Organization of DNA replication origins in the fission yeast genome

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Eukaryotic DNA replication initiates at multiple points along the chromosomes known as replication origins (ORIs). We have developed a strategy to identify ORIs directly from replication intermediates in the fission yeast *Schizosaccharomyces pombe*. Mapping of a selection of the novel ORIs onto the genome reveals their preferential localization at intergenic regions upstream from genes. These results are supported by the observation that a large proportion of regions overlapping gene promoters contain active ORIs. Mapping of the genomic *arsI* replication origin at nucleotide resolution shows that replication initiates at a defined position immediately upstream from the *hus5*⁺ promoter. Deletion analysis indicates that the regulatory elements required to initiate transcription and replication lie in close proximity, suggesting a possible relationship between both processes *in vivo*.

Keywords: DNA replication/gene transcription/promoters/replication origins/*Schizosaccharomyces pombe*

Introduction

Eukaryotic replication origins (ORIs) have been particularly well characterized in *Saccharomyces cerevisiae* because they are easy to isolate in plasmids as autonomous replication sequences (ARSs). The presence of a consensus sequence 11 bp long has allowed the isolation of an origin recognition complex (ORC) that binds to it throughout the cell cycle (Bell and Stillman, 1992; Diffley *et al.*, 1994). Homologous ORC genes, as well as other initiator proteins initially identified in *S.cerevisiae*, have been found in the fission yeast *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, *Drosophila* and vertebrates, suggesting significant conservation of the replication machinery during eukaryotic evolution (reviewed in Dutta and Bell, 1997). In contrast to this, ORIs seem to be specific to each organism as they show no homology across species and they are non-functional in heterologous systems.

Plasmid maintenance assays have also been applied to *S.pombe*, in which detailed analysis of a number of ARSs has uncovered significant differences to *S.cerevisiae*. All the elements required to drive replication initiation in *S.cerevisiae* span <150 bp, whereas the shorter fragments

capable of sustaining ARS activity in *S.pombe* range from 500 to 1500 bp (Maundrell *et al.*, 1988; Dubey *et al.*, 1994; Wohlgemuth *et al.*, 1994; Clyne and Kelly, 1995; Dubey *et al.*, 1996; Kim and Huberman, 1998). In addition, mutation of the consensus element in *S.cerevisiae* ARS1 completely abolishes replication activity both in plasmids and in the chromosome (Marahrens and Stillman, 1992, 1994). This contrasts sharply with the situation in *S.pombe*, in which no specific sequences seem to be required for replication initiation as shown by mutational analysis in plasmids (Maundrell *et al.*, 1988; Clyne and Kelly, 1995; Dubey *et al.*, 1996; Kim and Huberman, 1998). Despite the absence of conserved elements, DNA replication seems to initiate at discrete sites at genomic loci as analysed by two-dimensional gel electrophoresis (Caddle and Calos, 1994; Dubey *et al.*, 1994; Okuno *et al.*, 1997), leaving open the question of which signals this specificity relies on.

A close relationship between transcription and replication has been established in several eukaryotic systems in which ORIs have been found to localize in the vicinity of gene promoters. Examples include *Physarum* (Pierron *et al.*, 1999), *Drosophila* (Sasaki *et al.*, 1999) and mammalian cells (Kitsberg *et al.*, 1993; Giacca *et al.*, 1994; Delgado *et al.*, 1998; Antequera and Bird, 1999). In *S.cerevisiae*, some auxiliary elements at ORIs bind transcription factors (Marahrens and Stillman, 1992), and heterologous transcriptional activation domains are capable of promoting replication initiation in the chromosome (Li *et al.*, 1998). The situation in *S.pombe* is unknown because only a small number of chromosomal ORIs have been identified to date. As an initial step in our study, we have developed a strategy to isolate chromosomal ORIs directly from replication intermediates, which circumvents the plasmid maintenance assay. This has provided us with a number of chromosomal ORIs that we have mapped onto their genomic context by taking advantage of the fact that a large fraction of the *S.pombe* genome has already been sequenced. Our results reveal a strong preference of ORIs to localize at intergenic regions overlapping gene promoters. Detailed dissection of the *arsI* genomic region shows that transcription and replication initiate very close to each other, suggesting a possible relationship between both processes *in vivo*.

