

Mapping of early firing origins on a replication profile of budding yeast

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Abstract

Background: Understanding of the firing time determination of replication origins in the entire genome will require a genome-wide survey of replication origins and their mapping on chromosomes. A microarray technology was applied to obtain a genome-wide profile of DNA replication and to classify early firing origins.

Results: A total of 260 potential replication origins (PROs) were identified in the entire budding yeast genome: 247 as defined peaks on the replication profile and 13 as regions located in the chromosomal termini. Based on the firing time, the 247 PROs

were classified into 143 early PROs and 104 late PROs, that were not randomly distributed on chromosomes but formed separated clusters. Most of the early PROs were found to fire in the presence of hydroxyurea, indicating that they were free from the control of the intra-S-checkpoint mediated by Mec1 and Rad53.

Conclusions: The monitoring method of DNA replication and the analysis method of microarray data used in this study proved powerful for obtaining a genome-wide view of the initiation and progression of DNA replication.

Introduction

The budding yeast genome, which comprises 16 chromosomes, replicates from a number of replication origins in the S phase. The S phase lasts 25–30 min under normal culture conditions and DNA strands of 13.5M bp in total are duplicated. The mechanism of both initiation and regulation of DNA replication has been extensively studied (Campbell & Newlon 1991; Sugino 1995; Kelly & Brown 2000; Diffley 2001). Replication origins have specific nucleotide sequences, to which several proteins bind to form an initiation/replication complex. These origins have their own time of firing in the S phase and are classified into early origins and late origins. The early origins fire in the presence of hydroxyurea (HU), while the late origins do not. HU is an inhibitor of ribonucleotide reductase, which is required for the synthesis of dNTPs. The nucleotide depletion caused by HU would activate the intra-S-checkpoint and block the firing of the late origins (Santocanale & Diffley 1998; Shirahige *et al.* 1998; Desany *et al.* 1998). The molecular mechanism

determining the time of origin firing has not been well understood. A genome-wide fractionation of early and late origins and their positioning on the chromosomes would be necessary for a more complete understanding of the timing mechanism of origin firing in relation to the replication of the entire genome. Here, we show a genome-wide replication profile on which early origins, being able to fire in the presence of HU, are mapped.

Results

Copy number changes detected by a microarray technology

DNA microarray technology was used to obtain a replication profile of the yeast genome. Recently, Fangman's group analysed the replication profile by combining a density-separation method and a DNA microarray technology (Raghuraman *et al.* 2001). Their strategy was based on isotopically labelling of DNA in order to differentiate newly synthesized DNA from not-yet-synthesized DNA in the progression of DNA replication. We took a more direct approach: monitoring the change of copy number from one to two during DNA replication

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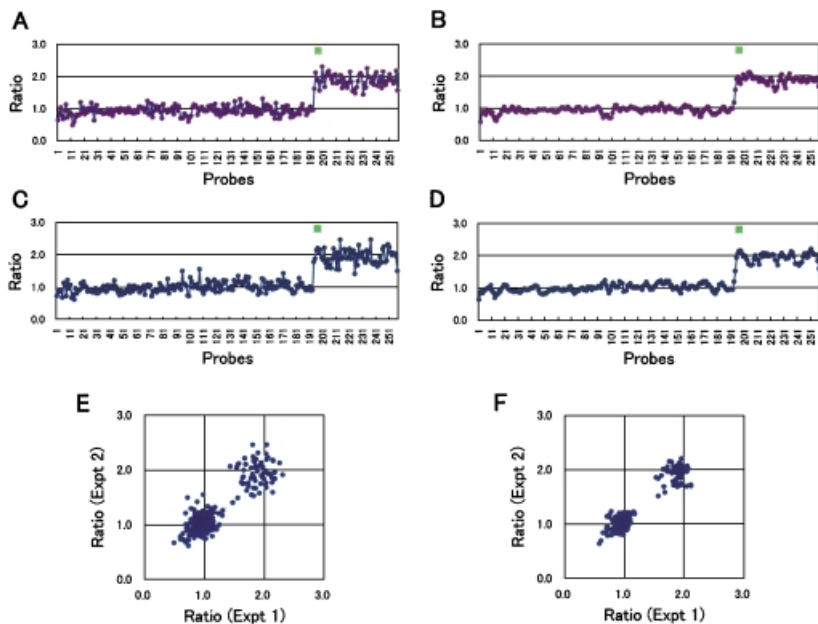


