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Identification of a predominant replication origin in fission yeast

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ABSTRACT

We have identified five autonomously replicating sequences (ARSs) in a 100 kbp region of the *Schizosaccharomyces pombe* chromosome II. Analyses of replicative intermediates of the chromosome DNA by neutral/neutral two-dimensional gel electrophoresis demonstrated that at least three of these ARS loci operate as chromosomal replication origins. One of the loci, *ori2004*, was utilized in almost every cell cycle, while the others were used less frequently. The frequency of initiation from the respective chromosomal replication origin was found to be roughly proportional to the efficiency of autonomous replication of the corresponding ARS plasmid. Replication from *ori2004* was initiated within a distinct region almost the same as that for replication of the ARS plasmid. These results showed that the *ori2004* region of ~3 kbp contains all the *cis* elements essential for initiation of chromosome replication.

INTRODUCTION

Replication origins are physically defined as the sites where DNA synthesis is initiated, while the regions required for initiation of chromosome replication are called replicators. Initiation of replication in eukaryotic cells is tightly regulated during the cell division cycle. The regulation presumably involves interactions of various proteins with unique DNA sequences constituting the replicators.

The structures of eukaryotic replicators have not been clarified, except in the budding yeast, *Saccharomyces cerevisiae.* Certain chromosomal fragments of the yeast have been shown to be capable of autonomous replication (autonomously replicating sequences, ARS) (1,2). All the yeast ARSs contain a match to an 11 bp sequence, called the ARS consensus sequence (ACS), that is essential for ARS function (3,4). In addition to the ACS, distinct elements within a 100 bp region are required for ARS function (5). Some of the ARS segments function as chromosomal replication origins (6,7). An origin recognition complex (ORC) containing six protein subunits has been purified and shown to bind the ACS (8). *In vivo* footprinting yields a pattern similar to that found by footprinting with ORC *in vitro* (9). Moreover, analyses of temperature-sensitive *orc2-1* and *orc5-1* mutants by 2D gel techniques have shown that binding of ORC to the ACS is essential for initiation of replication of the yeast genome (10).

Physical mapping of the replication origins of higher eukaryotic chromosomes has suggested that replication is initiated from restricted regions, ranging in size from 0.5 to 55 kb (11). The replication origins for the human β-globin and *Drosophila* chorion genes are located within 2 and 3 kb regions respectively (12,13). For their function, however, regions apart from the actual initiation sites are also required (13,14). The elements involved in the initiation of replication remain to be identified. In contrast to the budding yeast, no short chromosome fragments capable of autonomous replication have yet been isolated from mammalian cells, although human chromosome fragments in the 10 kb range do exhibit significant autonomous replication activity in human cells (15–17). These results suggest that the replicators in higher eukaryotes have more complex structures than the budding yeast replicator.

In *Schizosaccharomyces pombe*, certain chromosome fragments can replicate autonomously (18–22), but the regions required for ARS function are much longer than those in the budding yeast (19,23). Although a match to an 11 bp sequence similar to the budding yeast ACS exists in the *S.pombe* ARS fragments, the sequence is not essential for ARS activity (19). Detailed analyses of two ARS elements, namely *ars1* and *ars3002*, suggest the importance of an asymmetric A+T-rich sequence clustered within several hundred base pair regions (24,25). Thus, the fission yeast replicators appear to more closely resemble those in higher eukaryotes with regard to the level of complexity. Physical mapping of the fission yeast replication origins using two-dimensional (2D) gel techniques has shown that replication of a 6 kb region upstream of the *ura4* gene is initiated from multiple sites (26). The origin region contains three ARS elements that are required for initiation from the corresponding replication origins (23). Although these replication origins do not individually operate in every cell cycle, initiation from the region is achieved in every cell cycle by their concurrent action. It remains unknown whether the *ura4* origin region is characteristic of fission yeast replicators.

In this report, we describe identification of five distinct ARS elements in a continuous 100 kb region of *S.pombe* chromosome II and provide evidence that three of the ARS loci function as chromosomal replication origins. Our results suggest that a single ARS element can promote chromosome replication in every cell cycle.

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MATERIALS AND METHODS

Strains and media

The *S.pombe* haploid strain used was HM123 *h⁻ leu1* (27). It was cultured in YPD complete medium (1% yeast extract, 2% polypeptone, 2% glucose) and EMM minimal medium (28). *Escherichia coli* DH5α (29) was grown in LB (0.5% yeast extract, 1% polypeptone, 1% NaCl, pH 7.5). For EMM and LB plates, agar was added to 2 and 1.5% respectively. Plasmid DNA was prepared from *E.coli* transformants as described previously (30).

