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Enforcement of Late Replication Origin Firing by Clusters of Short G-rich DNA Sequences*[S]♦

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Previous studies in budding yeast suggested that the default firing time of most DNA replication origins is early in S phase and that origins can be forced to fire later by proximity to certain *cis*-acting sequences. However, these *cis*-acting sequences were not well defined. We have attempted to characterize *cis*-acting sequences that affect replication timing in the fission yeast. We identified a stretch of 200 bp that was sufficient to compel nearby origins to fire late. The 200-bp stretch was able to force an origin to fire late whether adjacent to the origin or ~800 bp away in opposite orientation. The stretch contains a cluster of three close matches to a G-rich, 10-bp late consensus sequence (LCS). The three LCS elements cooperate with each other and with other sequences within the 200-bp stretch to enforce late replication. Although only a few origins that fire in very late S phase have been identified in fission yeast, all of them are located close to a cluster of LCS elements.

DNA replication origins in most eukaryotic cells fire at specific times during S phase, from early to late. Consequently, each segment of the genome replicates at a precise time during S phase. It is thought that this DNA replication timing program may be important for gene regulation, because changes in gene expression are in some cases associated with changes in replication timing (1). Maintenance of replication timing may also be important for preventing cancer, because disruptions of the replication timing program in human peripheral blood lymphocytes are tightly correlated with renal (2), prostate (3), and perhaps other cancers.

For these reasons, it is important to understand the mechanisms that control origin firing times. Unfortunately, little is currently known about them. Studies in budding yeast (*Saccharomyces cerevisiae*) have suggested that the default firing time of most origins is early, and origins can be forced to fire later in S phase by flanking (4) and internal (5) *cis*-acting sequences or by chromosome ends (6). However, the mechanism by which chromosome ends control replication timing has not yet been elucidated, and, with the exception of sequences that affect the affinity of origin recognition complex for its binding sites at origins (7), other *cis*-acting sequences that can force origins far from telomeres to fire late have not yet been defined.

Previous results suggest that chromatin structure plays a major, but apparently not a completely determining, role in regulating replication timing. Euchromatic regions frequently replicate early, while heterochromatic regions usually replicate late (8, 9). Because there are significant exceptions to this correlation (10), better evidence for the importance of chromatin structure for replication timing is provided by recent studies in budding yeast, which have shown that genetic alterations affecting chromatin structure can have dramatic effects on the replication times of many genomic regions (11–14). Thus it is reasonable to suggest that the ill-defined *cis*-acting sequences that are capable of forcing origins to fire in late S phase (see above) may act by promoting the formation of chromatin structures that favor late replication. Determining whether this hypothesis is correct will require better definition of the *cis*-acting sequences that affect replication timing.

To help identify and characterize such *cis*-acting sequences, we turned to the fission yeast, *Schizosaccharomyces pombe*. DNA replication origins in fission yeast are larger (500–1500 bp) than those in budding yeast (100–250 bp) (15). We suspected that *cis*-acting sequences controlling replication timing might be included in such larger fission yeast origins. Here we report that *ars727*, a fission yeast origin that normally replicates in late S phase, does indeed contain *cis*-acting sequences that are responsible for its late replication. Furthermore, when placed near *ars3001*, a fission yeast origin that normally replicates in early S phase, these sequences proved capable of forcing *ars3001* to fire in late S phase. We localized the important *cis*-acting sequences in *ars727*, and we found that they are contained in a stretch of 200 bp. Within this stretch, we noticed that three matches to a 10-bp late consensus sequence (LCS)[1] are especially important determinants of replication timing.

EXPERIMENTAL PROCEDURES

Strains and Media—We used the *Escherichia coli* strains DH5α (Invitrogen) for general subcloning, XL1-Blue (Stratagene) for construction of plasmid pLS1.2, and INVαF′ (Invitrogen) for propagating the pCRII vector. We used the *S. pombe* D18 strain (*ura4*-D18 *leu1–32 end1 h*−) (16) grown in YES (complete) medium (17). To select for cells transformed to ura+, we used EMMS-Ura medium (17).

Yeast Transformation—D18 cells were transformed as described (18).

Construction of Mono- and Bi-autonomously Replicating Sequence (ARS) Plasmids—pARS3001 contains a 573-bp version of *ars3001* cloned between the BamHI and ClaI sites of YIp5 (GenBank™ accession number L09157) and was previously constructed in our laboratory

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[S] The on-line version of this article (available at http://www.jbc.org) contains Supplemental Procedures, Tables 1 and 2, and Fig. 1.

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[1] The abbreviations used are: LCS, late consensus sequence; ARS, autonomously replicating sequence (a replication origin); HU, hydroxyurea.

(19). To construct other mono- and bi-ARS plasmids, we used standard molecular techniques. The procedures and oligonucleotides employed for these constructions are described in detail in the Supplemental Procedures and in Supplemental Table I.

Generation of Mutant Versions of Mono- and Bi-ARS Plasmids—Deletion, linker substitution, and other types of mutations were generated by standard molecular techniques and are described in detail in the Supplemental Procedures and in Supplemental Table I.

Determination of Plasmid Structure in S. pombe—After transfection of mono- or bi-ARS plasmids into fission yeast cells, the Ura+ transformants were cultivated in 10 ml selective medium (EMMS-Ura) with shaking at 30 °C until saturation. The cells were pelleted, and DNA samples were prepared by the "Smash and Grab" method (20). The DNA pellets were resuspended in 40 μl of TE (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0) containing 100 μg/ml RNase A. Half of the sample (20 μl) was digested with PstI. Undigested DNA (20 μl) and digested DNA were analyzed by electrophoresis in a 0.8% agarose gel in TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0) (65 volts, 3 h). The gel was then blotted to a nylon membrane, which was hybridized with a YIp5 probe to detect plasmid molecules. In all cases, only the predicted PstI fragments (3.1 and 2.4 kb) were detected, and these were detected in equimolar ratio, indicating that the only possible plasmid rearrangments were multimerizations. The sizes of the bands generated by mono-ARS plasmids indicated that multimerization had taken place, while the sizes of the bands generated by bi-ARS plasmids in most cases indicated that monomeric plasmids were retained.

