ORC- and Cdc6-dependent complexes at active and inactive chromosomal replication origins in *Saccharomyces cerevisiae*

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We have developed a genomic footprinting protocol which allows us to examine protein–DNA interactions at single copy chromosomal origins of DNA replication in the budding yeast *Saccharomyces cerevisiae*. We show that active replication origins oscillate between two chromatin states during the cell cycle: an origin recognition complex (ORC)-dependent post-replicative state and a Cdc6p-dependent pre-replicative state. Furthermore, we show that both post- and pre-replicative complexes can form efficiently on closely apposed replicators. Surprisingly, ARS301 which is active as an origin on plasmids but not in its normal chromosomal location, forms ORC- and Cdc6p-dependent complexes in both its active and inactive contexts. Thus, although ORC and Cdc6p are essential for initiation, their binding is not sufficient to dictate origin use.

Keywords: ARS/genomic footprinting/origin interference/origin recognition complex/replicator

Introduction

DNA replication in eukaryotic cells initiates from multiple replication origins throughout S phase, with some origins firing early and others late (Huberman and Riggs, 1966; Fangman and Brewer, 1992). In addition to this temporal complexity, the same origins are not necessarily used in all cell types. For example, in early metazoan embryos, virtually any sequence can act as a replication origin; however, origin use becomes restricted later in development (Harland, 1981; Mechali and Kearsley, 1984; DePamphilis *et al.*, 1988; Martinez Salas *et al.*, 1988; Crevel and Cotterill, 1991; Hyrien and Mechali, 1992, 1993; Mahbubani *et al.*, 1992; Hyrien *et al.*, 1995). Understanding the regulation of origin usage at these levels will require characterization of protein–DNA interactions at these sequences in their normal chromosomal locations.

We previously have characterized protein–DNA interactions at two budding yeast replication origins, ARS1 and the 2 μ m origin, on high copy number plasmids using genomic footprinting (Diffley and Cocker, 1992; Diffley *et al.*, 1994; Dahmann *et al.*, 1995; Rowley *et al.*, 1995; Cocker *et al.*, 1996; Piatti *et al.*, 1996). In this technique, cells are permeabilized and immediately treated with DNase I. The position of nuclease cleavage sites is determined after DNA purification by multiple rounds of primer extension with *Taq* DNA polymerase (Perkin-

Elmer) using an end-labelled oligonucleotide which anneals outside the region of interest. This analysis has revealed a number of important features. (i) Origins on high copy plasmids exist in two chromatin states during the cell cycle, a post-replicative state which first appears at about the time replication initiates and persists until the end of mitosis, and a pre-replicative state present from the end of mitosis until DNA replication initiates (Diffley *et al.*, 1994). (ii) The pattern of DNase I protection and hypersensitivity in the post-replicative state is very similar to that produced *in vitro* with purified origin recognition complex (ORC) (Diffley and Cocker, 1992; Diffley *et al.*, 1994). (iii) Footprints at ARS1 *in vivo* as well as ORC binding *in vitro* require both the A element containing the ARS consensus sequence (ACS) and the adjacent B1 element (Rao and Stillman, 1995; Rowley *et al.*, 1995). (iv) The pre-replicative complex (pre-RC) which generates an extended region of protection across the B domain requires the product of the *CDC6* gene (Cdc6p) for both its establishment and maintenance (Cocker *et al.*, 1996). (v) In the absence of Cdc6p, the 2 μ m origin in G₁ reverts to a state resembling the post-replicative state, arguing that ORC is also a component of pre-RCs (Cocker *et al.*, 1996). (vi) Cdc6p-driven pre-RC formation can normally only occur during G₁ and is blocked by the action of the Cdc28–Cyclin B kinase later in the cell cycle (Dahmann *et al.*, 1995; Piatti *et al.*, 1996).

