Physical Mapping of Origins of Replication in the Fission Yeast *Schizosaccharomyces pombe*

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We isolated four fragments from the *Schizosaccharomyces pombe* genome that mediate autonomous replication. A two-dimensional gel analysis revealed that in each case initiation could be mapped to within the *S. pombe* sequences. In three of the fragments, initiation could be mapped to one discrete location. In the fourth fragment, subcloning and two-dimensional gel analysis suggested that two discrete origins of replication were located within 3 kb of each other. When in proximity, usually only one of these origins fired, suggesting origin interference. Two-dimensional gel analysis of the four origin fragments at their genomic locations demonstrated that each is used in the chromosomes, but in only a subset of cells or cell divisions. The *S. pombe* genome appears to contain many discrete origins, not all of which fire in any given cell and some of which are closely spaced. *Not* I/*Sfi* I mapping of the five origins from this and a previous study indicates that they are randomly distributed throughout the genome and appear to be representative of chromosomal origins of replication in this organism. We compare the features of *S. pombe* replication origins with those of *S. cerevisiae* and animal cells.

INTRODUCTION

There is a dichotomy between the types of replication origins that have been characterized in the budding yeast *Saccharomyces cerevisiae* and those of animal cells. In *S. cerevisiae*, replication initiates at discrete sites distributed throughout the genome. Replication origins are characterized by the presence of an 11 basepair (bp) consensus sequence, together with a more heterogeneous set of flanking elements (Newlon, 1988; Campbell and Newlon, 1991; Newlon and Theis, 1993). Mutational analysis has established that the consensus sequence is a necessary determinant of chromosomal origin function (Deshpande and Newlon, 1993). Two-dimensional (2-D) gel analyses that physically map initiation confirm that initiation is localized to a small region at each origin (Brewer and Fangman, 1987; Huberman *et al.*, 1987, 1988; Fangman and Brewer, 1991).

By contrast, in animal cells it has not been possible to identify a consensus sequence that is present at all origins. Autonomous replication studies suggest that a wide variety of fragments can serve to initiate replication in mammalian cells (Heinzel *et al.*, 1991). Two-dimensional gel analyses of initiation on autonomous replicons

(Krysan and Calos, 1991; Krysan *et al.*, 1993) and on the chromosomes (Vaughn *et al.*, 1990; Dijkwel and Hamlin, 1992) indicate that initiation occurs in a dispersed manner at many sites throughout a sizeable region.

To better understand the mode of replication in higher eukaryotes and how it may have evolved, we have turned to the fission yeast *Schizosaccharomyces pombe*. *S. pombe* preserves the small genome size and ease of analysis of budding yeast, but its chromosomal properties suggest a closer relationship to higher eukaryotes. It has three larger chromosomes, sizeable centromeres composed of repeat sequences, a mode of cell division by fission, and features of gene organization and expression that are reminiscent of animal cells (Russell and Nurse, 1986; Clarke, 1990). Replication origins in fission yeast may also be more closely related to those of higher eukaryotes.

Previous studies of replication in *S. pombe* have used autonomous replication assays similar to those used to isolate origins in *S. cerevisiae* (Hsiao and Carbon, 1979; Stinchcomb *et al.*, 1979). Notably, this type of assay was used by Maundrell *et al.* (1988) to isolate *S. pombe* fragments that could mediate autonomous replication

in the organism. A comparison of the sequences of nine such fragments revealed an 11-bp consensus sequence. However, deletion of the sequence appeared to have no effect on replication (Maundrell *et al.*, 1988). These studies left open the question of how *S. pombe* origins were structured and whether initiation occurred at discrete sites or in a dispersed manner. Application of 2-D gel techniques to the *ura4* region of the *S. pombe* genome suggested that initiation might occur in a dispersed manner over a region of several kilobases (kb) (Zhu *et al.*, 1992). By contrast, a detailed 2-D gel mapping study of initiation from an *S. pombe* origin fragment mediating autonomous replication gave clear evidence of tightly localized initiation (Caddle and Calos, 1994).

Studies of more examples of *S. pombe* origins are needed to determine with confidence the nature of initiation in fission yeast. To that end, we have isolated several more origins of replication from *S. pombe* and have performed 2-D gel analyses of initiation mediated by these origins on autonomous replicons and in the chromosomes. We have also localized the origins within the genome in an effort to begin building a complete replication map of the organism. The data we have derived from this study has begun to give us a picture of how replication origins function in *S. pombe* and how they are distributed. This information allows us to begin to compare and contrast the nature of replication origins in *S. pombe* with those of *S. cerevisiae* and animal cells.

MATERIALS AND METHODS

Strains and Plasmids

DNA for cloning and gel analysis was obtained from the *S. pombe* strain 972h⁻. This strain was grown in YEA media (Moreno *et al.*, 1991). Yeast transformations were done using the *S. pombe* strain sp806 as a host. This strain is *ura4*⁻ and was grown in Edinburgh minimal media (Moreno *et al.*, 1991). The sp806 *ura4*⁻ mutation can be complemented by the *S. cerevisiae* URA3 gene carried by the vector YIp5 (Struhl *et al.*, 1979) that was used for cloning and transformation of yeast. The plasmids that were examined in this study were generated by digestion of *S. pombe* DNA with the restriction enzyme *Hind*III and size fractionation of the digest on an agarose gel, followed by cloning of the fragments excised from the gel into the unique *Hind*III site in the YIp5 vector. Transformations were carried out by electroporation with 1 µg DNA as described (Prentice, 1991).