Figure 1 Identification of two-copy regions on chromosome IX. Genomic DNA was isolated from the mutant strain SH5752 which has an extra minichromosome originating from the entire right arm of chromosome IX and its wild-type strain W303-1A and separately hybridized to oligonucleotide microarrays. Hybridization signals from 251 probes from the mutant strain are shown as ratios to the wild-type strain. Two-repeated experimental results are shown: (A) and (B) from the first experiment and (C) and (D) from the second experiment. In (B) and (D), the signal ratios were converted into means of the neighbouring three ratios. Numbers in (A–D) on the horizontal axes indicate probes placed in the nucleotide sequence order on chromosome IX. Squares indicate the centromere position. (E) and (F) show relationships between the two experimental results, before and after the smoothing procedure, respectively.

using DNA microarray technology. As a beginning, the sensitivity of the microarray technology was examined. Genomic DNA was extracted from a mutant strain which had an extra minichromosome constructed from the entire right arm of chromosome IX, labelled with a biotinyl nucleotide and used for hybridization to oligonucleotide microarrays. Hybridization signals of 251 probes on chromosome IX from the mutant strain were compared with those from the original wild-type. Only the probes in the right arm of chromosome IX exhibited higher signals and their ratios to the wild-type strain reached around 2.0 (Fig. 1). Signal ratios in the probes on the left arm of chromosome IX and all the other chromosomes remained around 1.0. The use of mean ratios, calculated from three neighbouring ratio values, can smooth the data. These results were reproducible in repeated experiments. We concluded that the microarray technology we used allowed us to monitor small changes in copy number of the yeast genome.

DNA replication profile of the whole yeast genome

The cell cycle of yeast cells was synchronized by treatment with α -factor. After release from the G1 block, the cells were harvested in every 2.5 min and their genomic DNAs were hybridized to microarrays. A data set of hybridization signals from the 8105 probes was obtained at each sampling time, from 0 to 55 min after the release. The relative DNA content of the cells was measured by flow cytometry and a logistic curve was obtained (Fig. 2). The DNA content on the curve was used for scaling of

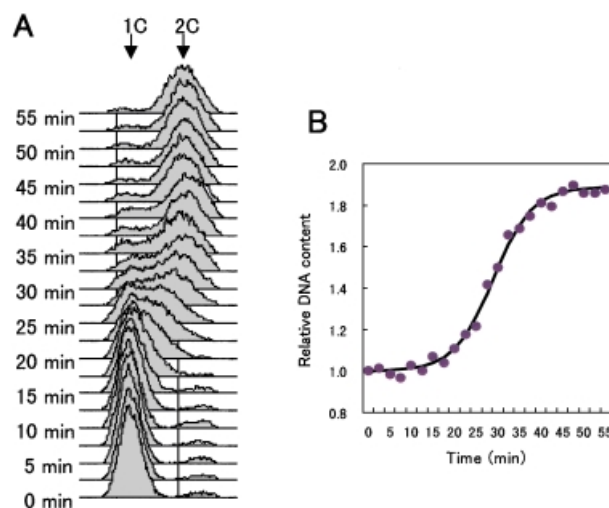


Figure 2 Flow cytometric analysis of cell cycle progression. Cells arrested at the G1 phase with α -factor were released from the block and incubated at 26 °C. Samples were collected every 2.5 min and analysed with flow cytometry (FACS). 1C and 2C in (A) indicate the DNA contents. Relative DNA contents in (B) were calculated from the FACS image in (A). A logistic curve fitting the change in the relative DNA contents with time was obtained, using a regression formula: $C = 1 + 0.89 / (1 + \exp(-0.55(x + 0.43)))$, where C is the relative DNA content and x is standardized time (= sampling time (min)/2.5 – 12).

the hybridization signals. The signals were converted into ratios relative to the signals of the G1 arrested cells. To smooth the data, each ratio value was replaced with the mean of nine neighbouring ratio values in a 3×3