Results

Single copy genomic footprinting

In order to begin to study the regulation of initiation at chromosome replication origins, we have made several modifications which have increased the sensitivity of our primer extension reactions and have allowed us to examine sequences present at single copy in the yeast genome. The two most important changes were, first, use of Vent Exo[−] DNA polymerase (New England Biolabs) instead of *Taq* DNA polymerase and, second, reduction in the amount of labelled oligonucleotide primer by ~10-fold. Methods of cell lysis and chromatin digestion with DNase I have not been altered since they previously had been optimized for examination of unstable chromatin structures at replication origins (Diffley *et al.*, 1994). The first four lanes of Figure 1 show the products of sequencing reactions on genomic DNA using an ARS1-specific labelled oligonucleotide. The sequence of a single copy of ARS1 in its chromosomal location can be seen, demonstrating the sensitivity and specificity of this new protocol.

These footprinting conditions were first used to examine the well-characterized replicator, ARS1. In Figure 1, we compare footprints at ARS1 when present as a single copy in its normal chromosomal location (lanes 5–13) and when present on a high copy number plasmid (YRp7) (lanes

Fig. 1. Chromatin organization of the ARS1 replicator on a high copy plasmid and in its normal chromosomal location during the cell cycle. Cells (W303-1a) either with or without a high copy plasmid (YRp7) containing ARS1 were subjected to genomic footprinting using the new protocol for primer extension. Positions of the A, B1, B2 and B3 elements (Marahrens and Stillman, 1992) are indicated by the white boxes on the left of the figure. The DNase I-hypersensitive site in the B1 element is indicated with an asterisk. Protection adjacent to the B1 element is indicated with a white box, while the extended G₁-specific protection is indicated with the grey box on the right of the figure.

14–22). We have shown previously that both ARS1 and the 2 μ m origin on high copy number plasmids are in the pre-replicative state in cells blocked with the α -factor mating pheromone and are in the post-replicative state in cells blocked with the microtubule inhibitor nocodazole (Diffley *et al.*, 1994). Examination of footprints in Figure 1 lanes 14–22 indicates that this conclusion is independent of the method of primer extension. Using the new protocol, DNase I-hypersensitive sites (asterisks) can be seen

extending into domain B and an additional region of protection can be seen between the B1 and B2 elements (white box) in nocodazole-blocked cells (Figure 1, lanes 17–19). On this strand (T-rich), the A element containing the ACS is generally cleaved quite poorly by DNase I even in naked DNA in most ARSs, making determination of protection in this region difficult. Because of the strong DNase I hypersensitive sites, however, this strand is usually the most informative. The protections and hyper-

Fig. 2. Chromatin organization of the histone H4ARS. The DNA samples from W303-1a in (A) were subjected to primer extension using primer CS4 specific for H4ARS. The position of the ACS is indicated on the left and the DNase I-hypersensitive site adjacent to the ACS is indicated with the asterisk. The extension of the G₁-specific protection is indicated with the grey box. Cells carrying plasmid pDK368-7 (Hogan and Koshland, 1992) were arrested in G₂/M (Noc) or in G₁ (α-factor) and subjected to footprinting analysis. Primer extension was performed with primer CS4 (H4ARS). In (B) the ratios of naked DNA and post-replicative chromatin (Noc) (lanes 1–4) or post-replicative (Noc) and pre-replicative (α) chromatin were subjected to primer extension as described in Materials and methods.

sensitive sites seen in the post-replicative state are very similar to those produced with purified ORC. In G₁-blocked cells (lanes 20–22), the pre-RC can be seen which is characterized by suppression of the ORC-induced hypersensitive sites and extended protection across the B1 and B2 elements (grey box). Both the post- and pre-replicative states shown with this new protocol are very similar to those seen previously (Diffley *et al.*, 1994).

In Figure 1 lanes 5–13, the single chromosomal copy of ARS1 was examined using identical conditions. Although the overall signal is weaker and hence requires longer exposure times, the patterns of DNase I cleavage in both α factor- and nocodazole-blocked cells are virtually identical to those seen on high copy plasmids, indicating that chromosomal ARS1 forms both post- and pre-RCs.

