with Klenow enzyme prior to ligation.

For chromosomal integration, pRS306-CS *ARS1* was cleaved with *Cl*aI and *S*aI, and a 3.13-kb fragment containing the *ARS1* insertion was isolated by electroelution. This fragment was transformed into strain RM14-3a, and Trp⁺ transformants were screened for the correct insert to yield strain RM14-3a *ARS1*int.

pRS306-*ARS ACS2* was linearized with *M*luI and transformed into strain RM14-3a *ARS1*int. Ura⁺ transformants were streaked on plates containing 5-fluoro-orotic acid (5-FOA) to select for cells in which the vector was excised by recombination between repeated sequences. 5-FOA resistant colonies were screened by Southern hybridization for presence of the engineered *Acc*I site.

2D agarose gel electrophoresis

DNA was extracted from pooled S-phase cells (Huberman et al. 1987; Brewer et al. 1992), and 2D agarose gels were performed as described (Friedman and Brewer 1995). The fragments that were detected are listed in the legend to Figure 4. Autoradiographs were scanned using an Apple OneScanner and printed on an Epson Stylus Color printer at a resolution of 720 dots per inch.

Replication time analysis

The replication times of *Eco*RI fragments were determined in the yeast strain RM14-3a using the density-transfer method as previously described (McCarroll and Fangman 1988). The sizes of the *Eco*RI fragments are listed in the legends to Figures 1 and 5. Replication kinetics were determined after quantitation at the PhosphorImager Facility of the Markey Molecular Medicine Center at the University of Washington.

Replication times of all plasmids were determined using probes unique to the vector sequences. p13 and its deletion derivatives were detected using a 1.166-kb *P*vuII-*D*raI or 3.99-kb *Eco*RI-*B*amIII fragment of pBR322. p12 and its deletion derivatives were detected using *Eco*RI-linearized pBR322.

Sequence analysis

Insert sequences from p12 and p13 were compared with telomeric $[C_{1-3}A]_n$ sequences using Intelligenetics sequence analysis programs. The 118-bp telomeric sequence used for the comparison was derived from locus YSCYELOQ (GenBank accession number M58718) on yeast chromosome VII or XV (Louis and Haber 1992). The insert sequences were searched for perfect matches to telomere sequences of at least 6 bp. As early replicating control sequences, we also tested sequences from the *ARS1* region near the centromere of chromosome IV [insert of Sc4120; Stinchcomb et al. 1982] and the insert of pR151 (Ferguson and Fangman 1992). Many small matches were found in all of the test sequences, the largest being 9 bp. Comparing the late inserts with early sequences, no clear differences were observed between the number of matches or their distribution relative to origins. Because Rap1 protein [Rap1p] is essential for telomere function, sequences were also searched for matches to the 13-bp Rap1p consensus binding site that is found within telomeric $[C_{1-3}A]_n$ sequences (5'-A/G T A/G CACCCANNC C/A CC-3'; the core sequence critical for binding is underlined; Graham and Chambers 1994). Matches to at least 9 of 13 bases were scored, and matches to the core sequence were identified. No perfect matches to the 13-bp consensus were discovered.

The number of matches to the core sequence in each tested sequence were p12, 3; p13, 2; pR151, 1; and Sc4120, 0.

We searched for sequences conserved between regions of chromosome XIV that affect origin activation time. Because many matches can be found even between early and late-replicating sequences at the stringency of 13/15 bp, we limited our analysis by selecting perfect matches at least 11 bp in length between the two *ARS1413* flanking sequences. Comparing all combinations of the flanks yielded 121 matches. These matches were used to search the insert sequences of p12 and pR151. Only one of these 121 matches was found in p12, and a different matching sequence was found on either side of the origin on the early replicating plasmid pR151.

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Multiple determinants controlling activation of yeast replication origins late in S phase.

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