Cell Synchronization by Hydroxyurea Block and Release—The Ura+ plasmid-containing cells from freezer stocks were grown in EMMS-Ura with shaking at 30 °C. One liter of log phase culture (0.8–1.4 $\times$ 10^7 cells/ml) was prepared and harvested. The cell pellets were resuspended in 1 liter of prewarmed (30 °C) YES medium and incubated with shaking for 30 min. Then 500 μl of the cell suspension were collected and fixed in 1 ml of ethanol for flow cytometric DNA content analysis. The culture was treated with 25 mM hydroxyurea (United States Biochemical) for 3 h at 30 °C with shaking. Cells were harvested and washed twice with sterile prewarmed (30 °C) water. The cell pellets were resuspended in 1 liter each prewarmed (30 °C) YES medium, and five samples of 200 ml each were collected at either 10- or 15-min intervals. Each of the samples was immediately mixed with 20 ml of prechilled (0 °C) solution containing 1/3 (v/v) glycerol, 2/3 (v/v) 0.2 M EDTA, and 0.1% NaN_3. Five-hundred μl of each chilled cell suspension were then fixed in 1 ml of ethanol for flow cytometric analysis. The cells were then harvested by centrifugation and washed once with cold sterile double-distilled water. The cell pellets were kept at −80 °C.

Two-dimensional Gel Analyses—DNA preparation and neutral/neutral two-dimensional agarose gel electrophoresis were carried out as described previously (21). Five μg of each DNA sample were digested with a 5-fold excess of AlwNI and NcoI.

Flow Cytometry—Evaluation of cell DNA content and cell cycle position by flow cytometry was carried out as described (21).

Hybridization Probes—To determine the structures of plasmids inside transformed yeast cells, HindIII-digested YIp5 was used as probe. To detect plasmid replication intermediates, the 2.39-kb AlwNI-EcoRI fragment of YIp5 was used as probe.

ARS Assays—ARS activity was measured as described previously (19). Two-hundred ng of each plasmid were used for each transformation. At least three independent repeats of each experiment were carried out.

Computer Analysis of LCS Clusters—We used MacVector software to do subsequence searches for RKK**GGGGG**AW (where R = A or G, K = T or G, and W = A or T). We required perfect matches at all five central Gs (boldface), and we required perfect matches at three or more of the remaining five positions. Searches were run against regions of 20 kb or more centered on each of the origins indicated in Fig. 8 and Supplemental Table II. In the cases of extended origin-containing regions (such as the centromeres and the mating type region), we searched against the extended region plus an additional 10 kb at each end. LCS clusters were defined as three or more LCS matches (according to the above criteria) within an interval of 1,500 bp or less. Accession numbers for the searched sequences are provided in Supplemental Table II.

RESULTS

Replication Timing of Mono-ARS Plasmids—Our first goal was to determine whether large fission yeast replication origin sequences can contain *cis*-acting sequences that affect their replication timing. For this purpose, we constructed two plasmids (Fig. 1*A*), each containing a single ARS element. ARS

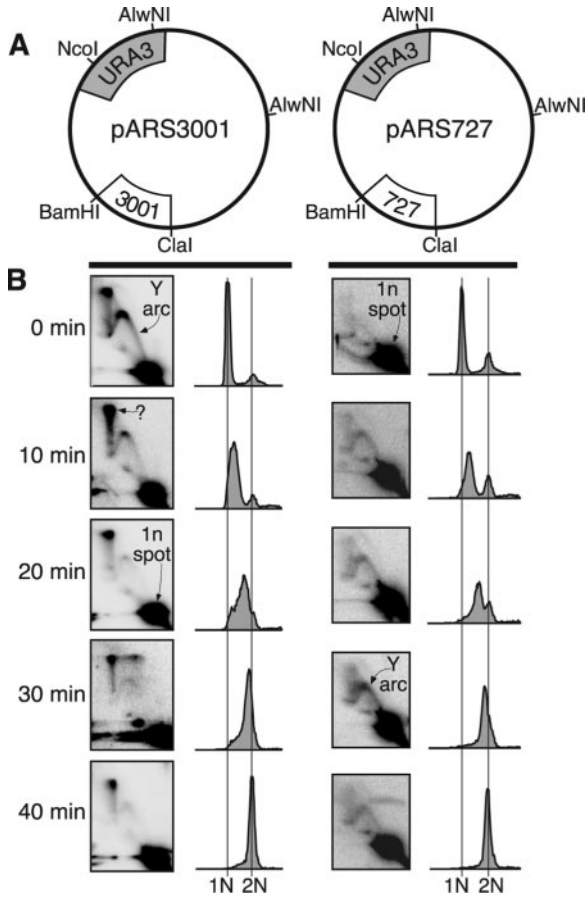

FIG. 1. **Replication timing of plasmids containing single replication origins (ARS elements).** *A*, structures (not to scale) of mono-ARS plasmids containing *ars3001* or *ars727*. Restriction sites used for cloning and for two-dimensional gel electrophoresis are indicated. *B*, two-dimensional agarose gel analysis (26) of the abundance of Y-shaped replication intermediates (Y arc) generated by the large NcoI-AlwNI fragments from pARS3001 and pARS727 during a synchronized S phase. pARS3001 replicates in early S phase, while pARS727 replicates in late S phase. "*?*" indicates a signal of unknown source. "*1n spot*" indicates the signal due to the linear double-stranded (non-replicating) form of the restriction fragment.

elements are stretches of chromosomal DNA that can serve as replication origins in plasmids. One of these mono-ARS plasmids (pARS3001) contained *ars3001*, which is the fission yeast rDNA replication origin. One copy of *ars3001* is found in each of the 150–200 tandem ~10-kb rDNA repeat units (19, 22), which are arranged in two clusters at the ends of chromosome III (Fig. 8). In its chromosomal location, *ars3001* is replicated early in S phase by a combination of active origin firing and passive replication by forks coming from neighboring rDNA repeat units (21). The second mono-ARS plasmid (pARS727; Fig. 1*A*) contained *ars727*, which is a single-copy ARS element (see Fig. 2 for sequence) (23) located on chromosome II (Fig. 8). Replication timing measurements had shown that, in its normal chromosomal position, *ars727* is passively replicated in mid to late S phase (21). The fact that *ars727*, a potential replication origin, is passively replicated in its normal chromosomal location led us to suspect that, like *ARS301* and *ARS302* in budding yeast (5, 24), *ars727* might be programmed to fire so late in S phase that it would always be passively replicated by forks from neighboring earlier firing origins before it had a chance to fire on its own.

