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Early initiation of a replication origin tethered at the nuclear periphery

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Summary

Peripheral nuclear localization of chromosomal loci correlates with late replication in yeast and metazoan cells. To test whether peripheral positioning can impose late replication, we examined whether artificial tethering of an early-initiating replication origin to the nuclear periphery delays its replication in budding yeast. We tested the effects of three different peripheral tethering constructs on the time of replication of the early replication origin *ARS607*. Using the dense-isotope transfer method to assess replication time, we found that *ARS607* still replicates early when tethered to the nuclear periphery using the Yif1 protein or a fragment of Sir4, whereas tethering using a Yku80 construct produces only a very slight replication delay. Single-cell microscopic analysis revealed no correlation between peripheral positioning of *ARS607* in individual cells and delayed replication. Overall, our results demonstrate that a replication origin can initiate replication early in S phase, even if artificially relocated to the nuclear periphery.

Key words: Replication timing, Subnuclear organization, Time-lapse imaging

Introduction

DNA replication in eukaryotic cells is normally initiated from a large number of origins located at intervals along the linear chromosomes. Individual origins initiate replication according to a temporal program, with some origins initiating early and others later in S phase (Friedman et al., 1995). In metazoan cells, replication timing correlates with subnuclear localization and gene activity of chromosomal domains. Inactive chromatin regions usually replicate late and are frequently located at the nuclear periphery or close to a nucleolus (Cimbora and Groudine, 2001). The establishment of the replication-timing program occurs in early G1 phase, coincident with the re-establishment of the spatial organization of chromatin following mitosis (Raghuraman et al., 1997; Dimitrova and Gilbert, 1999; Li et al., 2001). The established late replication context of a peripherally positioned chromosomal locus can be maintained during S phase, even if the sequence was released from the nuclear periphery in late G1 phase (Heun et al., 2001).

In the budding yeast *Saccharomyces cerevisiae*, origin activation time appears to depend on chromosomal context. Replication origins near telomeres are a typical class of late-replicating origins in yeast (Ferguson and Fangman, 1992) and the telomeres are localized to the nuclear periphery during most of interphase. Moreover, disruption of the telomere-binding protein complex Ku effects not only telomere localization (Laroche et al., 1998), but also replication-timing control (Cosgrove et al., 2002). Despite these observations, there is no clear evidence that peripheral localization directly causes late replication.

The histone modification state of chromatin surrounding an origin does influence replication timing of the origin. For example, establishment of silent chromatin has been shown to cause hydroxyurea sensitivity of replication initiation, which is characteristic of late origins (Zappulla et al., 2002). Acetylation of histones close to a normally late-replicating origin makes the origin initiate earlier (Vogelauer et al., 2002; Goren et al., 2008; Knott et al., 2009). However, manipulating histone acetylation causes fairly small changes in replication timing. There is moreover no clear correlation between origin initiation time in *S. cerevisiae* and acetylation level of the surrounding nucleosomes (Nieduszynski et al., 2006), highlighting our limited understanding of the controls over the replication-timing program.

These potential mechanisms that might influence replication timing are not mutually exclusive, and one mechanism could positively or negatively affect the others. To obtain a full understanding of the molecular control(s) over replication timing, it is necessary to dissect the effects of the potential control mechanisms. To test the possibility that localization at the nuclear envelope delays origin firing, we artificially tethered the early replicating origin *ARS607* to the nuclear envelope and examined the replication time of the repositioned origin. Here, we show that peripheral positioning of *ARS607* is not sufficient to delay firing of this origin. Therefore, peripheral positioning of an origin is not sufficient to establish a late-replicating chromosomal region.