The subclones pYars1-1 (pYars1-1HN and pYars1-1EN) were created by restriction enzyme digestion of the pYars1-1 plasmid to release the 2.2-kb *Hind*III-*Nco* I *ars1*-1HN and 2.4-kb *Nco* I-*Eco*RI *ars1*-1EN fragments. The fragments were then treated with DNA polymerase I large fragment (Klenow, New England Biolabs) to create blunt ends. *Hind*III linkers (New England Biolabs) were then ligated to the blunt ends, and the insertions, with *Hind*III ends, were ligated into the unique *Hind*III site of the YIp5 vector.

Yeast DNA Isolation

S. pombe DNA for cloning, 2-D gel analysis, and Southern blot analysis was isolated as described in Caddle and Calos (1994).

Two-Dimensional Gel Electrophoresis and Southern Blot Analysis

2-D gel electrophoresis was carried out by the method originally described in Brewer and Fangman (1987), with modifications for fragments >5 kb as described in Krysan and Calos (1991).

Zetaprobe (Bio-Rad) membrane was used for blotting and hybridization by the methods described previously (Krysan and Calos, 1991). Radioactive DNA probes were prepared by the random priming method using $\alpha^{32}\text{P}$ -dATP and $\alpha^{32}\text{P}$ -dCTP (Feinberg and Vogelstein, 1983).

CHEF Gel Analysis

S. pombe DNA plugs for CHEF gel analysis were made from the 972h⁻ strain. Ten-milliliter yeast cultures were grown in YEA at 30°C to saturation. Yeast from 3-ml aliquots of culture were resuspended in 75 ml ET (50 mM EDTA; 10 mM Tris(hydroxymethyl)aminomethane [Tris]-HCl, pH 7.5) and treated with 0.5 mg of zymolyase 20T (ICN, Biomedicals, Inc.) at 37°C for 4 hours. The yeast were then mixed with 1% Sea Plaque GTG agarose (FMC BioProducts) in 0.125M EDTA and allowed to solidify in CHEF plug molds (Bio-Rad). The plugs were further treated with 1 mg zymolyase 20T in 1.8-ml LET solution (500 mM EDTA; 10 mM Tris-HCl, pH 7.5) at 37°C for 24 hours. The LET buffer was then replaced by 1.8 ml of NDS (500 mM EDTA; 10 mM Tris-HCl, pH 7.5; 1% *N*-lauroyl sarcosine) and the plugs were treated with 3.6 mg Proteinase K (EM Science) at 50°C for 24 hours. Plugs were washed three times, with slow shaking, at room temperature in 40 ml ET buffer containing 7 mg phenylmethylsulfonyl fluoride (Sigma).

For restriction enzyme digestion, one-half of one plug was incubated in 600 µl appropriate 1× enzyme buffer with 160 units of restriction enzyme (New England Biolabs) at the suggested temperature for 24 h. Plugs were then run in agarose gels on the CHEF-DR II pulsed field electrophoresis system (Bio-Rad). To resolve a desired fragment size range, we used conditions described in the maker's instruction manual and applications guide.

RESULTS

Isolation of *S. pombe* Clones with High Transformation Efficiencies

S. pombe chromosomal fragments were generated by digestion with *Hind*III and were ligated into the vector YIp5 (Struhl *et al.*, 1979). The *S. cerevisiae* URA3 gene in the YIp5 vector can complement *S. pombe* *ura4* mutations and thus serves as a selectable marker in the *ura4*⁻ *S. pombe* strain sp806. Forty-seven inserts carried by YIp5 (representing 241 kb of *S. pombe* DNA), as well as YIp5 alone, were transformed into the *S. pombe* *ura4*⁻ strain sp806. The YIp5 vector alone had a barely detectable transformation rate, and the few resulting colonies were extremely small. Thirty-nine of the plasmids with *S. pombe* insertions yielded similarly small colonies and low transformation rates. However, four of the plasmids transformed at rates that were at least an order of magnitude higher. These plasmids had an average rate of transformation of 5640 colonies/µg DNA and were candidates to contain strong origins of replication. Another four plasmids had intermediate transformation frequencies averaging 800 colonies/µg DNA. These plasmids may contain weak or partial origin activity and were not studied further. The transformants produced by the four plasmids with highest transformation efficiencies grew to large colony sizes and had fast doubling times in liquid media (~3 h/generation; the wild-type generation time was ~2.5 h). The likely explanation for the greatly increased transformation efficiency

and vigorous growth was that the four plasmids were efficiently replicating and maintained in multiple autonomous copies per cell.

These four insertions were provisionally designated *ars1-1*, *ars2-2*, *ars2-3*, and *ars3-5*. In this nomenclature, *ars* indicates that the fragments are autonomously replicating sequences (ARS). The first number represents their chromosomal location (see RESULTS below), while the second number indicates their order of discovery for the given chromosome. The plasmids bearing these ARS insertions were given the names pYars1-1, pYars2-2, pYars2-3, and pYars3-5, denoting that these ARS insertions were carried by the vector YIp5.