To test the firing times of *ars3001* and *ars727* in the plasmid context (Fig. 1*A*), we used a hydroxyurea block-and-release synchronization procedure (21) (Fig. 1*B*). Hydroxyurea (HU)

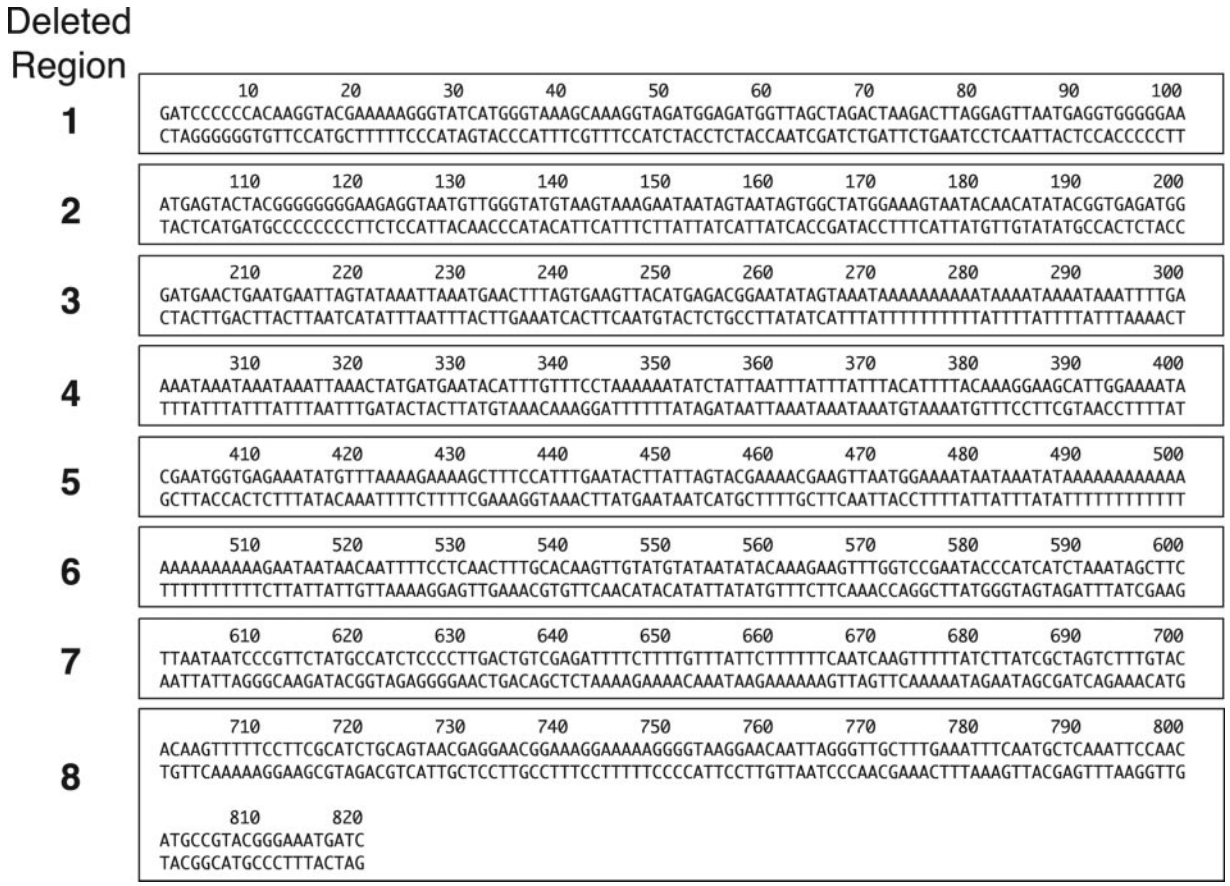

FIG. 2. **The complete nucleotide sequence of *ars727* (23).** This corresponds to nucleotides 3,338,897 to 3,339,716 on the complementary strand of *S. pombe* chromosome II (GenBank™ accession number NC_003423). The regions deleted in our consecutive deletion scan of *ars727* are indicated. All of these regions contain 100 bp except for region 8, which contains 120 bp.

inhibits ribonucleotide reductase and prevents the synthesis of dNTPs. When cells enter S phase in the presence of HU, endogenous pools of dNTPs are rapidly exhausted. Only early origins fire, and replication forks move variable distances, usually no more than a few kb, away from the early origins (21, 25). For the experiments reported here, we treated unsynchronized cells with HU for 3 h to permit them to accumulate in early S phase with early origins fired. Then, at 0 min, we removed the HU so that cells could continue through S phase. In the experiment in Fig. 1*B*, both cell lines completed S phase by 40 min after HU removal, as indicated by the flow cytometry profiles. Next, replication intermediates were separated from non-replicating DNA by two-dimensional agarose gel electrophoresis (26) (Fig. 1*B*). Because both *ars3001* and *ars727* are weak, the plasmids containing them multimerized under selection pressure to increase the number of replication origins per plasmid. Under these conditions, only one origin fires per multimer; the other origins are replicated passively (27). Consequently the major signal from replication intermediates has the form of a Y arc (as indicated in Fig. 1*B*). It is evident that the strongest Y arc signal from pARS3001 was detected in the 0-min sample (before release from the HU block) indicating that in the plasmid context, as in chromosome III, *ars3001* fires in early S phase. In contrast, few or no replication intermediates were evident in the 0-min sample from pARS727. The maximum Y arc signal for pARS727 was detected in the 30-min time point, indicating that in the plasmid context *ars727* fires in late S phase, even later than the time when it is passively replicated in chromosome II (21). Since pARS3001 and pARS727 differ only in their ARS elements, these results suggested that *cis*-acting sequences controlling replication timing must be present in one or both of the ARS elements.

Replication Timing of Bi-ARS Plasmids—To learn more about these putative timing control sequences, we constructed four bi-ARS plasmids, representing the four possible relative orientations of the two ARS elements (*ars3001* and *ars727*) side-by-side (Fig. 3*A*). Of these, one orientation (pBiARS#2; Fig. 3*A*) proved to replicate poorly in yeast cells due to diminished origin activity (data not shown). The other three orientations displayed enhanced origin activity, which permitted these plasmids to remain monomeric (as indicated by bubble arcs in two-dimensional gels, Fig. 3*B*, and by investigations of plasmid structure, data not shown). One of these orientations (pBiARS#1) replicated in early S phase but slightly later than pARS3001 (Fig. 3*B*). The other two orientations (pBiARS#3 and pBiARS#4) replicated in late S phase, similar to pARS727 (compare Fig. 1*B* with Fig. 3*B*). In pBiARS#3 and pBiARS#4 (but not in pBiARS#1), the *cis*-acting sequences within *ars727* that enforce late replication timing appeared to be dominant over any *cis*-acting sequences within *ars3001* that might encourage early replication.