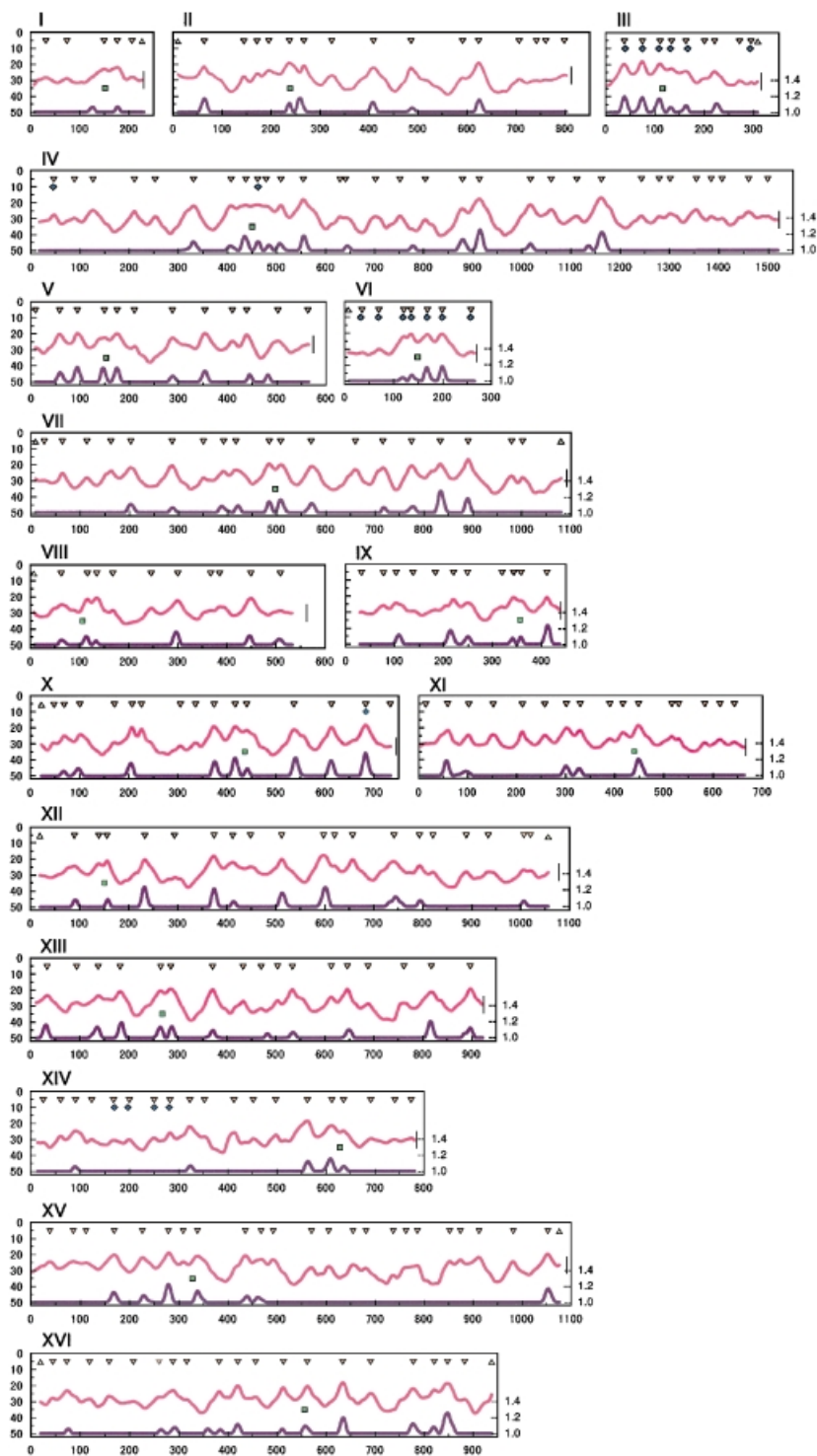


Figure 3 Replication profiles of all 16 yeast chromosomes. Red curves indicate the 50% replication time along the chromosomes. Numbers on the horizontal axes represent nucleotide sequence positions (in kb) and numbers on the left vertical axes represent the time (in min) after release from the G1-block with α -factor. Orange coloured downward triangles indicate positions of potential replication origins (PROs) appearing as peaks in the replication curves. Yellow upward triangles indicate chromosomal termini with PROs. Blue diamonds indicate chromosomal positions of locus-known ARSs: *ARS305*, *306*, *307*, *309*, *310* and *HMRE* on chromosome III; *HO-ARS* and *ARS1* on chromosome IV; *ARS601/602*, *603*, *603.5*, *605*, *606*, *607* and *609* on chromosome VI; *ARS121* on chromosome X; *ARS1411*, *1412*, *1413* and *1414* on chromosome XIV. Green squares indicate chromosomal positions of the centromeres. Purple curves represent the changes in copy number of genomic DNA in the presence of hydroxyurea (HU). Cells arrested at G1 with α -factor were released in the presence of HU and the change in copy number of genomic DNA was analysed with microarrays. Numbers on the right vertical axes represent relative copy numbers for the purple curves.

matrix. The time at which half a probe sequence replicated was determined for each probe and connected by a fitting curve obtained from a local polynomial regression analysis. The resulting curves (called replication curves, representing the time of 50% replication along

the nucleotide sequence) of all the yeast chromosomes are shown in Fig. 3.