ARS Plasmids Are Shown to Exist Autonomously by Southern Blot Analysis

Southern analysis was employed to demonstrate that the four plasmids existed autonomously and were not integrated into the *S. pombe* genome (Figure 1). DNA samples from *S. pombe* that were transformed by each of the four plasmids were digested with the restriction enzyme *Not* I and separated on an agarose gel next to the same plasmids that were uncut and isolated from *Escherichia coli*. The gel was blotted and probed with the vector backbone (YIp5), so that only the plasmid would be detected and not the endogenous ARS sequences in the *S. pombe* genome. Because none of the plasmids had a *Not* I restriction site, plasmid DNA that was integrated into a yeast chromosome would comigrate with large yeast chromosomal DNA fragments that were generated by *Not* I digestion and were identified on the ethidium bromide-stained gel before blotting (lane M, arrow). In each case, the predominant plasmid species from transformed yeast comigrated with pure uncut supercoiled plasmid DNA, indicating that the transformant contained monomeric autonomous plasmid. Linear forms of the plasmids were also observed. Relatively more supercoiled DNA was detected in the plasmid DNA preparations, probably because of the comparative ease of purifying plasmid DNA from bacteria. In each case, the size of the band above the supercoiled band was confirmed as linear monomer by its correspondence to the expected size when compared to 1-kb ladder size markers present on the gel. Bands above the position of linear monomers were also present in the transformants and may correspond to nicked monomers and plasmid multimers. In contrast, plasmid DNA from yeast transformed with the plasmid pYAM9.0 (lane M) migrated with the large *Not* I chromosomal fragments. This transformant had previously been shown to have the plasmid pYAM9.0 integrated into the yeast genome (Caddle and Calos, 1994).

Two-Dimensional Gel Analysis Specifically Localizes Replication Initiation on the ARS Plasmids

The four ARS plasmids appeared to replicate autonomously in *S. pombe*. Neutral-neutral 2-D gel analysis

Figure 1. Southern analysis of ARS plasmid transformants. For each pYars plasmid, 0.1 ng of pure uncut plasmid DNA isolated from *E. coli* (P) was run on a 0.6% agarose gel next to 15 μ g of *Not* I-digested DNA from fission yeast transformed with the same plasmid (T). The gel was run for a prolonged time to improve resolution, then was blotted and probed with 32 P-labeled YIp5 vector. The bracket at bottom right denotes the region of the gel where the comigrating supercoiled forms of each of the plasmids are seen in the pure plasmid and transformant lanes. DNA (15 μ g) from *S. pombe* that was transformed by pYAM9.0 (see text) was digested with *Not* I and included on the far right in the lane labeled M (marker). The position of the large genomic *Not* I fragments, as observed on the ethidium bromide-stained gel before blotting, is denoted on the far right by an arrow. 2-2, pYars2-2; 3-5, pYars3-5; 2-3, pYars2-3; 1-1, pYars1-1; M, pYAM9.0.

(Brewer and Fangman, 1987) was employed in order to physically map the site of replication initiation on the plasmids. DNA was isolated from cultures of actively growing *S. pombe* that were transformed with the ARS plasmids, digested with various restriction enzymes, and subjected to 2-D gel analysis (Figures 2-5).

For the plasmid pYars2-2, a *Hind*III digest released the entire 4.0-kb insertion (Figure 2-I). Upon probing with the same 4.0-kb fragment, a full bubble arc, denoting initiation of replication with the fragment, was detected. This result indicated that replication was initiating from a specific region within the insertion and not from an initiation site on the YIp5 vector backbone. Therefore, termination of bidirectional replication would be predicted to occur in the YIp5 vector backbone. To test this prediction, the same blot was stripped and re-probed with the YIp5 backbone fragment II (Figure 2-II). The result was a Y-to-double-Y transition arc, which indicated that the YIp5 vector backbone was being replicated passively by forks coming from the insertion from both directions and terminating off-center in the vector fragment.

To further map the site of replication initiation on the pYars2-2 insertion, two subsequent digestions were done that generated smaller, overlapping fragments within the insertion. The 3.2-kb fragment III generated a strong Y arc pattern with no easily detectable bubble signal (Figure 2-III), while the 2.9-kb fragment IV yielded a prominent bubble signal (Figure 2-IV). These results indicated that replication initiated towards the center of the 2.9-kb fragment IV and toward the left end of the bubble detection zone in fragment I. The bubble detection zone encompasses the middle one-third of a fragment and is considered to be the portion of a fragment in which initiation events (bubbles) can

Figure 2. Two-dimensional gel analysis of pYars2-2. DNA from actively growing *S. pombe* that were transformed with the pYars2-2 plasmid was isolated, digested with various restriction enzymes, and subjected to 2-D gel electrophoresis. The resulting Southern blots were probed with ^{32}P -labeled restriction fragments, yielding the autoradiographs seen above. The linear map at bottom is of the plasmid pYars2-2. The shaded box in the center is the 4.0-kb insert from *S. pombe*, while the remainder is the Ylp5 vector backbone with its *URA3* gene shown. Above the map are the restriction fragments (I–IV) tested and the resulting autoradiographs with corresponding Roman numerals. The middle third of each fragment is depicted as a box to denote the bubble detection zone of the fragment. I, *Hind*III-digested DNA was run on a 2-D gel and probed with the entire 4.0-kb insertion. II, the blot in I was stripped and probed with the 5.5-kb Ylp5 vector backbone. III, a 2-D gel generated by digestion with *Sac* I and *Hind*III was probed with the 3.2-kb *Sac* I to *Hind*III fragment from the insertion. IV, a 2-D gel generated by digestion with *Eco*RI and *Hind*III was probed with the 2.9-kb *Eco*RI to *Hind*III fragment from the insertion. Sm, *Sma* I; H, *Hind*III; S, *Sac* I; E, *Eco*RI.

be reliably detected by 2-D gel analysis (Linskens and Huberman, 1990). All four of the 2-D gels are consistent with a single initiation site within a delimited region of the 4.0-kb *S. pombe* insert.