Results and Discussion

Tethering of a replication origin to the nuclear periphery

To examine whether perinuclear localization affects the temporal program of replication origin activation, we used a system designed to allow *ARS607*, an early-replicating origin on chromosome VI, to be artificially tethered to the nuclear periphery. *ARS607* is located on the right arm of chromosome VI, approximately 51 kb from

CEN6 and 71 kb from the right telomere (Shirahige et al., 1993), and shows largely random subnuclear positioning (Taddei et al., 2004). *ARS607* is efficiently active (initiating replication in >85% of cells) and replicates early in S phase (Friedman et al., 1997; Yamashita et al., 1997). To enable controlled tethering of *ARS607* to the nuclear periphery, we used a strain in which four copies of the *lexA* operator sequence (*lexA*^{op}) are inserted 0.7 kb from *ARS607* (Fig. 1A) (Taddei et al., 2004). This insertion allows the *ARS607* locus to be directed to the nuclear periphery by expressing a 'tethering construct' in which the LexA DNA-binding domain is fused to a protein moiety that can mediate peripheral positioning [such as fragments of the Ku and Sir proteins implicated in telomere tethering (Taddei et al., 2004)]. The locus was visualized by inserting an array of *lacO* operator sequences (centered 5.9 kb from *ARS607*) and expressing GFP fused to LacI repressor protein (LacI-GFP) (Straight et al., 1996). In vivo, 5.9 kb corresponds to separation of less than 50 nm (Bystricky et al., 2004); because this distance is much shorter than the ~200 nm resolution limit of light microscopy, the position of the *lacO* array can be considered to reflect the position of *ARS607*. The strain additionally expresses the nuclear pore protein Nup49 fused to either GFP or mCherry protein (Iwase et al., 2006), allowing microscopic assessment of *ARS607* location relative to the nuclear envelope.

We employed three different LexA fusion tethering constructs: LexA-Sir4^{PAD}, LexA–Yku80-9 and LexA-Yif1, all of which were previously shown to mediate nuclear peripheral localization (Taddei et al., 2004). LexA-Sir4^{PAD} contains a fragment of the Sir4 protein. Sir4 forms part of the telomeric transcriptional silencing machinery, but because the Sir4^{PAD} fragment lacks the domain required for interaction with other Sir proteins, it cannot nucleate silent heterochromatin when tethered to a chromosomal locus (Ansari and Gartenberg, 1997; Taddei et al., 2004). Another tethering construct, LexA–Yku80-9, is formed from LexA fused to an allele of the telomere-binding Yku80 protein (Taddei et al., 2004). We also tested a construct containing LexA fused to the inner nuclear membrane protein Yif1, which can tether *ARS607* to the nuclear periphery independent of proteins involved in telomere clustering (Taddei et al., 2004). Importantly, none of these LexA fusions induces transcriptional silencing at the *ARS607* locus (supplementary material Fig. S1), although LexA–Yku80-9 was previously shown to induce moderate silencing at a crippled silencer (Taddei et al., 2004). All three LexA fusions mediate tethering of the *ARS607* locus to the nuclear periphery in G1 and early S phase cells (data not shown), as previously reported (Taddei et al., 2004; Hiraga et al., 2006; Ebrahimi and Donaldson, 2008). Yeast chromatin is highly mobile and it should be noted that the *ARS607* locus remains dynamic, even when positioned at the periphery by one of these constructs. Therefore, we observe *ARS607* at the periphery in 60-70% of cells in a population snapshot (compared to 33% of cells for a randomly positioned locus). This observation is consistent with previous data showing that endogenous perinuclear chromosome domains, such as telomeres, are peripheral in 60-70% of cells (Hediger et al., 2002; Brickner and Walter, 2004).