Nine and seven peaks were identified in the replication curves of chromosome III and VI, respectively (Fig. 4). The chromosomal positions of the peaks agreed

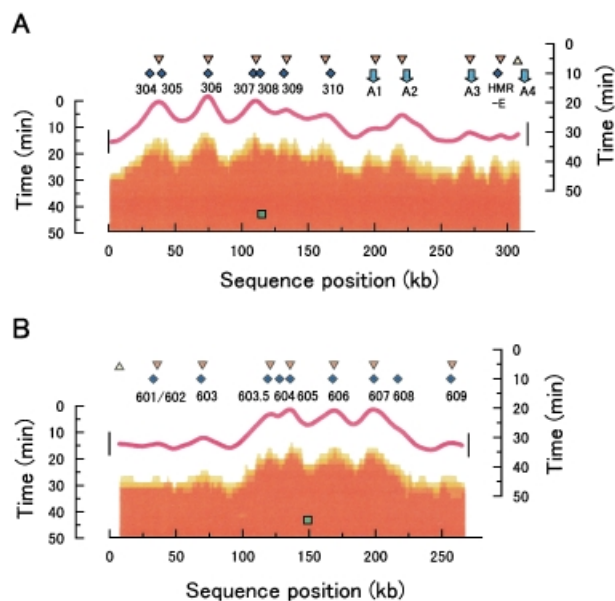


Figure 4 Replication profiles of chromosome III and VI, (A) for chromosome III and (B) for chromosome VI. The profiles are shown by two different methods: by a coloured tile image (lower) and by a curve (upper). Tile colours represent the levels of replication progression ranked by signal ratios (SRs): yellow, $1.3 \leq \text{SR} < 1.4$; dark yellow, $1.4 \leq \text{SR} < 1.5$; orange, $1.5 \leq \text{SR} < 1.6$; dark orange, $1.6 \leq \text{SR}$. Red curves represent the 50% replication, being equivalent to 1.5 SR in the tile image. Horizontal axes represent nucleotide sequence positions (in kb) and vertical axes represent the time (in min) after release from the G1 block with α -factor. Orange downward triangles indicate the sequence positions of potential replication origins (PROs) identified as peaks in the replication curves. Yellow upward triangles indicate chromosomal termini with PROs. Blue diamonds with numbers indicate sequence positions and names of known origins. Azure arrows with characters A1, A2, A3 and A4 indicate the loci of newly identified ARSs by the ARS-hunting assay. Green squares indicate centromere positions.

well with the loci of known origins examined. Peaks corresponding to all the active known origins were found, while no peaks appeared in the loci for inactive origins of *ARS304*, *ARS604* and *ARS608* (Newlon *et al.* 1993; Theis & Newlon 1997; Yamashita *et al.* 1997; Friedman *et al.* 1997). Besides these known origins, the replication curve of chromosome III demonstrated three additional peaks. An autonomous replication sequence (ARS)-hunting assay based on plasmid stabilization revealed ARS-positive fragments near these peaks' positions: fragment A1 of 0.80 kb (nucleotide sequence position 197250–198049), A2 of 0.86 kb (224640–225499) and A3 of 0.61 kb (272470–273077), with distances of 2.85, 4.28 and 1.18 kb from the peaks' positions, respectively. The transformation potencies of these

fragments were as high as that of a fragment containing the known *ARS607*. In addition to these peaks, upward lines near the margins of the replication curves suggested the existence of an origin at the right terminus of chromosome III and one at the left terminus of chromosome VI. In good agreement with this prediction, a previous report indicated the existence of an origin at the left terminus, but not at the right terminus of chromosome VI (Newlon *et al.* 1993). To examine the potential origin at the right terminus of chromosome III, an ARS-hunting assay was performed for 10 kb of the most right-terminus of chromosome III and a 0.66 kb fragment A4 (nucleotide sequence position 315397–316059) was found to have the ARS-activity. For other locus-known replication origins including *HO-ARS* and *ARS1* on chromosome IV (Kearsey 1984; Celniker & Campbell 1982), *ARS121* on chromosome X (Walker *et al.* 1990), and *ARS1411*, *ARS1412*, *ARS1413* and *ARS1414* on chromosome XIV (Friedman *et al.* 1996), peaks appeared in the positions corresponding to the known loci of these origins (Table 1). The replication curve provides the time at which peaks appeared, after release from the G1 block. The peak times of the known origins were correlated well to their initiation times determined by two-dimensional agarose gel electrophoresis techniques (Yamashita *et al.* 1997; Friedman *et al.* 1997) or a density-transfer method (Friedman *et al.* 1996; Heun *et al.* 2001) (Table 1). Thus far, we have been able to demonstrate that the positions and times of peaks in the replication curves are in good agreement with the chromosomal loci of known origins and their firing times. Furthermore, the replication curves have mapped novel replication origins on chromosomes. Therefore, it can be said that this replication curve (now also called a replication profile) provides us with a genome-wide image of the initiation and progression of DNA replication.