In the same way, the entire 8.2-kb insertion of pYars2-3 produced a strong bubble signal (Figure 3-I). This result indicated that initiation was occurring near the center of fragment I. The Ylp5 vector backbone produced a termination signal (Figure 3-II). Further localization of replication initiation in the *ars2-3* insertion was

achieved when a 3.7-kb subfragment of the insertion (Figure 3-III) gave a strong Y pattern. The adjacent 2.3-kb subfragment (Figure 3-IV) yielded a strong bubble arc. Replication thus initiated near the right end of the bubble detection zone of the 2.3-kb fragment IV of *ars2-3*, confirming the location of the origin in fragment I.

Two-dimensional gel analysis of pYars3-5 using as a probe the entire 8.1-kb insertion fragment I yielded a full bubble arc, indicating that replication was initiating from within the insertion (Figure 4-I). Upon stripping

Figure 3. Two-dimensional gel analysis of pYars2-3. A linear map of pYars2-3 is depicted at the bottom. Shown above this are restriction fragments used as probes for 2-D gels generated with DNA from *S. pombe* that was transformed with pYars2-3 (see Figure 2 legend for greater detail). I, a 2-D gel was generated by digestion of DNA with *Hind*III and was probed with the entire 8.2-kb insertion. II, the blot from I was stripped and probed with the 5.5-kb Ylp5 vector. III, a 2-D gel generated by digestion with *Bam*HI and *Hind*III was probed with a 3.7-kb *Bam*HI to *Hind*III fragment from the insertion. IV, a 2-D gel generated by digestion with *Bam*HI and *Cla* I was probed with a 2.3-kb *Bam*HI to *Cla* I fragment from the insertion. Sm, *Sma* I; H, *Hind*III; C, *Cla* I; B, *Bam*HI.

the blot and reprobing with the YIp5 vector backbone, a double fork termination signal was seen (Figure 4-II). Two further experiments were then carried out with adjacent, nonoverlapping fragments that were completely within the *ars3-5* insertion. The 3.8-kb fragment III (Figure 4-III) produced a full bubble arc pattern with some Y arc whereas the adjacent 2.8-kb fragment IV (Figure 4-IV) gave only a Y arc. These results suggest that replication initiates on the plasmid within the left side of the 3.8-kb fragment III and the right side of fragment I. Because of the limited overlap between the bubble detection zones of these two fragments, it is conceivable that two closely spaced origins occur, one in each bubble detection zone. However, since the designation of the bubble detection zone is only an approximation, it is probable that just one origin exists, located near the junction of the two bubble detection zones.

Two-dimensional gel analysis of pYars1-1 revealed that initiation of replication on this plasmid was more complex (Figure 5). A probe consisting of the entire 7.2-kb *Hind*III insertion yielded complete Y and bubble arcs (Figure 5-I). These results are consistent with initiation of replication at more than one location within the insertion or initiation at the edge of the bubble detection zone. The 5.5-kb vector backbone produced a Y and delocalized termination signal (Figure 5-II), suggesting more than one initiation site. A 3.5-kb fragment III from the insertion (Figure 5-III) produced only a Y arc, indicating that replication must be initiating from the other one-half of the insertion. In an attempt to further localize the site of initiation, a 6.0-kb fragment IV that crossed the junction between the insertion and vector was tested and gave full bubble and Y arcs (Figure 5-IV). Therefore, both fragment IV and fragment I produced bubble arcs, even though they had nonoverlapping

ping bubble detection zones. These results might be explained in two ways. Either there was a single origin of replication that resides just at the junction of the bubble detection zones of fragments I and IV, or there were two origins of replication on the insertion, one in the bubble detection zone of fragment I and the other in the bubble detection zone of fragment IV.

To resolve this question, a 3.7-kb fragment V with a bubble detection zone that covered the junction of the bubble detection zones of fragments I and IV was tested (Figure 5-V). This fragment produced a bubble, a Y, and a termination signal. This result was consistent with the presence of two origins of replication in the *ars1-1* insertion. The Y and bubble arcs were the result of a given initiation taking place at only one or the other of the origins, whereas the faint termination signal was the result of replication initiation occurring rarely from both origins at once, leading to termination of replication within fragment V. Figure 5-II also supported the theory that *ars1-1* carried two replication origins, because two distinct replication termination signals could be discerned in the autoradiograph, indicating two major distinct sites of replication initiation. The probable locations of these two origins are denoted at the bottom of Figure 5 by brackets labeled *ars1-1HN* and *ars1-1EN*. A 2-D gel was done with the 2.2-kb fragment VI (*ars1-1HN*). This gel revealed strong, full-length Y and bubble arcs (Figure 5-VI). A faint termination signal was visible in the original autoradiograph. This result strengthened the hypothesis that the *ars1-1HN* fragment carried an origin of replication, accounting for the bubble arc, and that another origin was active on the *ars1-1* insertion, accounting for the Y arc and termination signal.

In an attempt to isolate these two origins, the 2.2 kb *Hind*III to *Nco* I fragment from the *ars1-1* insertion (see Figure 5) was subcloned into the *Hind*III site of the vec-

Figure 4. Two-dimensional gel analysis of pYars3-5. A linear map of pYars3-5 is depicted at bottom. Shown above this are restriction fragments used as probes for 2-D gels generated with DNA from *S. pombe* that was transformed with pYars3-5 (see Figure 2 legend for greater detail). I, a 2-D gel was generated by digestion of DNA with *Hind*III and was probed with the entire 8.1-kb insertion. II, the blot from I was stripped and probed with the 5.5-kb YIp5 vector. III, a 2-D gel generated by digestion with *Stu* I and *Hind*III was probed with a 3.8-kb *Stu* I to *Hind*III fragment from the insertion. IV, a 2-D gel generated by digestion with *Stu* I was probed with a 2.8-kb *Stu* I fragment from the insertion. Sm, *Sma* I; H, *Hind*III; S, *Stu* I.