Pre- and post-replicative complexes at tandem replicators

To extend our observations, we have also examined the ARS associated with the histone H4 gene (H4ARS) in its normal chromosomal context (Figure 2). As with ARS1, DNase I-hypersensitive sites can be seen across the domain B-equivalent (Bouton and Smith, 1986) at this ARS in nocodazole-blocked cells (lanes 4–6). These sites are in

the same positions as those induced by purified ORC *in vitro* (Bell and Stillman, 1992). Again, as in the case of ARS1, the DNase I-hypersensitive sites are suppressed in α-factor-blocked cells, and an extended region of protection across domain B can be seen (lanes 7–9). Thus, post- and pre-RCs may be a general feature of yeast ARSs.

Many mutants with defects in initiating DNA replication including both *orc* and *cdc6* mutants exhibit a high rate of plasmid loss. In many cases, this plasmid loss rate is reduced significantly if the plasmid contains multiple origins. A plasmid widely used in these experiments, pDK368-7, contains seven tandem copies of the H4ARS as well as a single copy of ARS1 (Hogan and Koshland, 1992). We were interested in addressing whether post- and pre-RCs formed efficiently on all copies of the H4ARS in this plasmid, particularly in light of the fact that nearby origins appear to interfere with each others function in budding yeast (Brewer and Fangman, 1993, 1994; Marahrens and Stillman, 1994). The oligonucleotide primer used in Figure 2A anneals within each tandem repeat on pDK368-7; therefore, the footprint obtained represents a composite of all copies of the H4ARS. Since both the post- and pre-replicative state of these origins (lanes 10 and 11) are very similar to the single chromo-

Fig. 3. The post-replicative complex at the chromosomal replicator ARS1 is defective in an *orc2* mutant. (A) Logarithmically growing cultures of wild-type (W303-1b) and congenic *orc2-1* cells were grown at 25°C and split in half. Half the culture was held for 3 h at 25°C while the other half was transferred to 37°C for 3 h. (B) Cultures of wild-type (W303-1b) and congenic *orc2-1* cells were arrested in G₂-M with nocodazole at 25°C and then shifted to 37°C in the presence of nocodazole. At the indicated times after the temperature shift, aliquots of the cultures were withdrawn and subjected to footprinting analysis. Primer extension was performed with primer JD51 (ARS1). The asterisk indicates the hypersensitive site in B1 and the arrows indicate two cleavage sites between B1 and B2 which are protected in wild-type chromatin.

somal copy, it appears that formation of both post- and pre-RCs occurs normally at these tandem origins.

The validity of this conclusion depends on the sensitivity of the assay. We, therefore, performed mixing experiments to determine how much loss of either post- or pre-RCs would be detectable. In Figure 2A lanes 1–4, varying ratios of naked DNA were mixed with post-replicative chromatin and subjected to primer extension. In this experiment, the intensity of the ORC-induced hypersensitive site (asterisk) decreases linearly with increasing relative amounts of naked DNA. From this, we conclude that we could easily detect a 25% reduction in ORC binding *in vivo*. We have also performed mixing experiments with post- and pre-replicative chromatin (lanes 5–8). Again, the intensity of the ORC-induced hypersensitive site decreases with increasing amounts of pre-replicative chromatin. Moreover, several sites adjacent to the ACS (grey box) are specifically protected in the pre-RC (see Figure 2A). The intensity of these sites also decreases with increasing amounts of pre-replicative chromatin. Therefore, we conclude that we could easily detect reasonably small reductions (<25%) in either post- or pre-RCs. Consequently, we conclude that at least 75% and probably more of the tandem H4ARS elements contain post-RCs in G₂ and pre-RCs in G₁.