Results

Identification of *S.pombe* genomic regions enriched in DNA replication origins

Sucrose gradients have previously been used to isolate short DNA replication intermediates to enrich active ORIs in mammalian cells (Tribioli *et al.*, 1987; Zhao *et al.*, 1994; Tao *et al.*, 1997; Delgado *et al.*, 1998). We adapted this approach to *S.pombe* and tested its efficiency

Fig. 1. Incorporation of BrdU by *S.pombe* T6. (A) Total RNA from *S.pombe* wild-type (wt) and T6 strains was hybridized with the herpes virus thymidine kinase gene (Tk) or the *cdc2+* gene as a probe. (B) BrdU incorporates into *S.pombe* T6 DNA up to 18% substitution of thymidine at 100 μ M BrdU. Incorporation was undetectable in the wild-type strain. (C) BrdU-substituted DNA from asynchronous *S.pombe* T6 cells grown in the presence of 100 μ M BrdU for 10, 30 and 60 min or without BrdU (C) was subjected to two rounds (first and second) of immunoprecipitation with anti-BrdU antibodies and used as a template for PCR of the *cig2+* gene as a control of DNA recovery. (D) Total BrdU-substituted DNA was fractionated in an alkaline sucrose gradient and nine fractions were collected from top to bottom (1–9). A sample of each was electrophoresed, blotted and hybridized using total DNA as a probe to monitor correct fractionation.

by two-dimensional gel electrophoresis. A *S.pombe* strain capable of incorporating bromodeoxyuridine (BrdU) into DNA was constructed by inserting the herpes simplex virus thymidine kinase (Tk) gene driven by the SV40 early promoter into the *leu1+* gene in the genome. The Tk⁺ strain (T6) actively transcribed the Tk gene (Figure 1A) and incorporated BrdU into its DNA in a concentration-dependent manner (Figure 1B) such that BrdU-substituted DNA could be effectively recovered by two rounds of immunoprecipitation with anti-BrdU antibodies (Figure 1C). To isolate BrdU-labelled short nascent strands, asynchronous cells were pulsed with BrdU and total DNA was purified and fractionated in an alkaline sucrose gradient. DNA fragments up to 2 kb long were recovered from the top fraction (fraction 1 in Figure 1D), immunoprecipitated to remove possible contaminating parental DNA, made double stranded and cloned. Only clones longer than 500 bp were considered for further analysis. As the cloning procedure involved extension of a primer from the 3' end of the DNA leading strand (see Materials and methods), we did not expect the cloned fragments to overlap the ORI precisely, but rather to map 1–2 kb away from them.

Sixteen clones were sequenced and eight of them were selected for further analysis because of their homology to *S.pombe* cosmids in the DDBJ/EMBL/GenBank database. This facilitated choice of the appropriate restriction sites for two-dimensional electrophoresis (Brewer and Fangman, 1987). Restriction fragments including the eight

Fig. 2. DNA fragments derived from short nascent strands detect chromosomal ORIs. (A) Black boxes represent immunoprecipitated fragments and white rectangles indicate genes. Arrows show the direction of transcription. Vertical lines indicate restriction fragments analysed by two-dimensional electrophoresis. J, *HindIII* (4.2 kb); C27, *EcoRV* (5.0 kb); B3, *Clal*–*BamHI* (6.3 kb); C11, *EcoRV*–*BamHI* (4.1 kb); C10, *Sall*–*PstI* (4.6 kb). (B) Upon digestion, total DNA was fractionated by two-dimensional electrophoresis and hybridized using the immunoprecipitated fragments as a probe. Arrows point to arcs containing replication bubbles.

cloned sequences were tested for replication initiation (Figure 2A). Faint but clear bubble arcs were detected in five of them (Figure 2B), revealing the presence of active ORIs. Prominent arcs of passive replication are also evident in Figure 2B, indicating that origins are active only in a small fraction of cells within the population. To rule out the possibility that weak bubble arcs could be generated by a non-specific replication background, we tested 10 randomly selected 4- to 5-kb-long genomic regions. No bubble arcs were detected in any of them (data not shown), thus pointing to the efficiency of our approach at enriching genomic ORIs in *S.pombe*.