Figure 5. Two-dimensional gel analysis of pYars1-1. A linear map of pYars1-1 is depicted at the bottom. Shown above this are restriction fragments used as probes for 2-D gels generated with DNA from *S. pombe* that was transformed with pYars1-1 (see Figure 2 legend for greater detail). I, a 2-D gel was generated by digestion of DNA with *Hind*III and was probed with the entire 7.2-kb insertion. II, the blot from I was stripped and probed with the 5.5-kb YIp5 vector. III, a 2-D gel generated by digestion with *Hind*III and *Pvu* II was probed with a 3.5-kb *Hind*III-*Pvu* II fragment from the insertion. IV, a 2-D gel generated by digestion with *Pvu* II was probed with a 6.0-kb *Pvu* II fragment from the vector and insertion. V, a 2-D gel generated by digestion with *Hind*III and *Pvu* II was probed with a 3.7-kb *Hind*III-*Pvu* II fragment from the insertion. VI, a 2-D gel generated by digestion with *Hind*III and *Nco* I was probed with the 2.2-kb *Hind*III-*Nco* I fragment from the insertion (*ars1-1HN*). Sm, *Sma* I; H, *Hind*III; P2, *Pvu* II; N, *Nco* I; E, *Eco*RI.

tor YIp5, producing pYars1-1HN (Figure 6B). The adjacent 2.4-kb *Nco* I to *Eco*RI fragment of the *ars1-1* insertion (Figure 5) was also cloned into the *Hind*III site of YIp5, producing pYars1-1EN.

To test the ARS activity of the two fragments, sp806 *ura4*⁻ fission yeast were transformed by electroporation of these two plasmids, along with the intact parent plasmid pYars1-1 and pYars2-1 (previously called pYAM6.2) as a positive control that has been shown to have strong ARS activity (Caddle and Calos, 1994). Resulting transformants were streaked on minimal media plates and grown for 48 h at 30°C. The pYars1-1HN subclone conferred growth comparable to the full-length plasmid pYars1-1 and to the control plasmid pYars2-1 (Figure 6A). The doubling time in liquid culture for cells carrying pYars1-1HN was ~4.5 h, compared with 3.5 h for the parent pYars1-1 plasmid. The other subclone,

pYars1-1EN, conferred growth above background, but much less than the other three plasmids and showed very little growth in liquid culture. Thus, the fragment *ars1-1HN* had complete ARS activity, whereas the adjacent fragment, *ars1-1EN*, had weaker ARS activity. *Ars1-1EN* may simply be a weaker origin of replication, or perhaps some critical sequences required for efficient function of the origin were not included on the fragment that was subcloned. Because *ars1-1EN* transformants

Figure 6. Genetic and 2-D gel analysis of pYars1-1HN, a subclone of pYars1-1. (A) Growth of fission yeast transformants on a *ura*⁻ selective plate. Yeast from single colonies resulting from transformation of the four plasmids shown were streaked on a *ura*⁻ plate and allowed to grow for 48 hours at 30°C. (B) Two-dimensional gel analysis of pYars1-1HN. A linear map of pYars1-1 is depicted at the bottom. Shown above are restriction fragments used as probes for 2-D gels generated with DNA from *S. pombe* that was transformed with pYars1-1HN (see Figure 2 legend for greater detail). I, a 2-D gel was generated by digestion of DNA with *Hind*III and was probed with the entire 2.2-kb insertion. II, the blot from I was stripped and probed with the 5.5-kb YIp5 vector. III, a 2-D gel generated by digestion with *Eco*RI and *Nru* I was probed with a 1.9-kb fragment overlapping the insertion and vector. Sm, *Sma* I; H, *Hind*III; E, *Eco*RI; N, *Nru* I.

grew poorly in liquid culture, two-dimensional gel analysis of initiation could not be done.

Replication of pYars1-1HN was studied further by two-dimensional gel analysis (Figure 6B). The entire 2.2-kb insertion of pYars1-1HN yielded a full bubble arc, with a faint Y arc, and no termination signal (Figure 6B-I). This result confirmed that an origin of replication was present on the *ars1-1HN* fragment. The absence of the faint termination signal and of a full Y arc differed from the result obtained for this same fragment in the context of the parent plasmid, pYars1-1 (Figure 5-VI). This difference was likely due to the absence of another replication origin on the plasmid. It is conceivable that the faint early portion of the Y arc visible in Figure 6B-I may be partly due to signal contamination derived from the Y arc present at the chromosomal fragment (see below). A 2-D gel probed with the vector backbone (Figure 6B-II) showed a termination signal only, thus confirming that replication of the pYars1-1HN plasmid originated from the *ars1-1HN* fragment. Finally, fragment III, which came partially from the vector and the insertion, further limited the origin of replication on the *ars1-1HN* fragment to the left side, as it produced only a Y arc on a 2-D gel (Figure 6B-III).