ORC2 is essential for chromosomal post-replicative complexes

We have shown previously that the footprint associated with post-replicative origins appears very similar to that obtained *in vitro* with purified ORC (Diffley *et al.*, 1994). The cloning of genes encoding ORC subunits and the availability of conditional *orc* mutants has allowed us to examine this question more directly. The gene encoding the 72 kDa ORC2 subunit is essential for viability, and mutants with compromised ORC2 function show elevated plasmid loss rates and reduced initiation frequency (Bell *et al.*, 1993; Foss *et al.*, 1993; Micklem *et al.*, 1993; Liang *et al.*, 1995). We have examined whether the footprint at ARS1 is thermolabile in the *orc2-1* mutant (Foss *et al.*, 1993). In the first experiment, asynchronous cultures of a wild-type and an *orc2-1* mutant were either left at 25°C or shifted to 37°C for 3 h and subjected to footprinting analysis. As shown in Figure 3A, both the hypersensitive site in B1 (asterisk) and the protection between B1 and B2 (arrows) are relatively stable at ARS1 at 37°C in the wild-type (*ORC2*) parental strain (lanes 2 and 3). However, in the *orc2-1* mutant, even at the permissive temperature, the intensity of the B1 hypersensitive site and the protection between B1 and B2 is greatly reduced (lane 4), consistent with the fact that this mutant shows significant initiation

Fig. 4. The pre-replicative complex at the chromosomal replicator ARS1 is defective in a *cdc6* mutant. Wild-type and congenic *cdc6-1* strains were arrested at 25°C in G₂-M with nocodazole (Noc) or in G₁ with α -factor (α 25°C). G₁-arrested cells were then shifted for 1 h to 37°C (α 37°C). At the different stages, aliquots of the cultures were withdrawn and cells subjected to footprinting analysis. DNase I-digested naked DNA (ND) is shown as control. Labelling is as in Figure 1.

defects even at the permissive temperature. Moreover, after shifting to the restrictive temperature, the B1 hypersensitive site and the protection between B1 and B2 virtually disappear (lane 5). To refine our analysis, we repeated the experiment using cultures of cells that had first been blocked in G₂-M with nocodazole. As with the asynchronous culture, the hypersensitive site in B1 and the protection between B1 and B2 is stable at 37°C in the wild-type strain (lanes 4–12). Furthermore, both the B1 hypersensitive site and the protection between B1 and B2 are reduced in the *orc2-1* mutant at the permissive temperature (lanes 16–18) and are essentially absent at the restrictive temperature (lanes 19–24). Thus, the post-RC is thermolabile in the *orc2-1* mutant, providing direct evidence that ORC2 is required for the maintenance of this complex *in vivo*.

Cdc6p is essential for chromosomal pre-replicative complexes

We have shown previously that the Cdc6 protein is essential for the formation and maintenance of pre-RCs on the high copy 2 μ m plasmid. We have used a similar approach to examine the role of Cdc6p in pre-RCs at ARS1 in its chromosomal location. Pre-RCs, evidenced by suppression of the ORC-induced hypersensitive site in B1 and by additional protection across domain B, are stable at ARS1 at 37°C (Figure 4, lanes 7–12). In the *cdc6-1* mutant, however, the pre-RC is largely lost after 1 h at 37°C (lanes 22–24), very similar to results seen at the 2 μ m origin (Cocker *et al.*, 1996). Thus, chromosomal origins of DNA replication form ORC-dependent post-RCs and Cdc6-dependent pre-RCs. Moreover, when pre-RCs are lost in G₁ by thermal inactivation of Cdc6, origins return to a state very similar to the post-replicative state, arguing that ORC is also a component of pre-RCs.

Protein complexes at the inactive ARS301

In budding yeast, there are a number of sequences that can act as ARSs on plasmids but do not support initiation in their normal chromosomal context (Dubey *et al.*, 1991;

Table I. Loss rates of ARS1- and ARS301-containing plasmids

ARS1	ARS301
4.3% (905)	7.3% (749)

ARS1 and ARS301 (see Materials and methods) were assayed for plasmid loss rates using the half sectorized colony method described by Hieter *et al.* (1985). The total number of sectorized colonies analysed is given in parentheses.