Widespread occurrence of ORIs at gene upstream regions

Complete bubble arcs are detectable only when the initiation site is located approximately within the central third of the restriction fragment analysed (Linskens and Huberman, 1990). In the case of ORIs identified by clones J, C27, B3 and C11, the central third encompasses

Fig. 3. Replication analysis at intergenic regions upstream from genes. (A) Restriction fragments containing regions upstream from the genes indicated (black rectangles) in their central third were analysed by two-dimensional electrophoresis. White rectangles represent nearby genes within the same chromosome region. Other symbols are as in Figure 2. Restriction fragments tested for replication were: *rum1+*, *XbaI* (4.6 kb); *rip1+*, *EcoRV* (5.0 kb); *cdc25+*, *HindIII* (3.5 kb); *nmt1+*, *BamHI* (4.7 kb); *tug1+*, *EcoRV* (3.3 kb); *rhp6+*, *XbaI* (3.5 kb); *pcr1+*, *BglII-PstI* (4.7 kb); *thr1+*, *EcoRI* (4.7 kb). (B) Two-dimensional electrophoresis analysis of the restriction fragments indicated above.

intergenic regions and the 5' end of adjacent genes (Figure 2A). The bubble arc detected by clone C10 is shorter than in the other four cases (Figure 2B), indicating that initiation is close to the edge of the analysed fragment and that the ORI may also map at either of the two intergenic regions within the restriction fragment (Figure 2A). In the light of these observations, we decided to test how general the association of intergenic gene regions with active ORIs was. We assayed eight genomic fragments containing intergenic regions upstream from active genes in their central third (Figure 3A) and another eight located between adjacent 3' gene regions (Figure 4A). All 16 regions were selected at random

Fig. 4. Replication analysis at intergenic regions downstream from genes. (A) Restriction fragments containing regions downstream from the genes indicated (black rectangles) in their central third were analysed by two-dimensional electrophoresis. Symbols are as in Figure 3. Restriction fragments tested for replication were: *sui1+*, *BamHI* (3.7 kb); *gpd1+*, *PstI-BglII* (4.2 kb); *mra1+*, *PstI* (3.0 kb); *spm1+*, *PstI-BglII* (5.1 kb); *scd1+*, *EcoRV* (4.2 kb); *rhp7+*, *XbaI* (3.7 kb); *hes1+*, *BamHI-ClaI* (3.8 kb) and TFIIB homologue gene (TFIIB), *ClaI-ScaI* (3.0 kb). (B) Two-dimensional electrophoresis analysis of the restriction fragments indicated above.

from cosmid in the DDBJ/EMBL/GenBank database. Figure 3B shows that sequences upstream from the *rum1+*, *rip1+*, *nmt1+*, *tug1+* and *pcr1+* genes contained active ORIs, while regions 5' to *cdc25+*, *rhp6+* and *thr1+* genes were always replicated passively. In contrast, none of the eight fragments spanning intergenic 3' gene regions detected any bubble arc (Figure 4B), suggesting a preference for *S.pombe* ORIs to map at intergenic regions close to gene promoters. In addition, these results also show that a large proportion of gene upstream regions (five out of eight tested) are associated with active ORIs, which is consistent with the bias of randomly selected ORIs to map upstream from genes (Figure 2).