Two-Dimensional Gel Analysis Demonstrates that the ARS Fragments Mediate Replication Initiation on the *S. pombe* Chromosomes

The 2-D gel analysis of the plasmids showed that the four insertions harbored active origins of replication in the context of YIp5 plasmids. It was important to discover whether they also mediated initiation of replication at their endogenous sites on the *S. pombe* chromosomes, thus qualifying them as chromosomal origins of replication. We performed a 2-D gel analysis in which DNA from actively growing *S. pombe* that did not carry a YIp5 ARS plasmid was isolated, digested with *Hind*III, electrophoresed in two dimensions, and blotted. The blots were probed with each of the *Hind*III ARS insertion fragments. *Ars3-5*, *ars2-3*, and *ars1-1* each yielded faint bubble arcs, along with Y arcs and termination signals (Figure 7). A similar result was obtained for *ars2-2*. However, the small size of the *ars2-2* fragment (4 kb), coupled with the lack of sensitivity of genomic 2-D gel analysis in detecting single-copy genomic sequences, produced only an extremely faint bubble arc after several weeks of autoradiographic exposure. The copy number of the plasmids relative to the chromosome was 10–20 per cell, based on the typical exposure times needed to detect replication of the plasmid and chromosomal fragments. This differential means that there would be minimal contamination from chromosomal sequences when probes were used that could detect both plasmid and chromosomal sequences. As we have demonstrated (Caddle and Calos, 1994), bubble arcs have not been observed for chromosomal fragments that lack ARS activity.

Figure 7. Two-dimensional gel analysis of ARS fragments at their endogenous sites in the *S. pombe* genome. Two-dimensional gels were generated by digestion with *Hind*III of DNA from fission yeast that were not transformed by an ARS plasmid. ³²P-labeled ARS insertions were used to probe the resulting Southern blots. Arrows designate bubble arcs.

The presence of a bubble arc indicated that replication initiated on the chromosomes from each of the four sequences. The simultaneous presence of Y arcs and termination signals may indicate that these sequences were also replicated passively in some cells and thus that they were not active as origins of replication in every cell cycle. Alternatively, initiation may have occurred over a more delocalized region in the chromosome, compared with the plasmid. These results demonstrated a context effect for *ars2-3* and *ars3-5* in which the ARS sequences were apparently more active or more localized as origins of replication when carried on the YIp5 vector than they were in the genome (see DISCUSSION). An alternative interpretation would involve breakage of replication bubbles at one fork to produce branched molecules that resemble Y-shaped replication intermediates. Even though the same method was used to prepare the DNA for the plasmid and chromosomal studies, breakage might preferentially affect chromosomal DNA because of its large size. Further experiments are required to definitively distinguish between these possibilities.

Each ARS was Localized to a Fragment of the *S. pombe* Chromosomes

To begin to define the chromosomal distribution of origins of replication in fission yeast, each of the ARS insertions was mapped to a segment of the *S. pombe* genome. This mapping was achieved by digesting the chromosomes in agarose plugs with the restriction enzymes *Not* I and *Sfi* I, separating the fragments with CHEF gel electrophoresis, blotting, and hybridizing with ³²P-labeled insert DNA from each pYars plasmid. The CHEF gel results were interpreted according to the *Not* I/*Sfi* I restriction map of Fan *et al.* (1991). Figure 8A provides an example of a CHEF gel autoradiograph that successfully localized the *ars2-2* insertion to *Not* I and *Sfi* I fragments. In this case, the fragment hybridized to the 446-kb *Sfi* I fragment L and the 1525-kb *Not* I fragment C from chromosome II. Interpretation of this and the other CHEF gels was aided by localization of the

fragments to the whole *S. pombe* chromosomes by CHEF gels that separated the uncut chromosomes.

Ars2-1 was described previously as the insertion in the plasmid pYAM6.2 (Caddle and Calos, 1994). It mapped to the same *Not* I and *Sfi* I fragments as *ars2-2*. These two origins are distinct and nonoverlapping, as shown by a comparison of their restriction maps. *Ars2-3* was localized to the 175-kb *Not* I fragment N and the 350-kb *Sfi* I fragment N, also on chromosome II. On chromosome I, *ars1-1* hybridized to the 900-kb *Not* I fragment F and to *Sfi* I fragment F or G. The neighboring F and G *Sfi* I fragments were too close in size (770 and 750 kb, respectively) to resolve. Finally, *ars3-5* was localized to chromosome III (*Not* I fragment A). This ARS appears to be on either the 383-kb *Sfi* I fragment M or the 242-kb *Sfi* I fragment P of chromosome III. These bands were not clearly resolved, possibly as a result of the presence of polymorphic rDNA repeats located at each end of chromosome III in *S. pombe* (Fan *et al.*, 1991). The *Sfi* I restriction fragments from chromosome III have been reported differently in a more recent publication (Mizukami *et al.*, 1993), but our pattern appears to match that of Fan *et al.* (1991) more closely. Figure 8B summarizes these results on a *Not* I/*Sfi* I restriction map of the *S. pombe* chromosomes, with the locations of the ARS fragments shown. Further localization could be done using the ordered *S. pombe* libraries that are now available (e.g., Mizukami *et al.*, 1993).

The names of the ARS fragments were assigned according to chromosomal location and order of discovery. The discovery of *ars3-5* as an ARS on *S. pombe* chromosome III was preceded by the discovery of several other chromosome III origins, which we have designated *ars3-1* (Toda *et al.*, 1984) in the rDNA repeats and *ars3-2*, *ars3-3*, and *ars3-4*, near the *ura4* gene (Zhu *et al.*, 1992). *Ars3-5* is distinct from these origins based on its restriction map. Perhaps the optimal system of nomenclature would be to use a four digit number to identify each origin, with the first digit representing the chromosome and the latter digits representing the position of the origin on the chromosome. When a full description of the locations of *S. pombe* origins is available, this system could be adopted.