A total of 247 peaks were identified in the replication profile as potential replication origins (PROs) (Fig. 3 and Table 2). Upward lines near the margins of the replication curves suggested the existence of at least additional 13 PROs in the most termini of chromosomes. In total, 260 PROs were mapped on the yeast genome and the mean PRO density was one per 46.4 kb, in a range from one per 11.2 kb to one per 104.8 kb. The 247 PROs exhibit various peak times in their replication curves, ranging from 16.8 to 33.1 min, with an average of 24.6 min, and are classified into two groups ($P < 0.01$): 143 as early PROs (21.7 ± 2.1 min) and 104 as late PROs (28.6 ± 1.6 min) (Fig. 5). These early PROs and late PROs form separated clusters on the chromosomes, potentially causing regional differences in the completion time of DNA replication (Fig. 6). All 16

Table 1 Known active origins and peaks in DNA replication curves

Chromosome	Name	Literature	Peaks in replication curves			Difference
		Locus (kb)	Position (kb)	Time (min)	d(ARS1)* (min)	Position† (kb)
III	ARS305	39.584	37.636	19.7	(−1.6)	1.948
III	ARS306	74.538	74.443	17.9	(−3.4)	0.095
III	ARS307	108.966	110.379	19.3	(−2.0)	1.413
III	ARS309	132.045	133.505	22.4	(+1.1)	1.460
III	ARS310	166.636	162.693	24.1	(+2.9)	3.943
III	HMRE	292.672	294.999	31.1	(+9.9)	2.327
IV	HO ARS	46.222	47.076	27.4	(+6.1)	0.854
IV	ARS1	462.597	461.488	21.3	(0.0)	1.109
VI	ARS601/2	32.965	36.050	32.1	(+10.8)	3.085
VI	ARS603	68.848	70.274	30.0	(+8.7)	1.426
VI	ARS603.5	118.792	121.319	21.9	(+0.7)	2.527
VI	ARS605	136.036	135.633	20.4	(−0.9)	0.403
VI	ARS606	167.723	168.820	20.7	(−0.6)	1.097
VI	ARS607	199.408	198.390	20.4	(−0.8)	1.018
VI	ARS609	256.368	257.413	31.8	(+10.5)	1.045
X	ARS121	683.706	683.328	18.3	(−3.0)	0.378
XIV	ARS1411	169.227	167.725	30.4	(+9.2)	1.502
XIV	ARS1412	196.269	198.986	30.1	(+8.8)	2.717
XIV	ARS1413	250.138	249.460	29.0	(+7.7)	0.678
XIV	ARS1414	280.431	282.062	25.9	(+4.6)	1.631

*Time difference (min) from the replication time of ARS1.

†Difference (kb) in positions of origins between this study and the previous studies.

centromere sequences were found to be in the clusters of the early PROs.

Mapping of origins firing in the presence of HU

To examine whether these 143 early PROs fire in the presence of HU, cells arrested at the G1 phase with α -factor were released in the presence of HU. After a 90 min incubation, genomic DNA was isolated from the cells and hybridized to microarrays. From the hybridization signals of the probes, copy number curves were obtained. To remove false positive peaks in the curves due to relatively low hybridization signals, the experiments were independently repeated four times and only peaks that appeared in all four repeats were scored as true positive peaks. In total, 122 peaks were identified (Fig. 3). Except for six peaks, 116 peaks were mapped on chromosomal positions corresponding to PRO loci mapped by the replication profile, and among them 112 peaks belonged to the group of the early PROs (Fig. 5). The PROs whose positions were nearest the centromeres were all involved in the group of the 112 PROs that fired in the presence of HU.

Discussion

We have identified 260 PROs in the yeast genome by microarray-based monitoring of the changes in copy number of the genomic DNA from one to two during DNA replication. 247 PROs were defined as peaks on the replication profiles, while 13 PROs were located as regions in the chromosomal termini. The frequency distribution of the 247 PROs ranged in the replication time shows two peaks and classifies these PROs into 143 early ones and 104 late ones. 112 of the early PROs were found to fire in the presence of HU, indicating that these PROs were free from the control by the intra-S-checkpoint induced by the depletion of dNTPs (Santocanale & Diffley 1998; Shirahige *et al.* 1998; Desany *et al.* 1998). The checkpoint requires the Mec1 and Rad53 proteins. Considering the use of a stringent cut-off-criterion in order not to take false positive peaks (see the data analysis in Experimental procedures), it is possible that some of the remaining 31 early PROs would also fire in the presence of HU. On chromosomes, early PROs are not randomly distributed but form clusters, resulting in regional differences in the time for replication