Plasmids

Restriction fragments (2–15 kb) of cosmid clones containing *S.pombe* chromosomal DNA, sp1029, 205, 1228 and 1558 (31), were subcloned into pYC11, a derivative of Bluescript KS(+) carrying the *S.cerevisiae LEU2* gene (32). Derivatives of pARS2004 with an additional insert at either the left or right end of the ARS segment were constructed as follows. A 564 bp *Hin*dIII fragment of bacteriophage λ and the 3.2 kb *Not*I–*Xba*I fragment of pARS2004 were inserted into the *Hin*dIII and the *Not*I–*Xba*I sites respectively of pYC11, resulting in pARS2004Hλ. The phage λ fragment was first cloned into the *Hin*dIII site of a pYC11 derivative whose *Xho*I site had been altered to a *Not*I site and then the *Not*I fragment of the resulting plasmid was inserted into the *Not*I site of pARS2004, resulting in pARS2004Nλ.

Transformation of *S.pombe* cells with genomic clones

The electroporation method (33) was employed to introduce plasmid DNA into *S.pombe* cells. HM123 cells (1×10^7 cells/ml) were washed three times and suspended in cold 1.2 M sorbitol at a concentration of 1×10^9 cells/ml. To the cell suspension (0.1 ml), 0.2 µg plasmid DNA was added with 5 µg sonicated salmon testis DNA. After electroporation at 2000 V, 200 Ω and 25 µF, one twentieth of the suspension was spread on an EMM plate and incubated for 4 days at 30°C.

Determination of the physical forms and copy numbers of ARS plasmids

For determination of the physical status and copy numbers of ARS plasmids, the total cellular DNA of transformants was prepared as described previously (34). The DNA was separated by agarose gel electrophoresis before or after digestion with a restriction enzyme and analyzed by Southern blot hybridization with the vector probe.

Stability of ARS plasmids

The stability of ARS plasmids was determined by the method described by Heyer *et al.* (35). Transformants grown in EMM medium to 1×10^7 cells/ml were diluted and plated onto YPD plates. Colonies which formed after 2 days at 30°C were replica-plated onto both EMM and YPD plates to determine the percentage of plasmid-containing cells under selective conditions (*A*). The cells in the EMM culture were then diluted to 1×10^3/ml with YPD medium and grown at 30°C for ~10 generations without selection. After scoring the cell number (*n*), diluted cells were plated onto YPD plates. The colonies formed were then replicated on EMM and YPD plates to determine the percentage of plasmid-containing cells under non-selective conditions (*B*). Plasmid loss rate per generation was calculated with the equation $1 - (B/A)^{1/N}$, where $N = 3.3 \log_{10} n - 10$.

Neutral/neutral two-dimensional gel electrophoresis

The neutral/neutral 2D gel electrophoresis method described by Brewer and Fangman (36) was employed to determine the initiation sites of replication on the *S.pombe* chromosome and ARS plasmids. Total cellular DNA was prepared as described by Shinomiya *et al.* (37) with some modifications (38). A culture of log phase cells (5×10^9 cells) in YPD medium was mixed with an equal volume of cold stop solution containing 3% toluene, 95% ethanol and 20 mM EDTA. The cells collected by centrifugation were washed twice with SE buffer (75 mM NaCl, 100 mM EDTA) and suspended in the same buffer at ~1×10^8 cells/ml. The cell suspension (5 ml) was then mixed with an equal volume of 1% low melting point agarose (SeaPlaque GTG Agarose; FMC BioProducts) and 4 vol. paraffin prewarmed to 42°C. After vigorous shaking for 1 min, emulsions were poured into 5 vol. cold SE buffer and stirred for 5 min. The agarose beads were collected by centrifugation, incubated at 37°C for 30 min in an equal volume of SE buffer containing Zymolyase 20T (Seikagaku Kogyo) at 5 mg/ml and then in 2 vol. 1% SDS and 25 mM EDTA for 10 min at room temperature. After further incubation at 37°C for 1 h in 2 vol. 1% Salkosyl, 25 mM EDTA containing proteinase K at 0.5 mg/ml and washing with TE buffer (10 mM Tris–HCl, pH 7.4, and 1 mM EDTA) containing 0.1 mM phenylmethylsulfonyl fluoride, the DNA in agarose beads was digested with appropriate restriction enzymes and electroeluted in a dialysis bag. After removal of agarose by centrifugation at 12 000 r.p.m. for 15 min at 4°C, DNA was concentrated with isobutyl alcohol and precipitated with 2-propanol. Electrophoresis for the first dimension was carried out in 0.35% agarose gels at 1 V/cm in TBE buffer (100 mM Tris, 100 mM boric acid and 2 mM EDTA) at 4°C and for the second dimension in 0.875% agarose gels at 8 V/cm in the presence of 0.3 µg/ml ethidium bromide. The DNA was blotted onto Hybond-N^+ membrane and hybridized with ^{32}P-labeled probes. The membranes were placed in contact with imaging plates for 2 days and the stored images were analyzed with an Image Analyzer Bas1000Mac (Fuji Film, Tokyo).