Newlon *et al.*, 1991, 1993; Greenfeder and Newlon, 1992; Dershowitz and Newlon, 1993). One of the best characterized of these is ARS301, also known as *HML E* (Dubey *et al.*, 1991) which has been studied in some detail (Dubey *et al.*, 1991; reviewed in Diffley, 1995). Dubey *et al.* (1991) have shown previously that ARS301 is <15% active in its chromosomal location. We examined the ability of a short (207 bp) fragment containing ARS301 to support plasmid maintenance. Table I shows that the ARS301-containing plasmid is lost at a rate of <8% per generation. This is slightly more than the loss rate of the full ARS1 but is similar to the loss rate of domains A and B of ARS1 alone (Diffley and Stillman, 1988; Marahrens and Stillman, 1992). Therefore, ARS301 is >90% active on a plasmid and there is at least a 6-fold difference in the activity of ARS301 on a plasmid and in the chromosome. We could examine this sequence when it is active (plasmid) and when it is inactive (chromosomal) in the same cells by using different oligonucleotide primers. Figure 5A shows that in nocodazole-blocked cells, two strong DNase I hypersensitive sites are found 3' to the T-rich strand of the ACS at positions identical to those produced by purified ORC *in vitro* (Bell *et al.*, 1993) on both the plasmid and chromosomal copies of ARS301 (compare lanes 4–6 with lanes 13–15). In addition, these hypersensitive sites are suppressed in α -factor-blocked cells in both contexts as well (compare lanes 7–9 with lanes 16–18). We note that at ARS301, this pre-RC occupies a shorter region of domain B than at either ARS1, H4ARS or the 2 μ m origin; however, it is

Fig. 5. Replicative complexes at ARS301 in its active and inactive states. (A) Strain W303-1a carrying plasmid pCS1 (ARS301) was arrested in G₂-M (Noc) or in G₁ (α -factor) and cells were subjected to footprinting analysis. DNase I digestions of naked DNA were included for control. Primer extensions were performed with primer CS8 specific for the chromosomal ARS301 locus (chromosome) or with primer JD63b specific for the plasmid sequence (plasmid). On the left, the binding site for RAP1 protein and the ACS are indicated with white boxes. Asterisks indicate the DNase I-hypersensitive sites adjacent to the ACS. (B) The middle lane in each of the DNase I titrations was scanned and plotted for both the chromosomal and plasmid copies of ARS301 in both G₁ (black line) and G₂-M (grey line). The ACS is indicated as a grey box. The ORC-induced hypersensitive sites are indicated as asterisks.

Fig. 6. ORC- and Cdc6-dependent protein complexes at ARS301. (A) ORC2-dependent post-replicative complexes at ARS301. The DNA samples from Figure 3A (*ORC*⁺ and *orc2-1*) were subjected to primer extension using primer CS8 (ARS301). (B) Cdc6p-dependent pre-replicative complexes at ARS301. *cdc6-1* mutant cells were arrested at 25°C in G₂-M (Noc) or in G₁ (α 25°C). G₁-arrested cells were shifted to 37°C for 2 h in the presence of α -factor (α 37°C). Cells were collected and subjected to footprinting analysis. Primer extension was performed with primer CS8 (ARS301).

identical in both the active and inactive copy. One difference that we have seen consistently between the plasmid and chromosomal copies of ARS301 is that the binding site for the RAP1 protein appears saturated in the chromosome but not in the plasmid. Whether this has any effect on ARS301 origin function is currently unknown. The data in Figure 5A have been quantified by densitometric scanning of the autoradiograms and are shown in Figure 5B. From this, it is apparent that both post- and pre-RCs form at the active and inactive copies of ARS301.