Fig. 5. Transcription is neither necessary nor sufficient for ORI activity. (A) Northern analysis of *nmt1*⁺ expression in *S.pombe* wild-type cells (wt) without thiamine (-T) or with 5 µg/ml thiamine (+T). The autoradiograph on the left was exposed for 1 h. The right panel shows that a low level of expression was detected in wild-type cells at longer exposure times (46 h). No expression was detected in a strain lacking the *ntf1*⁺ gene (*ntf1*Δ). Ribosomal RNA was used as a loading control (rRNA). (B) Two-dimensional electrophoresis shows that replication initiation at the region upstream from the *nmt1*⁺ gene is comparable in wild-type cells without (wt - T) or with (wt + T) thiamine and in the *ntf1*Δ strain. (C) Genomic map of the *mei2*⁺ gene region. The 5.8 kb *Pst*I-*Bgl*II fragment (bracket) was tested for replication. (D) Northern analysis of *mei2*⁺ expression in wild-type cells (wt) and the *cyr1*Δ strain. (E) Two-dimensional gel analysis in both strains.

Transcription is neither necessary nor sufficient for ORI activity

Given the widespread localization of ORIs near promoters, we wondered whether replication initiation depended on active transcription of the adjacent gene. We selected the ORI upstream from the *nmt1*⁺ gene (Figure 3) because of the lack of other promoters in the region that might hinder interpretation of the results. This gene is heavily repressed by thiamine (Maundrell, 1990), although a faint band of mRNA was visible in overexposed autoradiographs (Figure 5A). To make sure that repression was complete, we used a strain lacking the *ntf1*⁺ gene (*ntf1*Δ) that encodes an activator of the *nmt1*⁺ gene (Tang *et al.*,

1994). No *nmt1*⁺ RNA was detected in this strain even after long exposures (Figure 5A). We tested replication initiation in wild-type cells grown with and without thiamine and in the strain lacking the *ntf1*⁺ gene. As shown in Figure 5B, replication initiated with similar efficiency in the three strains, indicating that *nmt1*⁺ transcription is not required for replication initiation in the upstream region of the gene.

Next we tested the inverse possibility, namely, whether induction of high transcriptional activity might be sufficient to drive replication. We chose to study the *mei2*⁺ gene, whose transcription is undetectable in exponential wild-type cells but is heavily induced in a *S.pombe* *cyr1*Δ strain lacking the adenylate cyclase gene (Sugimoto *et al.*, 1991) (Figure 5D). The 5.8 kb fragment spanning the coding and upstream region of the *mei2*⁺ gene (Figure 5C) was passively replicated in wild-type and *cyr1*Δ cells (Figure 5E), despite the elevated rate of transcription in the latter (Figure 5D), indicating that induction of promoter activity, even at a high rate, is not sufficient on its own to trigger replication initiation. Together, these results indicate that transcription from a nearby promoter is neither necessary (*nmt1*⁺) nor sufficient (*mei2*⁺) for replication initiation.

DNA replication and transcription initiation at the *ars1* locus

To define further a possible relationship between transcription and replication, we analysed the *ars1* locus in the chromosome in detail. The *ars1* ORI is widely used in *S.pombe* vectors and its anatomy has been studied in plasmid assays (Maundrell *et al.*, 1988; Clyne and Kelly, 1995). In line with the results shown above, a 782-bp-long fragment capable of driving plasmid replication (Clyne and Kelly, 1995) mapped at a region upstream from *hus5*⁺ and a tRNA-Ser gene (Figure 7A). First, we ascertained that replication also initiates at this region in the genome (Figure 7C) and then we determined the transcription and DNA replication initiation points. Primer extension analysis using total RNA (Figure 6A and C) indicated that *hus5*⁺ transcription initiates 82 bp upstream from the ATG codon (Figure 6C). We also determined the DNA replication initiation point in the chromosome by using the approach recently developed by Bielinsky and Gerbi (1999) in *S.cerevisiae*. This method allows mapping of the 5' ends of DNA leading strands by primer extension using total DNA previously digested with λ-exonuclease. Only nascent strand DNA is protected by its RNA primer from the 5' to 3' λ-exonuclease activity, and this is used as a template to extend a labelled oligonucleotide to the junction with the RNA primer. The smallest detectable fragment indicates the position of the first deoxyribonucleotide synthesized. Bands of greater size represent Okazaki fragments joined to the leading strand from the replication fork proceeding in the opposite direction. Blank areas between bands are regions of continuous DNA synthesis. Figure 6B and C shows that extension of primers annealing to complementary strands defines two single initiation points three nucleotides apart from each other. The two major initiation points at ARS1 in *S.cerevisiae* are one nucleotide apart (Bielinsky and Gerbi, 1999). This indicates that replication initiates bidirectionally 261 bp upstream from the transcription initiation point. Identical