RESULTS

Autonomously replicating fragments of the fission yeast chromosome

To isolate chromosome fragments capable of replicating autonomously from *S.pombe*, we cloned overlapping fragments of 2–15 kb from a 100 kb region of chromosome II into a vector carrying the *S.cerevisiae LEU2* gene. Each clone of the library was introduced into *S.pombe leu1* cells to measure the ARS activity. Plasmids carrying five distinct fragments gave leu^+ transformants at a high efficiency of ~1×10^6/pmol DNA (Fig. 1). Plasmids with other fragments were unable to transform at a frequency higher than 1×10^4/pmol DNA. Thus, only distinct regions of the *S.pombe* genome had the ability for autonomous replication. The ARS regions identified were designated *ars2001–2005* from proximal to *cen2* (Fig. 1).

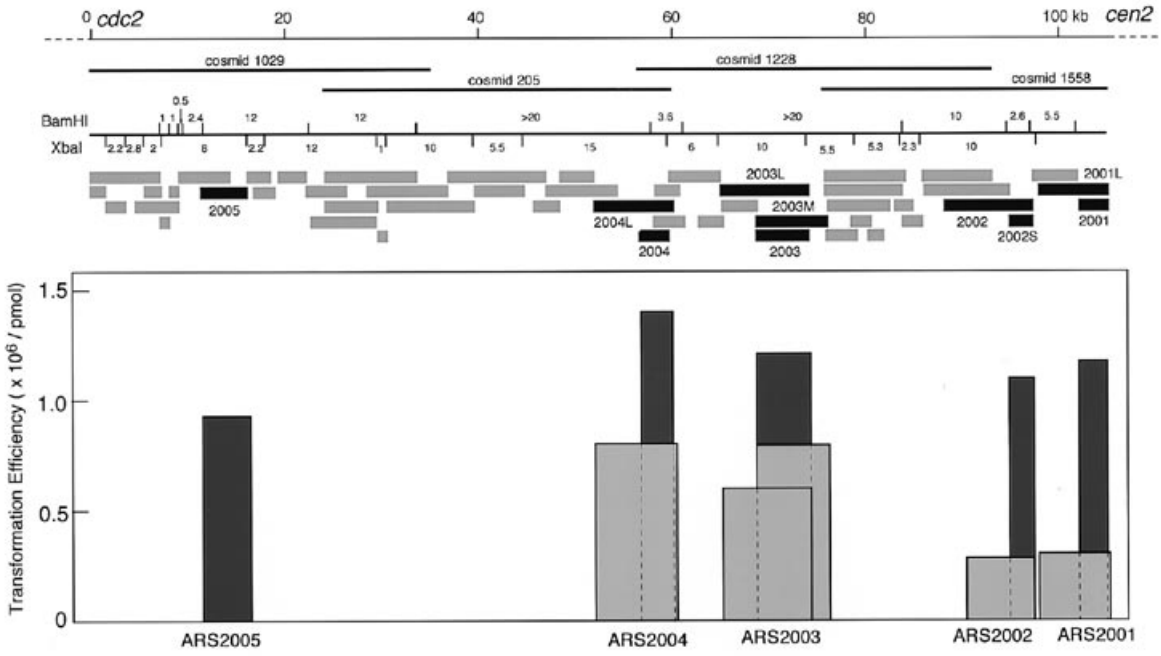

Figure 1. Autonomous replication activity of *S.pombe* genomic fragments. The positions of overlapping fragments from a 100 kb region of fission yeast chromosome II and their transformation efficiencies are presented. Plasmids carrying the chromosomal fragments shown by horizontal thick bars were introduced into HM123*leu1* cells and the numbers of leu$^+$ colonies were scored after incubation for 4 days at 30°C. The transformation efficiency, determined as the number of transformants per pmol plasmid molecule, is shown by the light gray columns. Fragments exhibiting high transformation activities are indicated by the darker bars. Cosmid clones are shown by thick lines.

Replication efficiency of ARS plasmids

Earlier studies have shown that *S.pombe* ARS plasmids are readily rearranged into large multimeric forms (21,39). To examine the physical status of newly isolated ARS plasmids, total cellular DNA extracted from the leu$^+$ transformants was analyzed by Southern hybridization with the vector fragment. Most of the ARS plasmids examined, except for pARS2001L and pARS2002S, existed in a monomeric form (Table 1). pARS2001L, a larger derivative containing *ars2001*, was found in a large multimeric form, while pARS2002S, a shorter derivative with *ars2002*, was in dimeric and trimeric forms. The transformants with pARS2001L and pARS2002S grew at a much slower rate than those with the other ARS plasmids (data not shown). Thus, plasmids that replicated efficiently were maintained as monomers, while those replicating inefficiently were rearranged into multimers.