Table 2 Potential replication origins (PROs) and their densities on chromosomes

Chromosome	Size* (kb)	Region not examined		Number of PROs		Total	Mean distance (kb)
		Left-end (kb)	Right-end (kb)	as peaks	as upward lines		
I	230.195	7.485	3.601	4	1	5	46.0
II	813.137	9.854	10.296	15	1	16	50.8
III	315.344	2.174	7.369	9	1	10	31.5
IV	1522.191	17.815	2.996	31	0	31	49.1
V	574.860	8.038	10.086	12	0	12	47.9
VI	270.148	7.500	5.178	7	1	8	33.8
VII	1090.936	8.893	10.807	19	2	21	51.9
VIII	562.638	7.031	29.950	10	1	11	51.1
IX	439.885	29.259	2.769	10	0	10	44.0
X	745.440	22.347	11.878	15	1	16	46.6
XI	666.448	3.714	3.036	16	0	16	41.7
XII	1078.172	19.485	21.507	19	2	21	51.3
XIII	924.430	11.730	2.185	17	0	17	54.4
XIV	784.328	13.896	3.986	19	0	19	41.3
XV	1091.282	2.425	15.372	23	1	24	45.5
XVI	948.061	19.534	9.685	19	2	21	45.1
Total	12057.495	191.174	150.702	245	13	258	46.7

*All the data of chromosome sizes and nucleotide sequences come from the yeast genome data base (YGD) (Cherry *et al.* 1997).

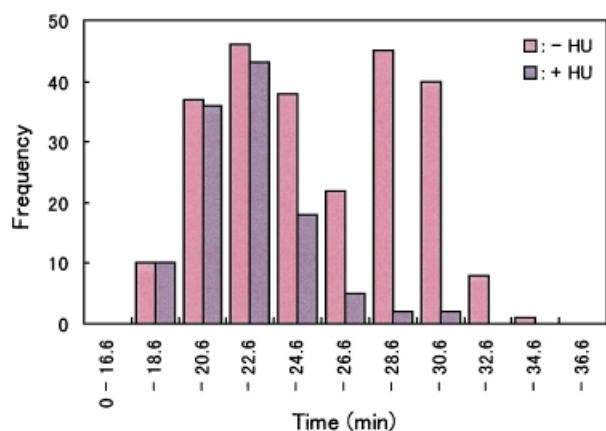
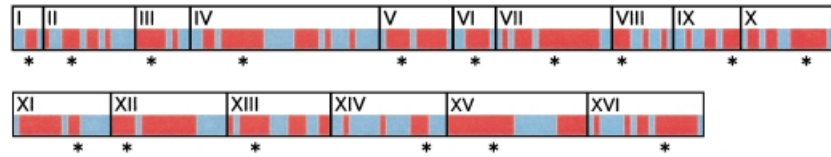


Figure 5 Histogram of potential replication origins (PROs) classified by their peak times. Red bars: 247 PROs can be separated into two groups by a borderline of 25.6 min, resulting in 143 of early firing PROs (21.7 ± 2.1 min) and 104 of late firing PROs (28.6 ± 1.6 min). Purple bars: 112 PROs activated in the presence of HU are superposed on the histogram of the 247 PROs identified in the replication profile.

completion. Clustered patterns vary from chromosome to chromosome and, as a simple case, chromosome XV with a size of 1091 kb exhibits a pattern comprising of two segments of early PROs and one of late PROs (Fig. 6). What is the factor which forms these clusters?

Sequence analyses revealed no clear-cut differences between early and late firing origins and replacement analyses of the origins on the chromosomes demonstrated positional effects on the origin firing time, indicating a participation of environments surrounding origins in determination of the origin firing time (Campbell & Newlon 1991; Fangman & Brewer 1992). Chromatin structure is one such environment. The binding of transcription factors and/or remodeling proteins changes chromatin structure, and as a consequence affects the origin firing time (Stevenson & Gottschling 1999; Muller *et al.* 2000; Windtersberger 2000; Lipford & Bell 2001). Considering the formation of large segments of more than 100 kb, cytological observations that the late origins were differently localized in nuclei at the G1/S phase from the early origins would be suggestive (Heun *et al.* 2001; Cimbara & Groudine 2001). A further study of the origins on chromosomes exhibiting distinct PRO clusters will provide insight into the controlling mechanism of the origin firing time over a genome-wide scale. All 16 centromere regions locate in such clusters of early PROs, indicating early replication, as was reported previously (McCarroll & Fangman 1988; Raghuraman *et al.* 2001). Furthermore, all the centromeres except for one on chromosome I had PROs that fired in the presence of HU as the nearest PRO.

Figure 6 Distribution of early and late PROs on chromosomes. Each PRO is shown as a tile with the same width. Early and late PROs are coloured red and blue, respectively. Asterisks under the tiles indicate PROs nearest centromere positions.