DISCUSSION

We isolated four origins of replication from *S. pombe* by using an autonomous replication assay. Among the 277 kb of *S. pombe* DNA surveyed in this and a previous study (Caddle and Calos, 1994), we found five strongly positive fragments, giving an approximate frequency of one origin every 55 kb. This figure may be an underestimate; several additional fragments had significant activity over background and may represent partial origins that were disrupted by the position of the restriction enzyme cut or weak origins that may still function in the chromosomes. Moreover, one of the fragments we examined

Figure 8. Chromosomal localization of ARS fragments. (A) Localization of *ars2-2*. *S. pombe* DNA was isolated, digested with *Not* I (left) and *Sfi* I (right) and separated by CHEF gel electrophoresis. The resulting blot was probed with 32 P-labeled *ars2-2* DNA. On the autoradiograph that is shown, *ars2-2* hybridizes to 1525-kb *Not* I fragment C and 446-kb *Sfi* I fragment L (see Figure 8B). Shown at right are the positions and sizes of the *S. cerevisiae* chromosomes that were used as size standards on the same gel. The bands seen on this autoradiograph were definitively identified by superimposing the autoradiograph with that of the same blot which had been probed with 32 P-labeled *S. pombe* total DNA and thus displayed all of the *Not* I/*Sfi* I fragments. (B) Positions of the ARS fragments on the *S. pombe* chromosomes. The locations of the ARS fragments are shown to the right side of their respective chromosomes with brackets denoting the resolution of their localization. The position of a fifth ARS (*ars1-1*) is also shown (Caddle and Calos, 1994; previously known as the insertion in pYAM6.2). The *Not* I fragments A–Q are shown along the left side of each chromosome, and the *Sfi* I fragments A–S are shown to the right. A few other loci are shown to the left of each chromosome. The map was adapted from Fan *et al.* (1991).

carried two discrete origins. Our origin frequency of one per 55 kb is lower than the estimate of one per 20 kb of Maundrell *et al.* (1988) and may be due to more stringent selection in our system. An origin frequency of one per 55 kb is roughly similar to the estimated frequency of one ARS fragment every 32–40 kb in *S. cerevisiae* (Beach *et al.*, 1980; Chan and Tye, 1980) and to the spacing of active origins on *S. cerevisiae* chromosomes of one per 36–42 kb (Newlon and Burke, 1980; Newlon *et al.*, 1991).

The 2-D gel analysis we performed on the four fragments mediating autonomous replication showed that,

in the cases of *ars2-2*, *ars2-3*, and *ars3-5*, initiation could be localized to one small region within the *S. pombe* fragment. The positions of bubble, simple Y, and termination arcs on each of these plasmids was consistent only with a unique, fixed initiation site. This site could be delimited to a region of several hundred bp in each case, which is the resolution attainable with the 2-D gel technique and similar to the degree of localization of origins in *S. cerevisiae* (Fangman and Brewer, 1991).

For *ars1-1*, we were not able to map initiation to one discrete location. The presence of a mixed simple Y and bubble pattern over a region of several kb could have been interpreted as dispersed initiation. A clue that initiation was instead occurring at two discrete, closely spaced sites was provided by the appearance of a faint termination signal within the initiation region. In cases where closely spaced origins have been created in *S. cerevisiae*, both in the chromosome or on an autonomous replicon, a faint termination signal has been observed between the origins (Brewer and Fangman, 1993, 1994). This signal has been interpreted to mean that in rare cases, both origins fire on the same molecule. The greater strength of the bubble and simple Y signals, in our case and in the *S. cerevisiae* examples, reflects the normal usage of only one of the origins per molecule. The term origin interference has been used to describe the phenomenon in which close proximity of two origins leads to a decreased efficiency of firing at each origin, relative to its efficiency in the absence of a closely spaced origin (Brewer and Fangman, 1993, 1994). Origin interference appears to operate in a similar fashion in *S. cerevisiae* and in *S. pombe*.

We confirmed that two origins were present in the *ars1-1* fragment by separately cloning the two regions where initiation had been localized and observing that both fragments were able to initiate autonomous replication. For one of the fragments (*ars1-1HN*), we were able to demonstrate that 2-D gel mapping now gave straightforward patterns indicative of a single initiation site. For the other fragment (*ars1-1EN*), replication was too weak to permit 2-D gel analysis, perhaps because not all of the replication signal was present on the fragment that we subcloned. We found previously (Caddle and Calos, 1994) that deletions into an origin region could affect replication efficiency and lead to the type of small colony size we observed here in the subcloned *ars1-1 EN* origin fragment.

Closely spaced origins appear to be relatively common in *S. pombe*. Among the first five origin fragments we have examined, one instance of two closely spaced origins has been documented. Furthermore, in the *ura4* region studied by another group (Zhu *et al.*, 1992), initiation over a region of several kilobases, that would be consistent with the presence of closely spaced origins, was found, and at least two fragments with ARS activity were present. A correlation of closely spaced origin locations with the positions of genes or other chromo-

somal features will be sought. Closely spaced ARS elements have been described in *S. cerevisiae* chromosome III (Newlon *et al.*, 1991). However, none of the three closely spaced ARS elements in the *HML* region are active as origins in the chromosome (Dubey *et al.*, 1991), suggesting that they may have other functions. Among nine ARS fragments detected in a 230-kb region of *S. cerevisiae* chromosome VI, two are very closely spaced, but whether they act as origins in the chromosome was not reported (Shirahige *et al.*, 1993).