To estimate the efficiency of replication of ARS plasmids that were present as monomers, we examined their copy numbers in the transformants. By hybridization analysis after linearization with a restriction enzyme, the copy number of pARS2004 was estimated to be ~14/cell (Table 1). pARS2002 and pARS2003 were present at about half the copy number of pARS2004, while pARS2001 and pARS2005 at about one fourth (Table 1). These results suggeste that pARS2004 replicated at a higher efficiency than the other plasmids isolated.

Since the vector used in this study did not contain a centromere, the stability of the plasmid during cell cycles would depend mainly on the efficiency of autonomous replication. The rate of mitotic loss of pARS2004 under the non-selective conditions (see Materials and Methods) was 2.3% per generation. Plasmids pARS2002 and pARS2003 were slightly less stable than pARS2004, while pARS2001 and pARS2005 were relatively unstable during cell cycles (Table 1). From comparison of the results on the copy numbers of plasmids and mitotic stability, the relative order of autonomous replication activity was thus judged to be ARS2004 > 2002 or 2003 > 2001 or 2005.

Identification of the chromosomal replication origins

To examine whether chromosomal replication was initiated from the ARS loci or from regions outside, we employed the neutral/neutral 2D gel electrophoresis technique (36). The total cellular DNA from exponentially growing HM123 cells was separated by 2D gel electrophoresis after digestion with appropriate restriction enzymes. By hybridization with the ARS2004 probe, a complete bubble arc that started from the 1N position and extended beyond the 2N position was detected (Fig. 2B), demonstrating that chromosomal replication had been initiated within the restriction fragment. Such a bubble arc was also observed with the ARS2003 and ARS2002 probes (Fig. 2D and E). The origins of replication in the *ars2002*, *2003* and *2004* regions of chromosome II were designated *ori2002*, *2003* and *2004* respectively.

When the ARS2001 probe was used, only a simple Y arc was detected (Fig. 2F). The failure to detect any bubble arc could have been either due to the absence of an efficient replication origin in the region or to the presence of the origin in proximity to either end of the restriction fragment. Because pARS2001 showed a much lower replication efficiency than pARS2002, pARS2003 or pARS2004, replication from the *ars2001* region on the chromosome seemed to be inefficient, if it occurred. Ten other chromosome fragments that did not contain ARS elements demonstrated only simple Y arcs (Fig. 2A and C and data not shown). These results suggest that replication in the fission yeast chromosome is initiated from distinct regions which are capable of autonomous replication as extrachromosomal elements.

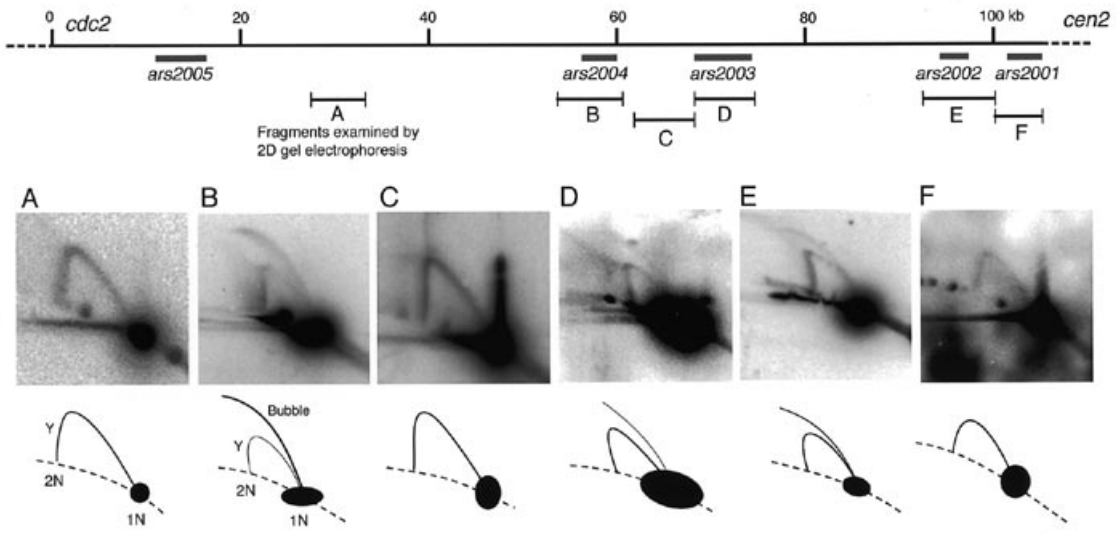

Figure 2. 2D gel analyses of *S.pombe* genomic DNA. Total cellular DNA from exponentially growing HM123 cells was digested with appropriate restriction enzymes and replicative intermediates were analyzed by the neutral/neutral 2D gel method. The results of hybridization are presented with traces of bubble and Y arcs. The restriction fragments examined are *Pvu*II–*Pvu*II (3.5 kb) non-ARS fragment (**A**), *Bgl*II–*Pst*I (6.0 kb) *ars2004* fragment (**B**), *Bgl*II–*Bgl*II (6.0 kb) non-ARS fragment (**C**), *Bgl*II–*Xba*I (7 kb) *ars2003* fragment (**D**), *Pvu*II–*Pvu*II (6.5 kb) *ars2002* fragment (**E**) and *Hin*dIII–*Hin*dIII (4.0 kb) *ars2001* fragment (**F**), whose positions are shown on the map of the 100 kb region.