Using a density-separation of synthesized DNAs followed by a microarray-based detection, Fangman's group has generated replication profiles for all chromosomes (Raghuraman *et al.* 2001). Although the preparation method of genomic DNA probes for hybridization to microarrays is different, the replication profiles obtained here are, as a whole, quite similar to those of the previous study. Related to this, the mean fork progression rate was calculated as 2.8 ± 1.0 kb/min from slopes between peaks and valleys on the replication curves, which is almost identical to the mean rate of 2.9 kb/min in the previous study. However, there are some differences. First, the mapping of PROs on the chromosomes would be more accurate in this study than the previous one. The mean difference between the predicted loci and the experimentally determined loci in known origins is 1.53 kb, with a range of 0.10–3.94 kb (Table 1). This is better, compared with the previous study (a mean of 3.65 kb with a range of 0.03–10.27 kb). All the origins that have been reported to be active on chromosome III and VI were identified. In addition, the replication profile allowed us to easily identify novel ARS-positive fragments (A1, A2 and A3) on chromosome III. The ARS consensus core sequence or its similar sequences were found in A1 (nucleotide sequence position 197539 TAAACATGAAT), A2 (224856 TTTTATGTTTT), and A3 (272623 ATTTACACTTC). Second, the distribution of the PRO firing times generated two peaks in this study (Fig. 5), but only one peak in the previous study. The difference could come from the numbers of PROs plotted in the replication profiles: 247 in this study and 332 in the previous one. The number of PROs identified is influenced by parameter setting in the analysis of the microarray data. The parameters were optimized to fit the calculated replication profile to the experimentally determined ARS-firing profile of chromosome VI (Yamashita *et al.* 1997; Friedman *et al.* 1997). We observed that an increasing number of PROs plotted in the replication profiles by changing the parameters is accompanied by an increase in the proportion of PROs firing in the middle of the early and late replication times.

The oligonucleotide microarray used here is designed for the analysis of gene expression (Lockhart *et al.* 1996). Therefore, the interprobe distances are sparse in some chromosomal regions, even though this averages one

per 1.4 kb for the whole genome. This partially seems to explain the reason for the relatively large difference between our predicted loci and the reported loci for some known origins examined. These are *ARS310* and one (A2) of the newly identified PROs on chromosome III. It would be possible to make a more precise DNA replication profile if a microarray designed to set probes every 1 kb or less was used. Combining the use of such an improved microarray and the data analysis method developed in this study will provide us with a more powerful tool for monitoring DNA replication and a genome-wide insight to DNA replication.

Experimental procedures

Strains and plasmids

Saccharomyces cerevisiae strain SH5752 (kindly provided by Dr Satoshi Harashima) harbouring an extra minichromosome constructed from the right arm of chromosome 9 and its original strain W303-1A (*Mata his3-11,15 leu2-3,112 trip1 ura3-1 ade2-1 can1-100*, Foss *et al.* 1993) were used for an examination of the sensitivity of the microarray technology. YPH499 (*Mata, his3 leu2 trip1 ura3*, Sikorski & Hieter 1989) was used for the ARS-hunting assay. W303-1Ab (same as W303-1A but *BAR1* is replaced with $\Delta bar1$, Masumoto *et al.* 2000) was used for the replication profile analysis experiments and the HU-treatment experiment. A plasmid vector pV34C was constructed by inserting a 0.86 kb fragment containing the *CEN4* sequence into the *AatII* site on YIplac204 (Gietz & Sugino 1988).

Synchronization of the cell cycle

Cells grown in YPD were treated with α -factor at 30 ng/mL for three hours at 26 °C. The treated cells were harvested by filtration, washed twice with YPD, and then suspended in YPD containing actinase (Kakenseiyaku, Japan) at 100 g/mL. The cell culture was incubated at 26 °C and samples were collected every 2.5 min for a flow cytometric analysis and isolation of genomic DNA.

Hydroxyurea (HU)-treatment

Cells arrested at G1 with α -factor were released in YPD containing 0.2 M hydroxyurea (Sigma). After incubation at 26 °C for 90 min, the cells were collected and used for a flow cytometric analysis and hybridization analysis with microarrays. The experiments were independently repeated four times.

Flow cytometric analysis

Samples were collected and processed according to a previously described method (Haase & Lew 1997) and analysed with a Becton-Dickinson FACScan.

ARS hunting assay

A fragment under test was inserted into an appropriate restriction site on pV34C and used for transformation of the yeast strain YPH499. After a 3 day incubation at 28 °C, colonies growing on agar plates were counted and the colony number per µg plasmid DNA was calculated. A fragment having a high transformation potency of more than 1000 colonies per µg DNA, which is comparable with that of known-ARS bearing plasmids, was regarded as an ARS-positive fragment.