Fig. 6. Mapping of transcription and replication initiation points at the genomic *arsI* region. (A) The primer 5'-CATGCTATTTTCTGATTATT complementary to the *hus5*⁺ transcript was annealed to total RNA (R) extended by reverse transcriptase and the extended products were electrophoresed next to the corresponding DNA sequence. The two transcription initiation points (TIP) are indicated. (B) Primers shown by long arrows in (C) were labelled, annealed to DNA replication intermediates (Ri), and the extended products were electrophoresed next to the corresponding DNA sequences. RIP, replication initiation point at each DNA strand. (C) Sequence of the *S.pombe arsI* region. White arrows show the replication initiation point in both strands. Initiation and direction of transcription are indicated by a short black arrow. Transcribed sequences are in lower case. The ATG translation initiation codon is shown. Sequence blocked by continuous or dashed lines indicates regions that were deleted in strains $\Delta 330$ and $\Delta 231$, respectively (see Figure 7).

results were obtained with three independent preparations of DNA (data not shown). This physical proximity between transcription and replication initiation suggested a possible relationship between both processes, despite the fact that mRNA synthesis is not required to initiate replication as shown above.

Sequence elements required for transcription and replication initiation are adjacent in the chromosome

We addressed this issue by deleting the regions encompassing the replication and transcription initiation points to see whether physical removal of promoter sequences of the *hus5*⁺ gene would affect replication initiation. As this gene has been reported to be required for normal cell growth (Al-Kodairy *et al.*, 1995), promoter deletions were made in a *S.pombe* strain in which we previously integrated into the *leu1*⁺ gene an extra copy of the *hus5*⁺ gene under the *nmt1*⁺ 81X promoter, which is repressible by thiamine (Forsburg, 1993). However, no effect on cell growth

Fig. 7. Replication analysis of the *arsI* region. (A) Diagram of the 3.7 kb *Bam*HI fragment analysed by two-dimensional electrophoresis. The striped bar on top represents the minimal sequence with ARS activity in plasmids (Clyne and Kelly, 1995). White boxes represent the tRNA-Ser gene (left) and the six exons of the *hus5*⁺ gene. The untranslated part of exon 1 is cross-hatched. A black arrow indicates initiation and the direction of transcription of *hus5*⁺. White arrows indicate replication initiation. ACS indicates the position of the 11 bp A/T-rich element. White bars represent the three deleted regions. (B) Expression of the *hus5*⁺ gene in wild-type cells (wt) and the three deletion mutants carrying an extra copy of the *hus5*⁺ under the *nmt1*⁺ promoter gene grown with (+) or without (-) thiamine. Filters were rehybridized with the *cdc2*⁺ gene as a loading control. (C) Two-dimensional electrophoresis analysis of the wild type (wt), ACS replacement (ACS) and deletion mutants $\Delta 231$, $\Delta 330$ and $\Delta 513$.

was observed, even under conditions of undetectable expression of the gene (see below). Deletion of the region between positions -409 and +104 (Figure 6C) removed the replication and transcription initiation points and the resulting strain was unable to transcribe *hus5*⁺ or initiate replication (Figure 7B and C, $\Delta 513$). Replacement of this region in the chromosome by an unrelated sequence 541 bp long did not restore transcription or replication (data not shown), suggesting that specific sequences were required and ruling out the possibility that the effect could be due to a gross structural alteration of the region. To define the regulatory elements better, we analysed the two halves of this region independently. Deletion from -128 to +103 (Figure 6C) completely abolished *hus5*⁺ transcription but did not affect replication (Figure 7B and C, $\Delta 231$). This result also shows that transcription is not required for replication, in agreement with our previous result with *nmt1*⁺ (Figure 5A and B). In contrast, removal of the sequence between -409 and -79 (Figure 6C) reduced transcription by a factor of three and replication by a factor of 10 relative to the wild-type level as measured in a phosphorimager (Figure 7B and C, $\Delta 330$), pointing to