The robust bubble arcs generated in late S phase by pBiARS#3 and pBiARS#4 indicated that the late replication of these bi-ARS plasmids could not be due to repression of firing of *ars3001*. If *ars3001* were not contributing to the replication of these bi-ARS plasmids, their replication efficiency would be determined solely by *ars727*, which on its own is a weak origin that cannot efficiently replicate monomeric plasmids. Plasmids dependent solely on *ars727* for their replication tend to multimerize, leading to loss of bubble arc signal (as in Fig. 1*B*). Thus the late replication-determining sequences within *ars727* must function at least in part by delaying the firing of *ars3001* until late S phase rather than by inhibiting it.

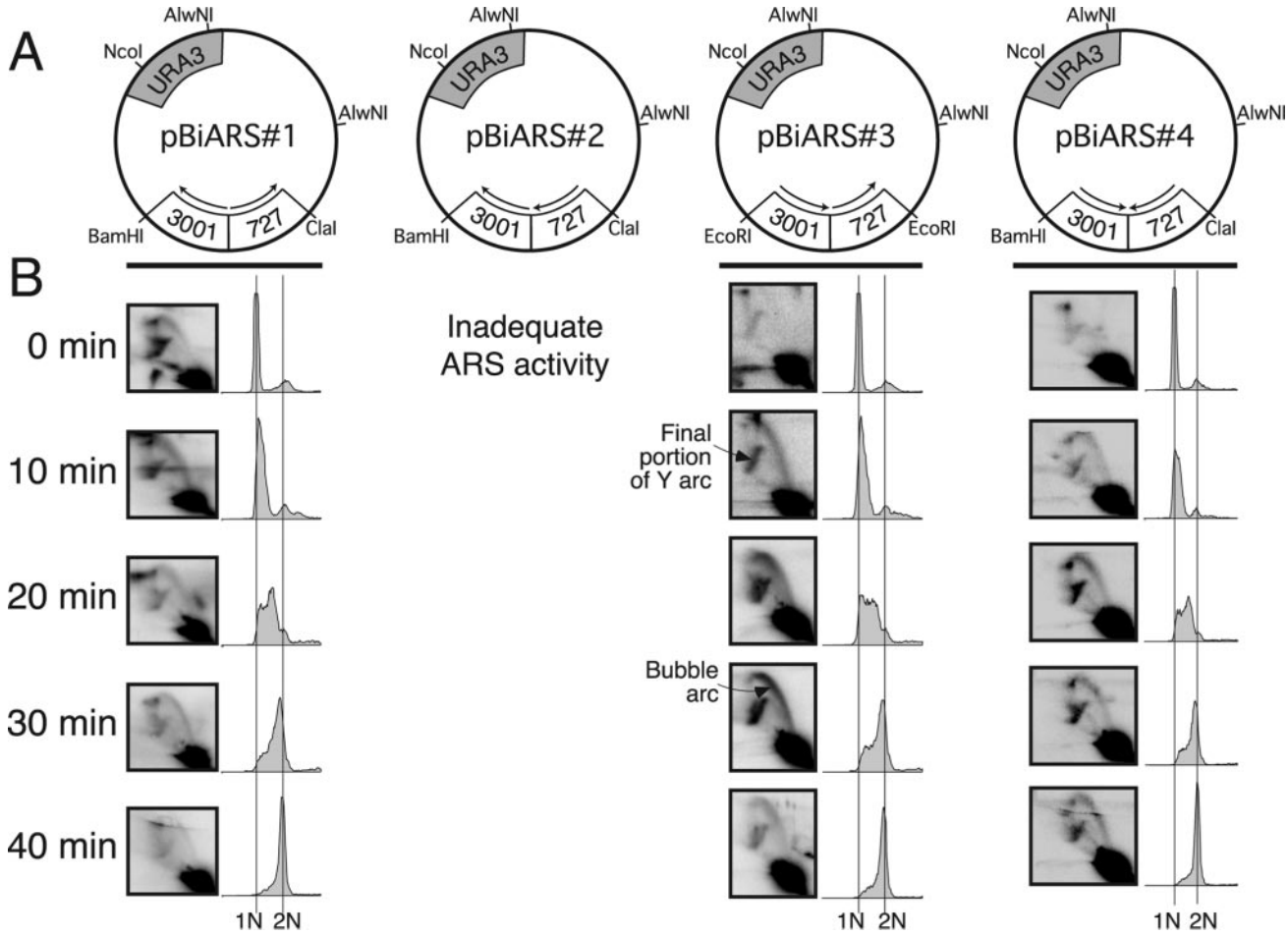

FIG. 3. **Replication timing of plasmids containing two replication origins.** *A*, diagrams showing the relative orientations of *ars3001* and *ars727* in the four bi-ARS plasmid constructs, pBiARS#1–pBiARS#4. The orientations indicated by the *arrows* correspond to the conventional numbering directions through these two ARS elements. *B*, two-dimensional gel analyses (26) of plasmid replication intermediates (from the indicated large NcoI-AlwNI restriction fragment) at the indicated times after release from a hydroxyurea block. pBiARS#1 replicates slightly later than pARS3001 (Fig. 1*B*). pBiARS#2 did not have sufficient ARS activity to generate viable transformed yeast strains. pBiARS#3 and pBiARS#4 replicated in late S phase, similar to pARS727 (Fig. 1*B*). The final portion of the Y arc is relatively intense in these autoradiograms, because the origin (the two ARS elements) is not located in the center of the restriction fragment (26).

Effects of 100-bp Deletions on the Origin Activity of ars727—Our next goal, therefore, was to identify the late replication-determining sequences within *ars727*. The fact that pBiARS#3 contains two ARS elements permitted us to carry out saturation mutagenesis of *ars727* and test the effect of each mutation on replication timing without losing the ability of the plasmid to replicate autonomously. In each case origin activity was supplied by the non-mutated *ars3001*.