That the DNase I-hypersensitive sites in the chromosomal copy of ARS301 are due to the binding of ORC is shown in Figure 6A; these sites (asterisks) are diminished in the *orc2-1* mutant at the permissive temperature (compare lanes 2 and 4) and are nearly completely lost in this mutant at the non-permissive temperature (lane 5). Additionally, the pre-RC seen in α -factor-blocked cells is dependent upon the Cdc6 protein as shown in Figure 6B; the ORC-induced hypersensitive sites which are suppressed in G₁-blocked cells reappear after shifting the *cdc6-1* mutant to the restrictive temperature (lane 3). Thus, the inactive ARS301 forms an ORC-dependent post-RC and a Cdc6p-dependent pre-RC.

Discussion

The results presented here demonstrate that active chromosomal replication origins form the same protein-DNA interactions as origins of replication on high copy plasmids. Previously we have shown that the post-replicative footprint on high copy plasmids is very similar to that generated by purified ORC *in vitro* (Diffley and Cocker, 1992; Diffley *et al.*, 1994). Here we have shown that this footprint at ARS1 is the same in its normal chromosomal location as it is on a high copy plasmid. Moreover, the post-RC is thermolabile in an *orc2* temperature-sensitive mutant, providing additional strong evidence that ORC is a major component of the post-RC. As other *orc* mutants become available, we should be able to examine the roles of the individual ORC subunits in the post- and pre-RCs. Taken together, our results suggest that the formation of ORC-dependent post-RCs and Cdc6p-dependent pre-RCs is a general feature of yeast replication origins.

Mutations in the *CDC6* gene as well as mutations in genes encoding ORC subunits cause increases in plasmid loss rates (Hartwell and Smith, 1985; Palmer *et al.*, 1990; Hogan and Koshland, 1992; Bell *et al.*, 1993; Foss *et al.*, 1993; Micklem *et al.*, 1993; Loo *et al.*, 1995) as well as decreases in the frequency of origin firing (Fox *et al.*, 1995; Liang *et al.*, 1995). Our results provide a molecular explanation for these results; namely, *orc2-1* and *cdc6-1* mutants show defects in levels of post- and pre-RCs respectively, even at the permissive temperature (Cocker *et al.*, 1996 and Figure 3). The defect in plasmid maintenance for *orc2-1* and *cdc6-1* mutants is greatly reduced in plasmids containing seven tandem copies of the H4ARS (Hogan and Koshland, 1992; Foss *et al.*, 1993). Since post- and pre-RCs can form efficiently on these adjacent replication origins (Figure 2), we propose that the suppression of plasmid instability occurs by simply increasing the probability that at least one origin on the plasmid will form both post- and pre-RCs.

That the seven tandem copies of the H4ARS appear to be able to form post- and pre-RCs efficiently is interesting for another reason. It has been shown previously that nearby origins interfere with each others function and this interference can occur over distances of several kilobases (Brewer and Fangman, 1993, 1994; Marahrens and Stillman, 1994). In the seven H4ARS plasmid, pDK368-7, the tandem ARSs are on fragments of <0.4 kb. We do not know whether each of these origins is active in this plasmid; however, these experiments indicate that mere proximity of origins is not sufficient to preclude efficient formation of either post- or pre-RCs. Thus, the phenomenon of origin interference or replicator dominance may not occur at the level of exclusion of either ORC or Cdc6p binding.

In a similar vein, our results indicate that a well-documented inactive replication origin, ARS301, also oscillates between ORC-dependent post-RCs and Cdc6p-dependent pre-RCs. Thus, although ORC and Cdc6p are essential for initiating DNA replication, their binding is not sufficient to guarantee the subsequent use of this origin. In trying to understand why this ARS is inactive, therefore, other steps in assembly of initiation complexes must be considered. The inactivity of ARS301 is not due to the fact that it lies within a domain of transcriptional

repression since it is still inactive in *sir1* and *sir4* mutants in which transcription of the *HM* loci is derepressed (Dubey *et al.*, 1991). Perhaps ARS301 in its normal chromosomal location is unable to interact with the MCM proteins, which enter the nucleus at about the same time that pre-replicative complexes form (Hennessy *et al.*, 1990; Yan *et al.*, 1993; Dalton and Whitbread, 1995). Alternatively, it may be unable to interact with Dbf4p, the regulatory subunit of the Cdc7 protein kinase previously shown to interact with active yeast ARSs *in vivo* (Dowell *et al.*, 1994).