All four of the origins tested here, as well as a previously studied origin (Caddle and Calos, 1994), have yielded mixed simple Y and bubble arc signals when probed in the chromosomes. This type of mixed signal appears to indicate that initiation occurs from the origin in only a fraction of cell cycles, with the size of the fraction roughly indicated by the strength of the bubble arc due to initiation, relative to the simple Y arc due to passive replication from another site. The one other case in which an *S. pombe* origin has been mapped in the chromosomes using 2-D gels gave a similar result (Zhu *et al.*, 1992).

We do not believe that initiation is ubiquitous on *S. pombe* chromosomes, because we have shown that in three of three cases where fragments that lacked the ability to mediate autonomous replication were probed on the chromosomes, they gave no evidence of a bubble signal (Caddle and Calos, 1994). Given that our autonomous replication studies show that initiation invariably occurs in a strongly site-specific fashion when only one origin is present on a molecule, it seems likely that the passive replication reflected by the simple Y signal at origins in the chromosomes is due to initiations at other discrete sites in the *S. pombe* chromosomes.

In this regard, the mode of chromosomal replication we observe in *S. pombe* may be intermediate between the type of replication observed in *S. cerevisiae* and that in mammalian cells. In *S. cerevisiae*, although some fragments that exhibit ARS activity are not used as origins in the chromosomes (Dubey *et al.*, 1991) and others are only inefficiently used (Deshpande and Newlon, 1992; Greenfeder and Newlon, 1992; Dershowitz and Newlon, 1993), initiation most commonly occurs at discrete sites that are close to 100% efficient (Newlon *et al.*, 1991; Brewer and Fangman, 1993; Dershowitz and Newlon, 1993). In mammalian cells, initiation appears to occur at a large number of sites dispersed over a region, none of which are very efficient (Vaughn *et al.*, 1990; Dijkwel and Hamlin, 1992). In *S. pombe*, while initiation appears to occur from discrete sites, each site has a relatively low efficiency. However, a careful survey of initiation on an *S. pombe* chromosome over a sizeable region, including analysis of direction of fork movement, will be required to confirm this view.

Low efficiency of usage of a given origin would be expected if the number of potential initiation sites on a molecule is higher than the number actually used and

if differing initiation sites are used on different molecules. This type of origin usage could reflect a greater spacing between actively used origins in *S. pombe* than in *S. cerevisiae* and/or a higher frequency of potential origins. The roughly equal frequency of fragments with ARS activity in the two organisms might support the former explanation. However, because the definition of what is scored as positive in the ARS assay is open to some interpretation, no firm conclusion can be drawn at this time.

It is instructive to begin to compare the characteristics we have found for *S. pombe* origins to those of the much better studied *S. cerevisiae* model. A striking similarity shared by both yeasts, and contrasting with animal cells, is that initiation of autonomous replication is highly localized, as demonstrated by the appearance of a pure bubble signal on 2-D gels, associated with a small region of several hundred basepairs.

However, there are two respects in which *S. pombe* origins appear to differ from those described in *S. cerevisiae*. As previously discussed, the efficiency of usage of any one origin in *S. pombe* appears to be low, contrasting with the high efficiency of most *S. cerevisiae* origins characterized to date. If the frequency of closely spaced origins is higher in *S. pombe*, it may reflect a higher frequency of regions in that organism that can serve as origins. Furthermore, origins appear to be larger in size in *S. pombe* compared with *S. cerevisiae*. In budding yeast, the origin is fairly compact, consisting of the 11-bp consensus and flanking regions. For ARS1, for example, the relevant regions encompass ~200 bp (Marahrens and Stillman, 1992). In *S. pombe* the signals for efficient replication appear to be spread over a larger region. Maundrell *et al.* (1988) were unable to clone autonomously replicating fragments that were <0.8 kb. Our deletion analysis of *ars2-1* (Caddle and Calos, 1994) also indicated that signals important for full replication efficiency were distributed over a region of ~1 kb. The nature of the sequence signals involved in specifying initiation in *S. pombe* is not yet clear and will require fine-structure mutational analyses of several origins.

S. pombe origins also offer points of similarity and contrast with animal cell origins, although our knowledge of origins is in both cases far from complete. As in 2-D gel studies of initiation in mammalian cells (Vaughn *et al.*, 1990; Dijkwel and Hamlin, 1992) and *Drosophila melanogaster* embryos and tissue culture cells (Shinomiya and Ina, 1991, 1993), a mixed bubble and Y signal is found at initiation regions on the chromosomes. This result suggests that there may be more potential initiation sites than sites actually used in any given molecule. However, in *S. pombe*, when an origin is isolated on an autonomous replicon, a discrete initiation site can be physically mapped. Autonomous replication studies in animal cells have failed to give this result, and instead show a dispersed pattern of initiation similar to that observed on the chromosome (Krysan

and Calos, 1991; Hyrien and Mechali, 1992; Mahbubani *et al.*, 1992) (Smith and Calos, unpublished results). This difference could reflect a lesser sequence specificity on the part of animal cell origins. *S. pombe* origins may have an intermediate degree of sequence specificity between *S. cerevisiae* and animal cells, particularly if they lack an essential consensus sequence and use a widely dispersed array of sequence signals, both of which are suggested by existing data (Maundrell *et al.*, 1988; Caddle and Calos, 1994).

A deeper understanding of origins of replication in *S. pombe* will require detailed mutational studies of origin sequences and more extensive mapping studies of initiation on the chromosomes. Such studies are likely to provide further illumination of the evolution of replication origins in eukaryotes.

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