First we constructed a series of eight consecutive ~100-bp deletions through the 820 bp of *ars727* (Fig. 2). Before testing the effects of these deletions on replication timing in pBiARS#3, we used two assays to determine their effects on the ARS activity (activity as a plasmid replication origin) of *ars727* in the mono-ARS plasmid, pARS727. Measurements of plasmid transformation frequency (Fig. 4*A*) and measurements of the growth rates of the transformed colonies as indicated by colony size (Fig. 4, *A* and *B*) indicated that deletion of region 1 (Δ1; the first 100 bp of *ars727*) slightly stimulated ARS activity; Δ2 and Δ8 had little effect; Δ3-Δ5 strongly inhibited ARS activity, and Δ6-Δ7 slightly inhibited ARS activity. Because regions 3–5 are particularly rich in asymmetric A+T-rich stretches (Fig. 2), which are known to form good binding sites for *S. pombe* origin recognition complex (28–30), these experimental results are not surprising.

Effects of 100-bp Deletions on the Replication Timing of pBiARS#3—Next we made the corresponding 100-bp deletions in the *ars727* portion of pBiARS#3, and we measured their effects on replication timing. As illustrated in Fig. 5*A*, deletion of region 1 produced a complete shift from late replication to early replication. The early replication was efficient, because the plasmids remained monomeric (data not shown). However, during incubation in HU, most of the earliest (bubble-shaped) replication intermediates disappeared, because most of the forks moved far enough from the origin to convert the replication intermediates from bubble-shaped to late Y-shaped. In contrast to deletion of region 1, deletion of region 2 (Fig. 5*A*) produced only a partial shift; replication intermediates were most abundant in the 10-min time point rather than at 20–30 min as in non-mutated pBiARS#3 (Fig. 3*B*). Deletions of regions 3–8 had no detectable effect on replication timing (Fig. 5*A* and data not shown). Thus region 1 appeared to contain *cis*-acting sequences that were essential for late replication timing, while region 2 appeared to contain additional *cis*-acting sequences capable of stimulating late replication timing.

Effects of Linker-Substitution Mutations on Replication Timing—To better localize these *cis*-acting sequences, we employed linker-substitution mutagenesis. Since deletion of region 1 had the most pronounced effect on replication timing, we constructed consecutive 10-bp XbaI linker-substitution mutations (GCTCTAGAGC) throughout region 1. Of these, pLS1.10, in which nucleotides 91–100 of *ars727* (Fig. 2) were replaced by the XbaI linker, had the most pronounced effect on timing, but

it was only a partial effect, similar to that of deleting region 2 (Fig. 5, *A* and *B*, and data not shown).

We noticed that nucleotides 91–100 (GGTGGGGGAA), whose loss by linker substitution had the greatest effect on replication timing (Fig. 5*B*), resemble nucleotides 2–11 (GTGGGGGGAT in the bottom strand) and 112–121 (GGGGGGGGAA) of *ars727* (Fig. 2). These three 10-bp sequences determine a consensus, GKKGGGGGAW, where K represents G or T, and W represents A or T. Because we found that the similar sequences in telomere-associated sequences, which replicate late (21) (Fig. 8), frequently have A in the first position, we modified the consensus to RKKGGGGGAW, where R represents A or G. There are two exact matches to this consensus in region 1 of *ars727* and one exact match in region 2. The fact that deletion of region 1 led to complete loss of late replication timing, while deletion of region 2 led to partial loss (Fig. 5*A*) suggested that three copies of the consensus might be required for maximal effect on replication timing, two copies might have a partial effect, and one copy might be insufficient for any detectable effect. To test this possibility, we constructed linker substitutions specifically targeted to the three consensus sequences. We named these three late consensus sequences LCS1, LCS2, and LCS3 according to their order in *ars727*. We found that individual linker substitutions of LCS1 and LCS3 (Supplemental Fig. 1) had effects no greater than the partial effect produced by linker substitution of LCS2 (Fig. 5*B*). However, double substitutions of LCS1 + LCS2, LCS1 + LCS3, and LCS2 + LCS3, and also triple substitution of LCS1 + LCS2 + LCS3, caused complete loss of late replication timing (Fig. 5*C* and data not shown). These results indicate that, as suspected, one copy of the LCS is insufficient to produce a detectable effect on replication timing, two copies are essential to produce a partial effect, and three copies are essential for a complete shift from early to late replication.

Our linker substitution analyses also suggested that non-LCS sequences immediately adjacent to the LCS elements can enhance the contributions of LCS elements to enforcement of late replication. For example, the results in Supplemental Fig. 1 show that linker substitution of nucleotides 1–20 permits

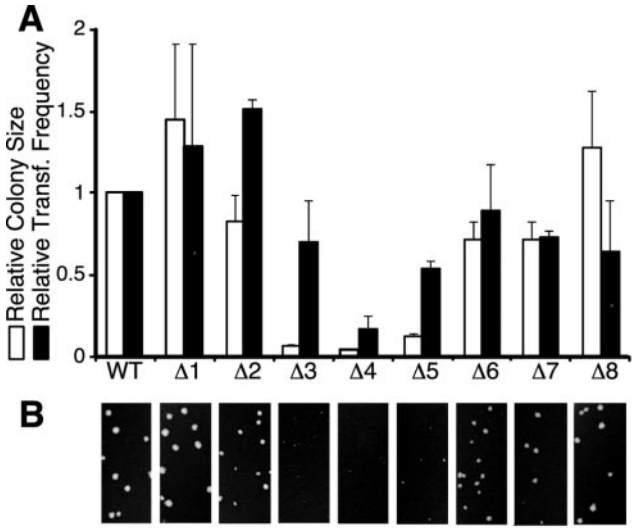

FIG. 4. **Effects of consecutive 100-bp deletions through *ars727* on its activity as a plasmid replication origin.** *A*, effects of the indicated deletions (Δ1–Δ8) of *ars727* in pARS727 on colony size (*white bars*) or on transformation frequency (*black bars*) relative to the corresponding measurements for wild-type pARS727. Three or more measurements were carried out in each case. The *error bars* show standard deviations. *B*, photographs of yeast colonies growing under selection for plasmids containing the indicated mutations. Under these conditions colony size depends on plasmid replication efficiency, which is proportional to replication origin efficiency.