We first checked that peripheral tethering does not repress origin activity, using neutral/neutral two-dimensional gel electrophoresis

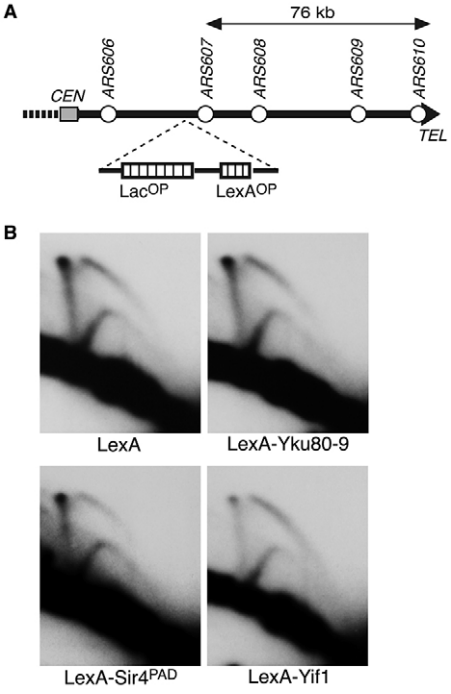

Fig. 1. Perinuclear tethering of a replication origin. (**A**) Schematic diagram of the tethering construct used in this study. Integrated next to *ARS607* are four copies of the *LexA* DNA-binding sequence and an array of *lacO* sites. (**B**) The origin activity of *ARS607* in asynchronous cultures was compared using two-dimensional gel electrophoresis of strains expressing LexA only (strain HE114), and LexA-Sir4^{PAD} (HE115), LexA–Yku80-9 (HE116) and LexA-Yif1 (HE87) tethering constructs.

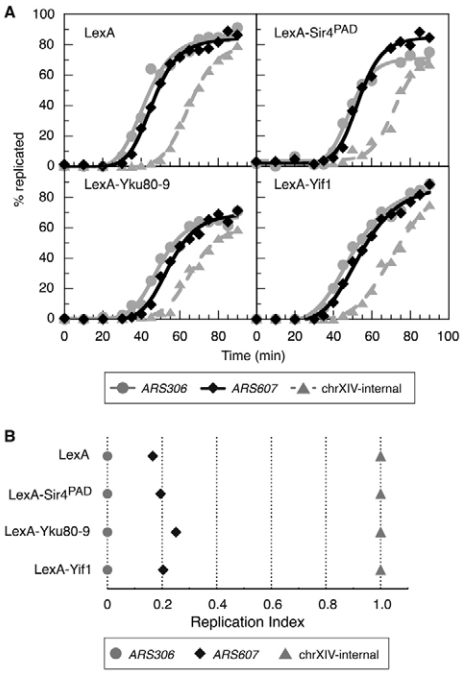

Fig. 2. Replication timing of the *ARS607* locus is unchanged when peripherally tethered. (**A**) Results of dense-isotope transfer experiments to measure replication timing in strains in which the *ARS607* locus was not peripherally tethered (LexA) or was tethered to the nuclear periphery (LexA-Sir4^{PAD}, LexA–Yku80-9, LexA-Yif1). The replication kinetics of the *ARS607* locus are compared with those of *ARS306* (early marker) and chrXIV-internal (late marker). (**B**) RI was calculated for *ARS607* in each of the four strains relative to very early and late replicating sequences (*ARS306* and chrXIV-internal, set to 0 and 1, respectively).