Table 1. Comparison of the replication activities of ARS plasmids

ARS plasmid	Length of insert (kb)	Colony size[a]	Form[b]	Copy number[c]	Loss rate per generation[d] (%)
2001	3.3	medium	monomer	3.7 ± 1.0	18.0 ± 11
2001L	8.0	small	multimer	ND	ND
2002S	2.6	small	dimer, trimer	ND	ND
2002	11	medium	monomer	6.0 ± 2.9	3.7 ± 1.3
2003	7.0	medium	monomer	7.3 ± 0.5	5.9 ± 2.8
2003L	10	medium	monomer	ND	ND
2004	3.2	large	monomer	13.5 ± 2.5	2.3 ± 0.9
2004L	9	large	monomer	ND	ND
2005	4.0	medium	monomer	3.2 ± 0.4	19.0 ± 4.0

[a]Transformants formed after incubation for 3 days were classified into three groups according to their colony sizes.
[b]Total cellular DNA of the transformants was separated by agarose gel electrophoresis and analyzed by Southern hybridization.
[c,d]The copy number and the mitotic loss rate were determined as described in Materials and Methods. ND, not determined.

In the *ori2004*, *2003* and *2002* regions, complete Y arcs that started from the 1N position and extended to the 2N position were also observed, together with the complete bubble arcs (Fig. 2B, D and E). The results suggested that these origin regions were passively replicated from the outside region in a fraction of the cells. The intensity of a bubble arc relative to that of the corresponding Y arc could represent the initiation frequency of replication within the segment. The bubble arc in the *ori2004* region was much stronger than the Y arc (Fig. 2B), indicating that replication was initiated from the origin in almost every cell cycle. On the other hand, in the *ori2003* and *ori2002* regions, Y arcs were stronger than bubble arcs (Fig. 2D and E), suggesting that initiation from these origins occurred once in a few cell cycles. Thus, these replication origins operated at different frequencies during cell cycles. *ori2004* was the most active origin in the 100 kb region.

The initiation site of chromosomal replication in the *ori2004* region

To determine the initiation site in the *ori2004* region, cellular DNA was digested with various restriction enzymes and analyzed by 2D gel electrophoresis. If the initiation site was located at the center of a restriction segment, a complete bubble arc would be expected. On the other hand, if replication was initiated from another position away from the center, a bubble arc would be detected near the 1N position and a Y arc would be formed with progression of replication. As shown in Figure 3A, the *Bgl*II–*Pst*I fragment formed a complete bubble arc, suggesting that replication was initiated near the center of the fragment. On the other hand, the *Bgl*II–*Xba*I fragment, lacking a 1 kb region at the right end of the *Bgl*II–*Pst*I fragment, formed a shortened bubble arc and a Y arc whose intensity near the 2N position was stronger than that near the 1N position (Fig. 3B). Furthermore, the *Kpn*I–*Pvu*II fragment lacking ~2 and 0.5 kb regions from the left and right ends of the *Bgl*II–*Pst*I fragment respectively formed a short bubble arc ending half way from the 1N to 2N positions and a strong Y arc starting at the middle and ending at the 2N position (Fig. 3C). These results show that replication in the *ori2004* region was initiated at a distinct site away from the center of the *Bgl*II–*Xba*I and *Kpn*I–*Pvu*II fragments. The *Bam*HI fragment, which overlapped with the right half of the *Kpn*I–*Pvu*II fragment,