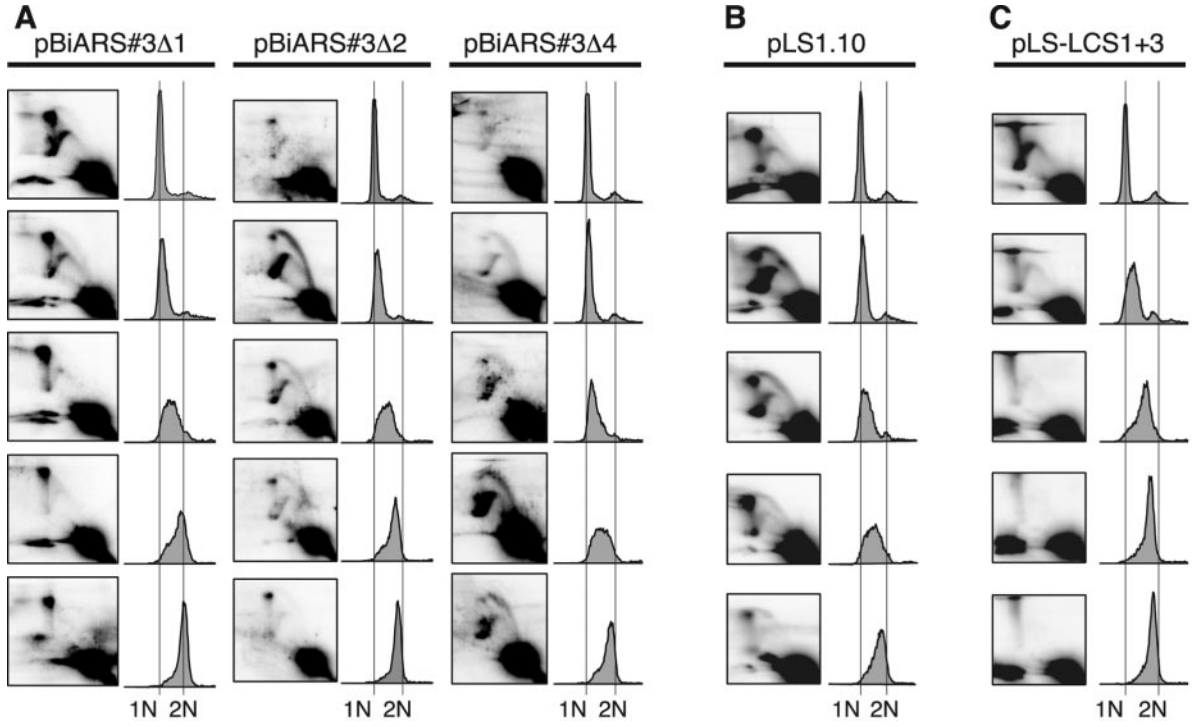

FIG. 5. **Effects on replication timing of mutations within the *ars727* component of pBiARS#3.** *A*, most of the 100-bp deletion mutations had no effect on replication timing. An example is provided by pBiARS#3Δ4. However, deletion of the first 100 bp of *ars727* (pBiARS#3Δ1) led to complete shift from late to early replication timing, and deletion of the second 100 bp (pBiARS#3Δ2) produced a partial shift toward early timing. *B*, linker substitution of nucleotides 91–100 in the *ars727* component of pBiARS#3 produced a partial shift toward early replication timing, similar to the shift in pBiARS#3Δ2. *C*, simultaneous linker substitution of LCS1 plus LCS3 produced a complete shift to early replication timing (as did simultaneous linker substitution of all additional pair-wise combinations of LCS1, LCS2 and LCS3; data not shown). The time points in *C* were at 0, 15, 30, 45, and 60 min after removal of HU; the time points in *A* and *B* were the same as in Fig. 1.

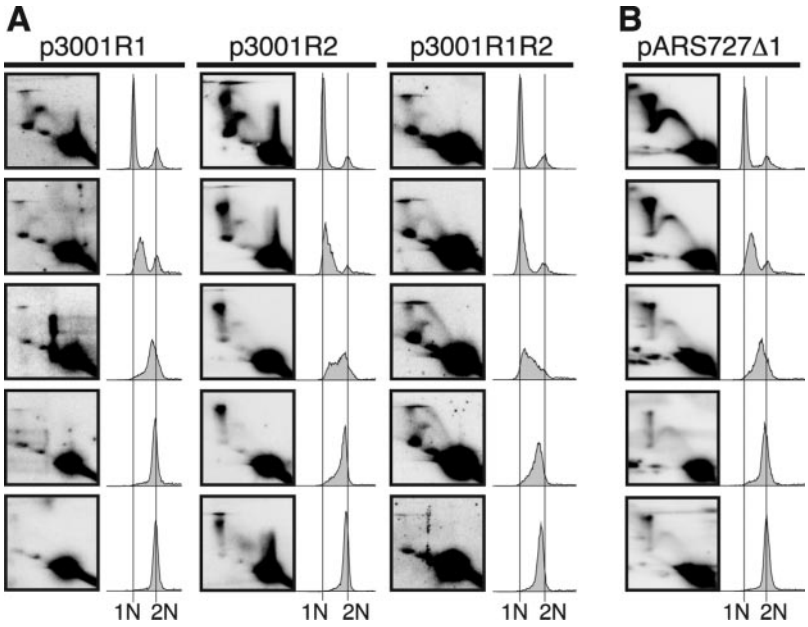

FIG. 6. **Region 1 is essential, and regions 1 plus 2 are sufficient, to force late firing on a nearby origin.** *A*, neither region 1 (*R1*) nor region 2 (*R2*) is sufficient by itself to force *ars3001* to fire late, but the combination of R1 plus R2 (in the absence of the rest of *ars727*) is sufficient. *B*, deletion of region 1 from *ars727* makes the mono-ARS plasmid, pARS727, replicate early. Thus *ars727* normally replicates in late S phase as a result of essential contributions from region 1. The time points in *A* were 0, 15, 30, 45, and 60 min after removal of HU; the time points in *B* were the same as in Fig. 1.