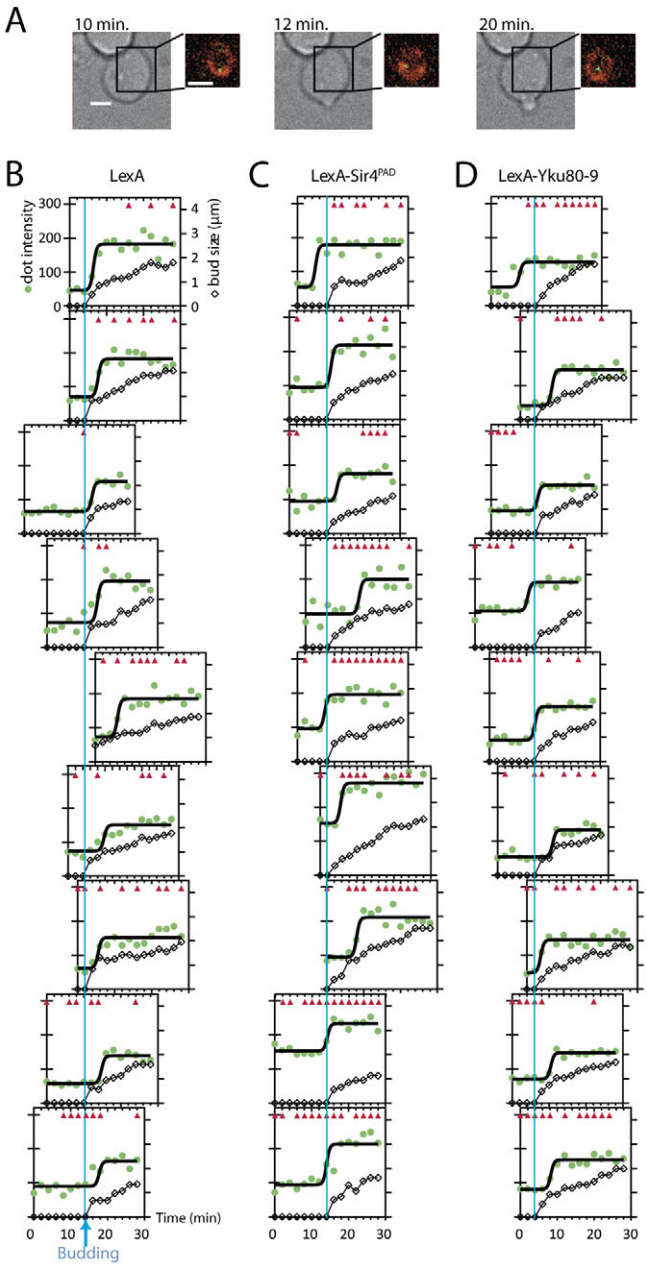

Fig. 3. Subnuclear positioning does not correlate with *ARS607* replication time in live-cell imaging experiments. (**A**) Images illustrating representative time points from a single time-lapse series. The gray-scale panels show DIC images of the cell at indicated time points. The position of *ARS607* (green GFP dot) within the nuclear rim (red mCherry) is shown in the colored insets. At each time point, the data sets collected consisted of 16 planes in each channel through the *z*-axis. At the time points shown, *ARS607* was close to the equatorial plane of the nucleus. The full data set for this cell is given in the bottom left plot in B. Scale bars: 2 µm. (**B**) *ARS607* positioning and intensity data measured every 2 minutes in nine cells expressing LexA (HE114). Fluorescence intensity of the GFP-tagged *ARS607* locus (green circles) is plotted (left *y*-axis). Open diamonds show bud size (right *y*-axis). Red triangles indicate time points at which *ARS607* was localized to the nuclear periphery (measured by calculating the three-dimensional distance between *ARS607* and the center of the nucleus, allowing estimation of dot-to-periphery distance). Graphs are aligned at the last time point when each cell was observed to be unbudded (vertical blue line). Graphs are sorted from top to bottom according to the degree of localization in each cell at the three time points preceding *ARS607* replication. (**C**) *ARS607* positioning and intensity data in nine cells expressing LexA-Sir4^{PAD} (HE115), displayed as in B. (**D**) *ARS607* positioning and intensity data in nine cells expressing LexA–Yku80-9 (HE116), displayed as in B.

(Fig. 1B) (Friedman and Brewer, 1995). A bubble arc indicative of *ARS607* origin activation was detected in the LexA-expressing strain. The presence of a similar bubble arc in the strains expressing LexA-Sir4^{PAD}, LexA–Yku80-9 and LexA-Yif1 indicated that *ARS607* remains active when tethered to the nuclear periphery.