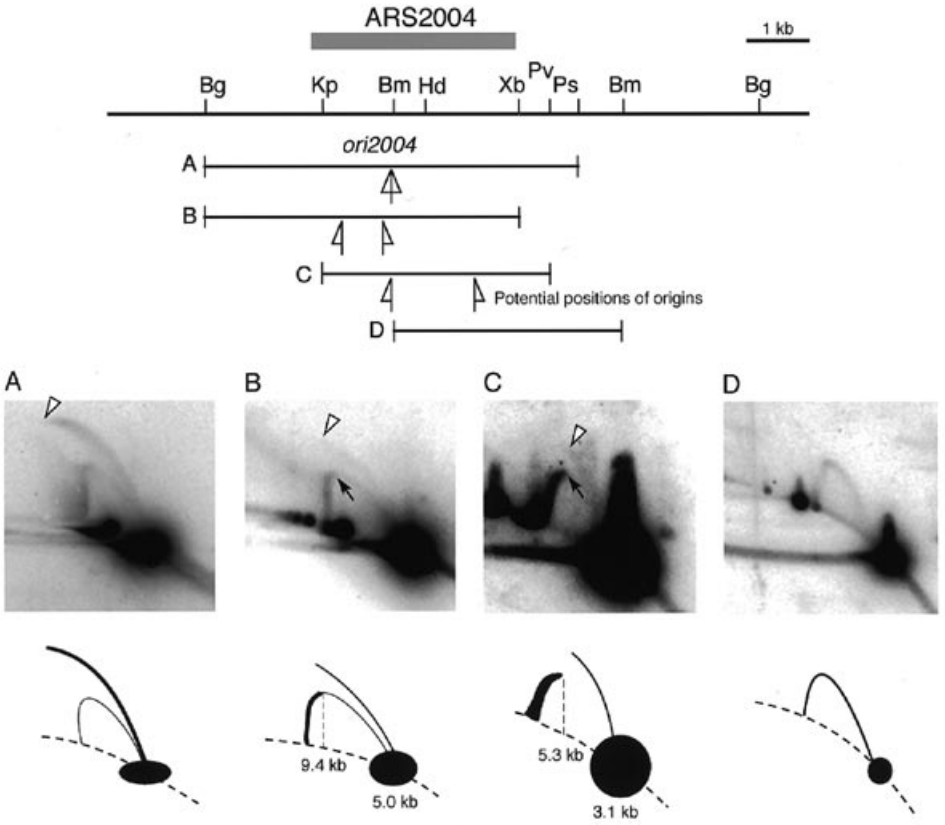

Figure 3. Mapping of the initiation site in the genomic *ori2004* region. The total DNA from HM123 cells was digested with *Bgl*II and *Pst*I (**A**), *Bgl*II and *Xba*I (**B**), *Kpn*I and *Pvu*II (**C**) or *Bam*HI (**D**) and analyzed by 2D gel electrophoresis. The positions of the fragments examined (top), the results of hybridization (middle) and their traces (bottom) are presented. The *Kpn*I–*Hin*dIII fragment of pARS2004 was used as the probe for hybridization in (A), (B) and (C) and the *Hin*dIII–*Xba*I fragment in (D). Arrows and open arrowheads in the panels indicate the positions of the smallest Y-shaped intermediates and the largest bubble-shaped molecules respectively. The molecular masses for the smallest Y-shaped molecules in (B) and (C) are shown on the traces. Potential positions of replication origins are indicated by half arrowheads below the maps of restriction fragments.

formed only a Y arc (Fig. 3D). These results suggest that replication was initiated from a site in the left half of the *Kpn*I–*Pvu*II segment.

It has been suggested that the initiation site of replication can be estimated from the molecular mass of the smallest Y-shaped intermediate that has been converted from a bubble-shaped molecule (7). Assuming that both replication forks proceed at the same rate, the distance (a) of the replication origin from the proximal end of a fragment can be estimated by the equation $a = (x - n)/2$, where n is the length of the restriction fragment and x is the molecular mass of the smallest Y-shaped molecule. From the results shown in Figure 3B and C, the molecular masses for the smallest Y-shaped intermediates derived from the *Bgl*II–*Xba*I (5.0 kb) and *Kpn*I–*Pvu*II (3.1 kb) fragments were calculated to be 9.4 ± 0.4 and 5.3 ± 0.2 kb respectively from the mobility in the first dimension. The initiation site was thus estimated to be at a position ~2.2 ± 0.2 kb from either end of the *Bgl*II–*Xba*I fragment or ~1.1 ± 0.1 kb from either end of the *Kpn*I–*Pvu*II fragment (half arrowheads in Fig. 3). From the results, we conclude that replication in the *ori2004* region was initiated within a restricted region of 0.4 kb around the center of the *Bgl*II–*Pst*I fragment.