ARS301 lies in a region of 'silent' chromatin which is largely inaccessible to factors such as those involved in RNA polymerase II- and III-dependent transcription as well as to endonucleases both *in vivo* and *in vitro* (Strathern *et al.*, 1982; Kostriken *et al.*, 1983; Klar *et al.*, 1984; Schnell and Rine, 1986; Laurenson and Rine, 1992; Loo and Rine, 1994). Thus, it is interesting that both ORC and Cdc6p are capable of gaining access to ARS301 efficiently in each cell cycle. Perhaps ARS301 lies in an accessible 'window' within *HML* or, alternatively, perhaps this region is not inaccessible to all *trans*-acting factors. In this regard, the first step in mating type switching (HO endonuclease cleavage) occurs at *MAT* but not the *HM* loci; however, the subsequent steps of recombination are not blocked at the *HM* loci. This recombination reaction, however, requires a number of gene products which appear to have a specific role in dealing with this inaccessible chromatin structure (Sugawara *et al.*, 1995).

Materials and methods

Plasmids and strains

Unless otherwise indicated, strains used were derived from W303-1a (*MATa ade2-1 ura3-1 his3-11MAT15 trp1-1 leu2-3,112 can1-100*) or W303-1b (*MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*).

A 207 bp fragment containing the ARS301 sequence was obtained by PCR (95°C for 30 s, 52°C for 30 s and 72°C for 1 min, 30 cycles) using A364a (*MATa ura1 ade1 ade2 tyr1 his7 lys2 gall-1*) genomic DNA as substrate, 2 U of Vent DNA polymerase (N.E.Biolabs), the 39mer oligonucleotide CS9 (5'-AAA AAG GAT CCT AGG TGT ATC GTA ATG GAA TGT AAT TTC-3') and the 37mer oligonucleotide CS10 (5'-AAA AAA AGC TTG GCC AAA TAC TAA GTA CAT TAA TTA G-3') as primers. The PCR product was digested with *Bam*HI and *Hind*III endonucleases and cloned in the vector pRS306 (Sikorski and Hieter, 1989), thus giving plasmid pCS1. This was also cloned into YRp14/CEN4 and the rate of loss of this plasmid was determined using the half sectoring assay (Hieter *et al.*, 1985).

Single copy genomic footprinting

Cell lysates for genomic footprinting, DNA digestions and DNA purification were made as previously described (Diffley and Cocker, 1992; Diffley *et al.*, 1994). Primer extension reactions were performed in a final volume of 30 µl using 400 ng of DNase I-digested DNA as substrate, 3 U of Vent Exo⁻ DNA polymerase (N.E.Biolabs), Vent buffer containing MgSO₄ at a final concentration of 15 mM, 200 µM dNTPs and 0.35 pmol of the 5' end-labelled indicated oligonucleotide. Direct genomic sequencing reactions contained 400 ng of *Eco*RI-digested yeast genomic DNA and the dideoxynucleotide mixtures as described in the CircumVentTM DNA sequencing Kit (N.E.Biolabs). After reaction (95°C for 1 min and 72°C for 1.5 min, 30 cycles), products were treated as previously described (Diffley and Cocker, 1992), separated on 5% sequencing gel and visualized by autoradiography.

Primers utilized in this study are JD51 (Diffley and Cocker, 1992) for ARS1, CS4 (5'-GTG TAG AAG ACC ACA CAC GAA AAT CCT GTG-3') for H4ARS, CS8 (5'-GAT TTG GCC CCC GAA ATC GAT AAT AAT GGC C-3') for chromosomal ARS301 and JD63b (Rowley *et al.*, 1995) for plasmid pCS1 analysis.

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