Tethering does not affect average replication time

Next, we examined the effect of perinuclear tethering on replication timing of *ARS607* using the dense-isotope transfer method. In this technique, the replication kinetics of specific sequences are monitored in a synchronized culture, by tracking the shift in density of genomic DNA fragments caused by the incorporation of specific carbon and nitrogen isotopes into nascent DNA (McCarroll and Fangman, 1988). The graphs in Fig. 2A show the replication kinetics of three different sequences: the early-replication origin *ARS306*; a late marker sequence, 'chrXIV-internal', centered at approximately 223 kb on chromosome XIV; and the *ARS607* locus. As expected, in the control strain (expressing LexA alone), *ARS607* replicated early in S phase, shortly after *ARS306*, which is one of the earliest-replicating sequences in the genome (Fig. 2A). Expression of either the LexA-Sir4^{PAD} or LexA-Yif1 localization constructs did not result in any noticeable change in the replication kinetics of *ARS607* (Fig. 2A, right hand graphs). Expression of LexA–Yku80-9 caused a very slight delay in the replication time of *ARS607* relative to the early marker *ARS306* (Fig. 2A, lower left panel), but this change might not be significant because it lies within the margins of error typically seen for this type of experiment (Friedman et al., 1996).

Replication time in these experiments is defined as the time at which a sequence has replicated in half of the cycling cells. The 'replication index' (RI) is the replication time of a sequence expressed relative to the early and late markers (whose replication times are assigned as 0 and 1, respectively). Calculating the RI normalizes the differences in the speed with which different cultures release from synchronization and proceed through S phase. Fig. 2B shows RI values for *ARS607* in the four experiments, and confirms that expressing LexA-Sir4^{PAD} or LexA-Yif1 caused no significant change in replication time, whereas LexA–Yku80-9 caused only a very slight change.

Single-cell analysis of replication timing

The results in Fig. 2 do not support the idea that perinuclear positioning mediates late replication. However, the replication timing values measured by the density-transfer method represent an average (mean) for all the cells in the population. Because the LexA fusion proteins typically cause perinuclear tethering in only 60-70% of cells at any moment (Taddei et al., 2004; Hiraga et al., 2006; Ebrahimi and Donaldson, 2008), it is conceivable that a delay to replication time caused by perinuclear localization could be obscured because *ARS607* is not localized in 30-40% of cells. To investigate this possibility, we examined the replication of the *ARS607* locus microscopically in individual cells, by monitoring the doubling of GFP fluorescence intensity (Kitamura et al., 2006; Ebrahimi and Donaldson, 2008). Briefly, time-lapse experiments

were carried out to measure three parameters in cells undergoing bud emergence: GFP fluorescence intensity of the *lacO-lexA*^{op}-*ARS607* locus, position of the locus relative to the nuclear envelope and bud size. Representative images are shown in Fig. 3A. The plots in Fig. 3C-D show the results for a series of individual cells. In these plots, the open diamonds show bud size at successive time points and red triangles indicate time points at which the *ARS607* locus was at the nuclear periphery. Measuring the midpoint of the increase in GFP fluorescence (filled green circles) allows assignment of the *ARS607* replication time in each cell.

In the control strain expressing LexA, the *ARS607* locus was consistently replicated 2-6 minutes after bud emergence (Fig. 3B, Fig. 4A). Replication time of the *ARS607* locus was also measured in cells expressing LexA-Sir4^{PAD} (Fig. 3C) and LexA–Yku80-9 (Fig. 3D). In neither case did we observe any consistent delay in replication timing relative to bud emergence. Importantly, even when examining these individual cells, no relationship was observed between *ARS607* replication time and its localization status at immediately preceding time points (Fig. 4A). Specifically, the *ARS607* locus showed no tendency to replicate later when it was localized at the periphery immediately before replication (Fig. 4A, grey and black circles do not cluster towards the top of the chart). Tethering of *ARS607* to the nuclear periphery by LexA-Sir4^{PAD} or LexA–Yku80-9 did result in replication timing that appears slightly more scattered relative to bud emergence than observed for the control strain (Fig. 4A), but the variation in replication timing relative to budding (s.d. less than 4 minutes) was much smaller than the length of S phase (15-20 minute difference in replication time between earliest and latest origins). Moreover, the *ARS607* locus replicated at a very similar average bud size, whether or not it was tethered to the nuclear periphery (Fig. 4B). To summarize, the results of single-cell analysis also indicated that peripheral localization of *ARS607* has no major impact on its replication time.