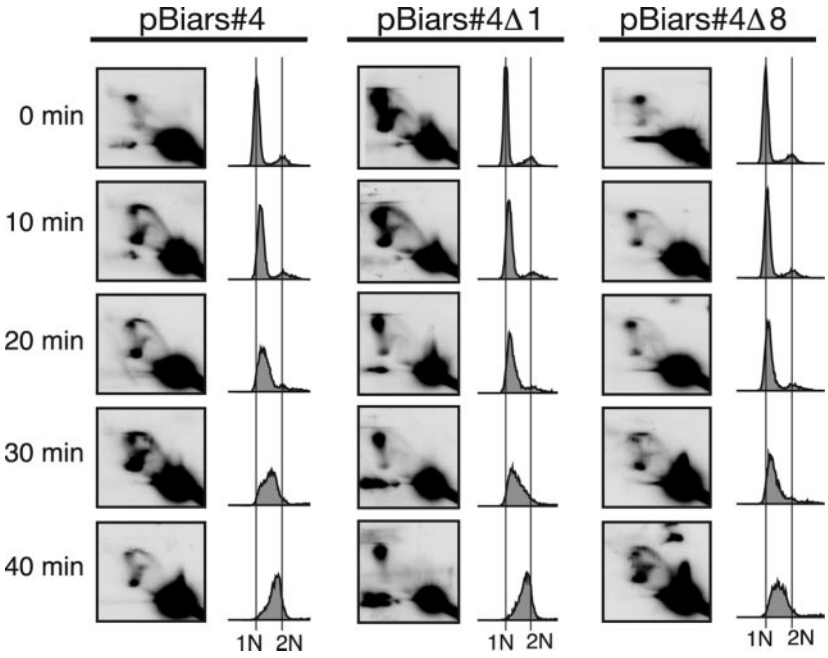

FIG. 7. **Region 1 is essential for forcing late replication on *ars3001*, even when its orientation is reversed and it is more than 700 bp away from *ars3001*.** In pBiARS#4, the orientation of region 1 with respect to *ars3001* is reversed, and region 1 is separated from *ars3001* by the rest of *ars727* (more than 700 bp; Figs. 2 and 3). Nevertheless, as shown here, deletion of region 1 permits pBiARS#4Δ1 to replicate in early S phase. In contrast, deletion of region 8 (which is located adjacent to *ars3001* in pBiARS#4) does not impede the ability of the other sequences in *ars727* to force pBiARS#4Δ8 to replicate in late S phase.

earlier replication than linker substitution of nucleotides 1–10, even though linker substitution of nucleotides 1–10 (which contain most of LCS1) should have inactivated LCS1 (nucleotides 2–11). Thus nucleotides 12–20 appear to have a small effect on replication timing in cooperation with LCS1. Similarly, Supplemental Fig. 1 shows that linker substitution of nucleotides 81–100 permits earlier replication timing than linker substitution of nucleotides 91–100 (LCS2), suggesting that nucleotides 81–90 have a small effect on replication timing in cooperation with LCS2. Note that linker substitution of nucleotides 11–20 or 81–90 alone has no detectable effect on replication timing (Supplemental Fig. 1 and data not shown).

Regions 1 and 2 Are Sufficient to Enforce Late Replication—To test whether a cluster of three LCS elements plus flanking sequences is sufficient to change the replication timing of *ars3001* from early to late, we constructed plasmids in which *ars3001* (with orientation as in pBiARS#3) was flanked on its right side by region 1 alone, by region 2 alone, or by regions 1 + 2. We found that regions 1 + 2 (total of 200 bp), but not region 1 or region 2 alone, were sufficient to force *ars3001* to replicate as late in S phase as it does in pBiARS#3 (Fig. 6*A*). Regions 1 + 2 have no ARS activity of their own (data not shown). Thus *ars3001* is the only ARS element in the plasmids studied in Fig. 6*A*, making it clear that regions 1 + 2 together *delay* the firing of *ars3001* until late S phase rather than inhibiting it.

We also found that deletion of region 1 from *ars727* in the mono-ARS plasmid, pARS727 (Fig. 1*A*), had little effect on ARS activity (Fig. 4) but led to complete loss of late replication timing (Fig. 6*B*). These findings indicate that a cluster of three LCS elements is essential, and may be sufficient, to force at least two different replication origins (*ars727* and *ars3001*) to fire in late S phase in a plasmid context.

Effects of Distance and Orientation—In pBiARS#3, the sequences in region 1 that contribute to forcing late replication on *ars3001* are located adjacent to *ars3001* (Fig. 3*A*). However, pBiARS#4, in which the orientation of *ars727* with respect to *ars3001* is reversed, replicates in late S phase like pBiARS#3

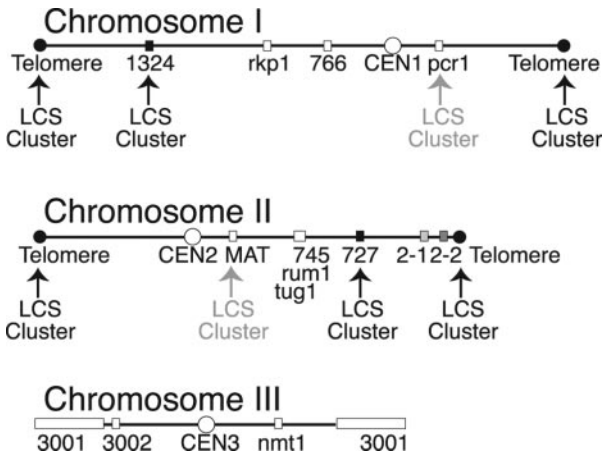

FIG. 8. **Chromosomal locations of all studied early and late replicating regions in fission yeast.** Early replicating regions are *white*; late regions are *black*. Regions with intermediate timing (*ars2-1* and *ars2-2*) have intermediate shades. Regions in which three or more 8/10 matches to the LCS consensus sequence are within 1.5 kb of each other and are also near a replication origin are indicated ("*LCS Cluster*"). In two cases, LCS clusters (indicated in *gray*) are separated from origins by transcribed regions. In the other cases (indicated in *black*), there is no transcribed region between the origin and the LCS cluster. For all other regions, there is no LCS cluster within the indicated region or within 10 kb on either side of it. See Supplemental Table II for detailed data. Note that simple sequence telomeric repeats are found at the ends of all chromosomes. However the telomere-associated sequences, which are more complex repeats, are found consistently only at the ends of chromosomes I and II (31) (www.sanger.ac.uk/Projects/S_pombe/telomeres.shtml). The LCS clusters that we have identified are in the telomere-associated sequences terminal HindIII fragments.

(Figs. 3 and 7). This raises a question. In pBiARS#4, are the *ars727* sequences that are closest to *ars3001* responsible for the late replication of *ars3001*, or does late replication require region 1, which is more than 700 bp from *ars3001* and reversed compared with its orientation in pBiARS#3? To answer this question, we created pBiARS#4Δ1 and pBiARS#4Δ8, which are identical to pBiARS#4 except for deletion of region 1 or region 8 (Fig. 2), respectively. We found (Fig. 7) that pBiARS#4Δ8 (which lacks the 120 bp of *ars727* closest to *ars3001*) replicates in late S phase like pBiARS#4. In contrast, pBiARS#4Δ1 (which lacks the 100 bp of *ars727* furthest from *ars3001*) replicates in early S phase. This result indicates that region 1 is essential for the late replication of *ars3001*, even when it is more than 700 bp away from *ars3001* and in reversed orientation. We conclude that the ability of a cluster of LCS elements to force late replication on a nearby ARS element can be independent of orientation of the LCS cluster (but not necessarily independent of orientation of the ARS element; Fig. 3) and can be independent of distance up to 700 bp or more.