The initiation site of replication on the pARS2004 plasmid

To examine whether replication of plasmid pARS2004 was initiated from a distinct site corresponding to the initiation site on the chromosome, cellular DNA from the plasmid-carrying HM123 cells was analyzed by 2D gel electrophoresis. Upon digestion of the DNA with *Pvu*II, which excised a 3.4 kb fragment containing *ars2004*, a strong bubble arc extending to nearly the 2N position was detected using the ARS fragment as probe (Fig. 4A), indicating that replication was predominantly initiated within the ARS segment. In addition to the bubble arc, a strong Y arc starting half way between the 1N and 2N positions and ending at the 2N position was observed (Fig. 4A). The transition from the bubble to Y arc indicated that replication was initiated from a distinct site located away from the center of the fragment. Based on the estimated molecular mass of the smallest Y-shaped molecule (6.0 ± 0.2 kb), the initiation site was mapped to 1.4 ± 0.2 kb from either end of the *Pvu*II fragment (half arrowheads in Fig. 4). For determination of the initiation site, a 0.5 kb fragment was inserted near the left or right end of the *Pvu*II fragment to shift the relative location of the initiation site. With insertion at the left end, the bubble arc was greatly extended and a transition to a very short Y arc was found (Fig. 4B), showing that the initiation site in the fragment was at a position near the center. In contrast, the fragment with the insertion at the right end formed a shortened bubble arc and a slightly extended Y arc, compared with those without insertion (Fig. 4C). The initiation sites estimated from the molecular masses of the smallest Y-shaped molecules were located at 2.0 ± 0.2 or 1.3 ± 0.2 kb from the *Pvu*II sites on plasmids with insertions at the left or right end of the ARS fragment.