Preparation of labelled genomic DNA and microarray experiments

Genomic DNA was isolated from cells using the genomic-tip 20/G isolation kit (Qiagen). Labelling of the genomic DNA was carried out as previously described (Lockhart *et al.* 1996). One µg of DNA was incubated with DNase I (Gibco BRL) at two units/mL for 20 min at 25 °C. After inactivation of the DNase I by EDTA addition and heating, the fragmented DNA was used for 3'-end labelling. The DNA was incubated with 50 nmol of biotin-N6-dideoxy ATP (Dupont/NEN) and terminal transferase (Roche Diagnostics) at 500 units/mL for 60 min at 37 °C. The reaction was stopped by heating for 5 min at 95 °C. The labelled DNA was hybridized to the YG-S98 microarray (Affymetrix), fluorescently stained, and subjected to scanning as previously described (Lockhart *et al.* 1996; Wodicka *et al.* 1997). Hybridization signals (in average different units) were calculated using Affymetrix GENECHIP software.

Selection of probes for analysis

The commercially available oligonucleotide microarray YG-S9 (Affymetrix), has been fabricated with 9210 probes covering more than all the yeast genes. We selected probes for this study based on two different contents: nucleotide sequences used for the designing probes and signal intensities in hybridization. First, all the sequences being in multicopy in the genome or showing a similarity to Ty elements were excluded. Probes with less than a 40-bp interprobe-distance on the chromosomes were also excluded. These filters eliminated 880 probes in total. Next, hybridization signals of the remaining 8330 probes were examined. In hybridization with the genomic DNA isolated from cells arrested at G1 with α -factor, probes exhibiting far stronger (> 1500 intensity units) or far weaker (< 50 intensity units) signals were excluded. The mean hybridization signal of the remaining 8149 probes was 328 intensity units, with a standard deviation of 130. The probes were further checked for their hybridization signals in a time-course experiment with 23 data points, resulting in the exclusion

of an additional 44 probes. These were probes exhibiting no changes (cutoff value: ≤ 2 samples with > 1.4 signal ratios) or pulse-like increases (cutoff value: ≤ 2 samples with > 4.0 signal ratios). In addition, eight probes exhibiting a < 1.0 signal ratio at sampling times of 52.5 or 55.0 min were also excluded. The remaining 8105 probes were used in this study. The probe sequences are mapped on chromosomes at an average distance of 1.46 kb, ranging from 0.04 to 19.79 kb. The yeast genome database (YGD) was used for nucleotide sequences of the yeast genome (Cherry *et al.* 1997).

Data analysis

Hybridization signals of the 8105 probes in 23 samples collected every 2.5 min after release from the G1 arrest with α -factor were analysed. The signals were scaled using the relative DNA contents of the samples measured with FACS and converted into ratios to the mean signals of the three samples at 0, 2.5 and 5 min. The resulting signal ratios were adjusted to a state where mean signal ratios at the start (0, 2.5 and 5 min) and at the end (52.5 and 55.0 min) were 1.0 and 2.0, respectively. For the smoothing, a mean ratio calculated from nine neighbouring ratios in a 3×3 matrix was used as the ratio in the centre of the matrix. The 50% replication time, defined as the time at which a signal ratio increases to 50% of the difference in mean ratios between the sampling start and the sampling end was calculated for each probe. A fitting curve for the 50% replication time was obtained by a local polynomial regression analysis using the software KYPLOT (generated by Koichi Yoshioka <http://www.qualest.co.jp/download/KyPlot/kyplot_e.htm>). A binomial regression program with the Gaussian kernel function in five bandwidths was applied and the result was output as a curve dotted in every 1.0 kb interval on the genome sequence. A peak was defined as a curve point where the sign of $\Delta_{\text{min}}/\Delta_{\text{kb}}$ changed from plus to minus and the same sign was kept at more than 3 kb from the change point.

Hybridization signals from the HU-treated cells were compared with those from the G1 arrested cells and signal ratios were calculated. After smoothing the signal ratios by replacing each ratio with a mean of three neighbouring values, a regression curve was obtained as mentioned above. The increase in signal ratios in this experiment was quite low, so that very small peaks appeared in regression curves. To avoid picking up false positive peaks, only peaks that satisfied two criteria were scored as true peaks: (i) peaks exhibiting more than a 10% increase in the ratio, and (ii) peaks appearing in all the four-time repeated experiments. A final regression curve was drawn as an average from the four independent experiments.

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Supplementary material

For the data used for drawing the replication curves and copy number curves in the presence of HU, see the Supplementary Table on web at <http://www.blackwell-science.com/products/journals/suppmat/GTC/GTC559/GTC559sm.htm>.

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