Several Very Late Replicating Chromosomal Origins Are Located Close to Clusters of LCS Elements—The preceding results demonstrate that LCS clusters can affect replication timing in plasmids. But do LCS clusters affect replication timing in chromosomes? Four observations suggest that LCS clusters may contribute to the lateness of many of the very late firing origins in the fission yeast genome. First, in its chromosomal context *ars727* is *passively* replicated in mid to late S phase. The fact that it is passively replicated suggests that it may be programmed by its LCS cluster to replicate so late in S phase that it becomes passively replicated by forks from neighboring earlier firing origins before it has a chance to fire on its own. Second, there are clusters of sequences similar to the LCS at the telomeres of chromosomes I and II, which replicate in very late S phase (21) (Fig. 8). Third, the only other very late firing origin discovered so far in fission yeast, *ori1324* [2] (Fig. 8), is also associated with a cluster of LCS elements. Fourth, only two of the known early firing replication origins in fission yeast are close (<10 kb) to a cluster of LCS elements, and in both of these cases the cluster of LCS elements is separated from the early firing origin by a transcribed region, which is likely to act as a barrier (Fig. 8). Although these observations (Fig. 8) are based on study of only a small portion of the origins in the fission yeast genome, the correlation between clusters of LCS elements and very late firing of nearby origins is striking.

DISCUSSION

The experiments presented here demonstrate that, in fission yeast, clusters of a 10-bp G-rich consensus sequence, the LCS, can enforce late firing on nearby origins that would otherwise fire in early S phase. This is the first high resolution localization, in any organism, of *cis*-acting sequences that affect replication timing.

The next step will be to figure out how LCS elements function to control replication timing, whether in plasmids or in chromosomes. Do they bind a protein and if so which one? Or do they act as structural elements? Answering these questions will require further experiments. *In vivo* footprinting studies will provide information regarding interactions between LCS elements and proteins. Additional mutagenesis studies will help to identify the nucleotides within LCS elements that are most important for their function. Oligonucleotides containing clusters of functional ("wild-type") or non-functional (mutant) LCS elements will provide important tools for purification of specific LCS-binding proteins. Studies comparing the chromatin structures of early firing origins (in the absence of LCS clusters) and the corresponding late firing origins (under the influence of LCS clusters) will assist in evaluating the potential role of chromatin structure in controlling replication timing.

Even with the limited information that is currently available, however, it is possible to reach some preliminary conclusions regarding the possible roles of LCS elements and their mechanisms of action. First, although our initial hypothesis was that fission yeast ARS elements contain internal sequences that affect replication timing (note: that hypothesis appeared to be supported by our initial experiments; Fig. 1 and data not shown), our subsequent results suggested that, as in budding yeast (4), fission yeast sequences controlling replication timing can be located in regions near ARS elements as well as within them. As an example demonstrating that replication timing sequences can be external to origins, consider the results in Fig. 4. These results demonstrate that deletion of *ars727* regions 1 and 2 (the regions containing the LCS cluster that enforces late replication of *ars727*) has no significant effect on the ability of *ars727* to function as a replication origin. Thus the portion of what was previously defined as *ars727* (23) that controls replication timing (regions 1 and 2) is in fact external to the portion of *ars727* that (according to Fig. 4) is important for origin activity. We suggest that, based on Fig. 4, the boundaries of *ars727* should be redefined to include regions 3–7 but exclude regions 1, 2, and 8 (Figs. 2 and 4). According to this new definition, the LCS cluster that controls its replication timing is adjacent to, not inside of, *ars727*. Additional examples of LCS clusters affecting replication timing from positions adjacent to or near (rather than within) ARS elements are provided in Figs. 3, 6, and 7.

Second, the results in Fig. 3 show that pBiARS#1 replicates early, while pBiARS#3 replicates late. The only difference be-

[2] D. L. Scott, C. Yompakdee, and J. A. Huberman, manuscript in preparation.

tween these two plasmids is the orientation of *ars3001* within the plasmid. In pBiARS#1, the "left" end of *ars3001* is adjacent to the LCS cluster in regions 1 and 2 of *ars727*, but in pBiARS#3, the "right" end of *ars3001* is adjacent to the LCS cluster. Thus the results in Fig. 3 show that the susceptibility of an ARS element to control by an LCS cluster can be affected by the orientation of the ARS element relative to the LCS cluster. A possible explanation of this phenomenon is that *ars3001* may contain a barrier sequence near its left end, and this barrier may prevent the spread of the late timing effect of the LCS cluster to the active internal portion of *ars3001*. Further experiments are needed to test for this hypothetical barrier sequence and, if it exists, to characterize it.

Third, although the orientation of an origin relative to an LCS cluster can be important, the results in Figs. 3 and 7 suggest that both the orientation and the distance of the LCS cluster relative to the origin can have *no* effect. In pBiARS#3, the LCS cluster is adjacent to *ars3001*, while in pBiARS#4, the LCS cluster is more than 700 bp away from *ars3001* and in the opposite orientation. Nevertheless, in both cases *ars3001* is forced to fire in late S phase (Fig. 3). Additional experiments are needed to determine precisely the distance over which an LCS cluster can control an origin.

Finally, our observations suggest that LCS clusters are not the only type of sequence that can control replication timing in fission yeast. In addition to testing the replication timing of mono-ARS plasmids bearing *ars3001* and *ars727* (Fig. 1), we also tested a mono-ARS plasmid containing *ars2-2*, which replicates in mid to late S phase (21) in its normal chromosomal location. We found that the plasmid with *ars2-2* also replicated in mid to late S phase (data not shown), suggesting that *ars2-2* contains internal sequences that determine its replication timing. However, *ars2-2* does not contain a cluster of LCS elements (Fig. 8). It is interesting that *ars2-2* replicates in mid to late S, while all of the origins known or suspected to be controlled by LCS clusters (*ars3001* and *ars727* in the plasmids described here and *ori1324* and telomere-associated sequences in chromosomes) replicate in very late S phase (Fig. 8). It is possible that different sets of *cis*-acting sequences regulate the firing of origins at different times during S phase.

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