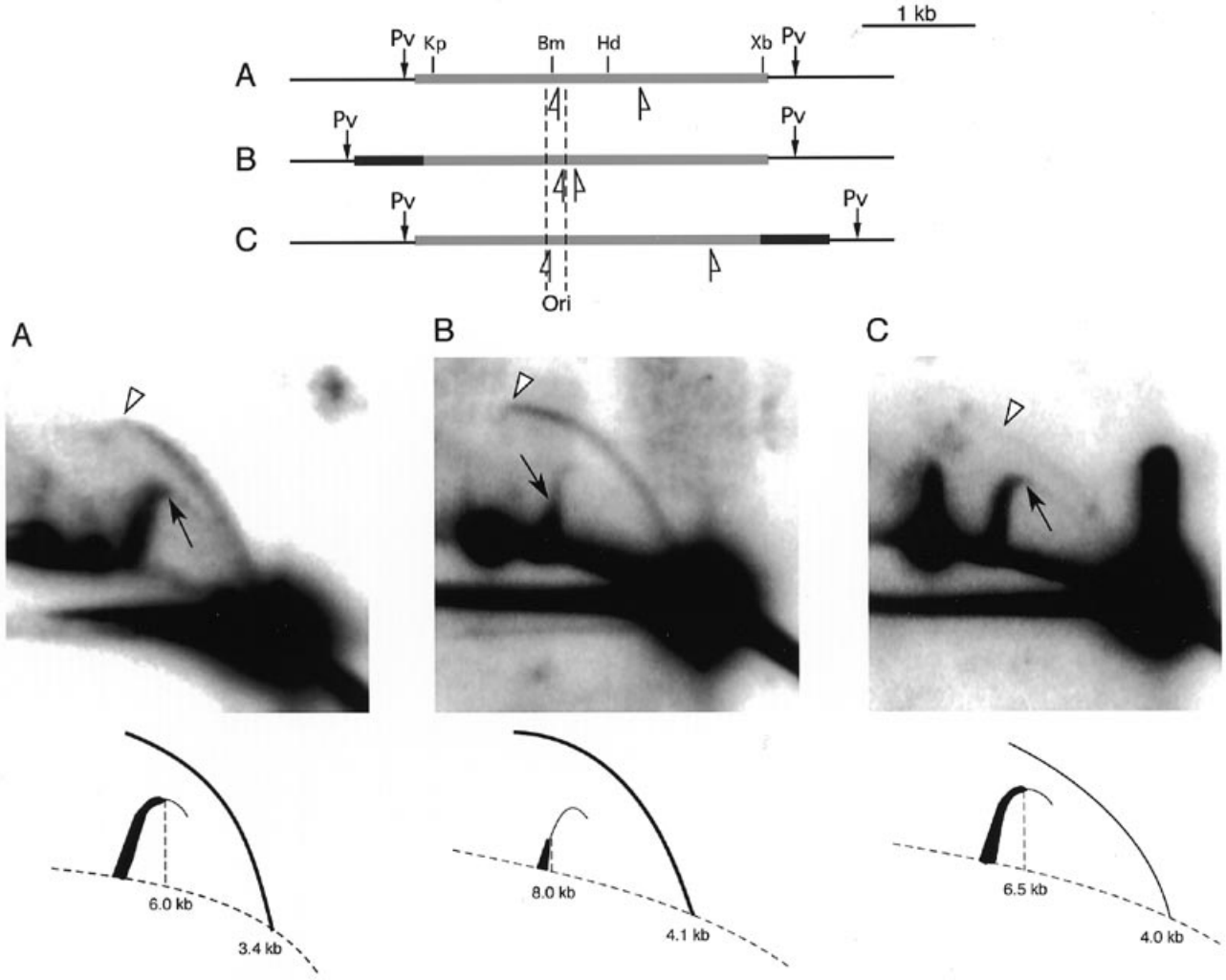

Figure 4. Localization of the initiation site for replication of the pARS2004 plasmid. Total cellular DNA from HM123 cells carrying derivatives of pARS2004 was digested with *Pvu*II and analyzed by 2D gel electrophoresis. The maps of relevant regions of plasmids (top), the results of hybridization (middle) and their traces (bottom) are presented. The plasmids used were pARS2004 (**A**), pARS2004Nλ (**B**), with an additional insert (black bar) at the left end of the ARS segment (shaded bar), and pARS2004Xλ (**C**), with an insert at the right end. The restriction enzyme sites *Pvu*II (Pv), *Kpn*I (Kp), *Bam*HI (Bm), *Hin*dIII (Hd) and *Xba*I (Xb) are indicated. Arrows and open arrowheads in the panels indicate positions of the smallest Y-shaped intermediates and the largest bubble-shaped molecules respectively. The molecular masses for the smallest Y-shaped molecules are given in the traces. Potential positions of initiation sites are indicated by half arrowheads on the maps and one of two possible sites on each plasmid is localized within a region shown by vertical broken lines.

From the results we conclude that replication on plasmid pARS2004 was initiated from a distinct location near the *Bam*HI site at 1.3 kb from the left end of the ARS fragment (see upper part of Fig. 4). These results, together with those presented in Figure 3, indicate that replication on the pARS2004 plasmid was initiated from the same site as that in the *ori2004* region on the chromosome.

DISCUSSION

We have identified five ARS elements in a 100 kb region of the fission yeast chromosome II, at least three of which function as chromosomal replication origins. The fact that ARS-lacking regions did not show any bubble arcs on 2D gel analyses indicates that chromosomal replication is likely to be initiated from distinct regions that are capable of autonomous replication. Initiation from *ori2004* occurs in every or almost every cell cycle, while that from *ori2002* and *ori2003* may take place once every few cell cycles. The efficiency of autonomous replication of the corresponding ARS plasmid is roughly proportional to the frequency of utilization of the corresponding chromosomal replication origin. These results suggest that elements involved in initiation of chromosomal replication are localized within limited regions and that the elements cloned into plasmids function as in the chromosomal locations.

The replication of pARS2004 is initiated from a unique site in the ARS fragment, which is identical, within the resolution of the 2D gel technique, to that in the *ori2004* region on the chromosome. We can thus conclude that the ARS2004 fragment contains all the *cis* elements required for initiation from the specific site. Analyses of deletion derivatives from either end of the 3.2 kb long ARS2004 fragment revealed that the initiation site is present within a 940 bp segment essential for ARS function (H.Satoh and H.Masukata, unpublished results). These results imply that the

essential region would be directly involved in reactions that promote replication.

It has been shown that the *ura4* origin region contains three clustered ARS elements that are functional as chromosomal replication origins (23,26). Although none of the origins operates in every cell cycle, initiation of replication in the *ura4* region does occur in every cell cycle. From the results, the authors proposed that the *ura4* origin region is a model for a replication initiation zone in higher eukaryotic chromosomes (40). In contrast, the *ori2004* region appears to contain a unique replication origin that operates in every cell cycle. Therefore, our results suggest that a single ARS element can function as a predominant chromosomal replicator and clustering of multiple origins as in the *ura4* region is not a general feature of fission yeast replicators.

It has been shown that the ARS elements of the fission yeast are several fold larger than those of the budding yeast. The ARS activity is gradually reduced by deletions from the ends of the fragments and no consensus sequence essential for ARS function has been identified (19,23). These characteristics are similar to those of human ARS fragments (15,17,41). However, the latter did not show any significant ARS activity in fission yeast and fission yeast ARS fragments did not replicate in human cells at a higher efficiency than non-ARS fragments (H.Satoh and H.Masukata, unpublished results). Presumably, the sequences required for initiation differ between these organisms. Extensive analyses of two ARS elements, *ars1* and *ars3002*, have suggested that asymmetric A+T-rich sequences clustered in one or two regions within a several hundred base pair segment are important for ARS activity (24,25). Detailed analyses of the ARS elements obtained in this study seem to be important to deduce the essential sequences and common structures of the *S.pombe* replicators.

In the budding yeast, recognition of the ARS consensus sequence by the ORC protein complex plays a crucial role in the initiation of replication (8,42). Recently, genes homologous to *ORC1* or *ORC2* have been identified in many organisms (43–45) and the homologs in fission yeast, *orp1*$^{+}$ and *orp2*$^{+}$, have been confirmed to be essential for cell growth (43,46,47). The Orp1 and Orp2 proteins of fission yeast, like those of the budding yeast (8), may interact with some element(s) in the replicator. The presently described *ars2004* that promotes efficient replication from a specific site could be used as a model replicator for studies of initiation of replication of fission yeast chromosomes.

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