The density-transfer method allows precise, standardized comparison of average replication times in a population, whereas single-cell imaging enables the simultaneous recording of replication time and locus subnuclear position in individual living cells. Assessing replication time using either technique revealed that perinuclear positioning of the origin does not impact its initiation time. In summary, our findings suggest that subnuclear localization is not the main determinant of replication timing in budding yeast. This reveals the reason for previous observations that telomeric origins can replicate late even when the origin is not properly localized to the nuclear periphery (Heun et al., 2001; Hiraga et al., 2006).

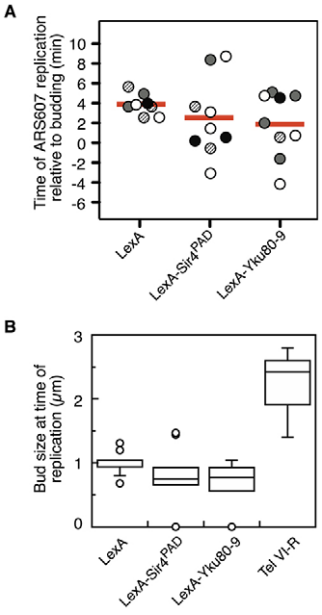

Fig. 4. Replication timing relative to bud emergence is not affected by perinuclear tethering. (**A**) Scatter plot showing *ARS607* replication time relative to bud emergence time, in live cells expressing LexA, LexA-Sir4^{PAD} or LexA–Yku80-9. Each data point represents *ARS607* replication time in one of the single cells shown in Fig. 3. Negative values indicate cells in which *ARS607* replicated prior to bud emergence. The horizontal red lines show mean replication time relative to bud emergence for each strain (LexA=3.52 minutes, LexA-Sir4^{PAD}=2.23 minutes, LexA–Yku80-9=1.65 minutes). *P* values determined by Welch's *t*-test are 0.35 and 0.11 for LexA versus LexA-Sir4^{PAD} and LexA versus LexA–Yku80-9, respectively, indicating no significant difference in mean replication time. The fill pattern of data points indicates the degree of peripheral localization of *ARS607* at the three time points immediately prior to replication. Black circles indicate perinuclear localization at all three time points; grey circles indicate localization at two time points; hatched circles indicate localization at one time point; open circles indicate no perinuclear localization at the three time points before replication. (**B**) Box plot showing bud size at the time of *ARS607* replication for the three strains in A. Bud size at time of replication for a late marker sequence (telomere VI-R) is shown for comparison (Ebrahimi and Donaldson, 2008).

Materials and Methods

Yeast strains and plasmids

Yeast strains are described in supplementary material Table S1. Primer sequences used in strain construction are available on request. Plasmids pAT4, pAT4-Sir2, pAT4-Sir4^{PAD}, pAT4–Yku80-9 and pAT4-Yif1 were described previously (Taddei et al., 2004).

Microscopy

Quantitative measurements of microscopic images were performed as described (Ebrahimi and Donaldson, 2008). To determine the time point at which *ARS607* replicated, a curve is fitted over the intensity data points; the midpoint of the intensity increase on the fitted curve is assigned as the replication time.

Density transfer

The replication timing analyses shown in Fig. 2 were carried out using the dense-isotope transfer technique, as previously described (Donaldson et al., 1998).

Two-dimensional gel

Genomic DNA was prepared as described (Huberman et al., 1987; Brewer et al., 1992). DNA fragments digested using *Nco*I and *Eco*RI were separated by neutral/neutral two-dimensional agarose gel electrophoresis (Friedman and Brewer, 1995) and transferred to neutral membrane (Qbiogene) by Southern blotting. The 1633-bp fragment containing *ARS607* was detected using a suitable ^{32}P-labeled probe.

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/7/1015/DC1

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