Table I. Localization of ORIs relative to adjacent genes

<<< ORI >>>	>>> ORI >>>	>>> ORI <<<
<i>Saccharomyces cerevisiae</i>		
ARS305 ^a	ARS306 ^a	ARS608 ^c
ARS1 ^b	ARS307 ^a	ARS1412 ^d
ARS606 ^c	ARS308 ^a	ARS501 ^b
ARS1411 ^d	ARS607 ^c	
	ARS609 ^c	
	ARS1413 ^d	
	ARS1414 ^d	
Observed (expected)		
28.5% (25%)	50% (50%)	21.4% (25%)
<i>Schizosaccharomyces pombe</i>		
Clone J	Clone B3	
Clone C27	<i>nmt1</i> ⁺	
Clone C11	<i>ars3003</i> ^c	
Clone C10		
<i>rum1</i> ⁺		
<i>rip1</i> ⁺		
<i>tug1</i> ⁺		
<i>pcr1</i> ⁺		
<i>ars1</i>		
<i>ars3002</i> ^c		
<i>ars3004</i> ^c		
Observed (expected)		
78.6% (25%)	21.4% (50%)	0% (25%)

<<< ORI >>>, ORI between the 5' ends of two adjacent genes;
 >>> ORI >>>, ORI between the 5' end and the 3' end of two
 adjacent genes; >>> ORI <<<, ORI between the 3' ends of two
 adjacent genes.

^aNewlon *et al.* (1993).

^bFerguson *et al.* (1991).

^cShirahige *et al.* (1998).

^dFriedman *et al.* (1996).

^eDubey *et al.* (1994).

the presence of elements involved in both processes within the deleted region.

Deletion Δ231 includes a 50 bp sequence reported to be essential for plasmid replication (Clyne and Kelly, 1995), indicating that the results obtained with plasmid assays may not always reflect the situation in the chromosome. We also tested the functional relevance of an 11 bp A/T-rich element (ACS) homologous to the consensus sequence of *S.cerevisiae* ARSs (Newlon and Theis, 1993) (Figure 7A). This element is not necessary for plasmid maintenance but it has been suggested to play a role in replication initiation within the chromosomal context (Maundrell *et al.*, 1988; Clyne and Kelly, 1995). Figure 7C (ACS) shows that replacement of the wild-type sequence (AATTTATTTAT) by an unrelated one (CGCGAAA-GCTT) of the same length in the genome affects replication very slightly if at all, implying that the consensus sequence is not necessary within the chromosome either.

Discussion

Genomic organization of *S.pombe* replication origins

Schizosaccharomyces pombe and *S.cerevisiae* have compact genomes with a high gene density. We have mapped 14 *S.cerevisiae* ARS elements previously found to be active in the chromosome onto its genome. The data in Table I indicate that they localize with the expected frequency at the three possible intergenic regions relative

to genes. In contrast, there is a strong preference for *S.pombe* ORIs to localize at intergenic regions containing promoters. The average size of intergenic regions is similar in all cases. All 11 novel genomic ORIs identified in this work map upstream from genes, with a preference for regions flanked by divergent transcription units (Table I). Despite the relatively small number of ORIs analysed, these data suggest that the organization of ORIs relative to genes is different in both yeasts.

Significant differences have also been found in the structure of ORIs. Previous studies in *S.pombe* have identified very degenerated consensus sequences and a marked tolerance to modifications (Maundrell *et al.*, 1988; Clyne and Kelly, 1995; Dubey *et al.*, 1996; Kim and Huberman, 1998). However, replication initiates at discrete sites, as previously suggested by two-dimensional gel electrophoresis results (Caddle and Calos, 1994; Dubey *et al.*, 1994; Okuno *et al.*, 1997) and as shown here at nucleotide resolution at *ars1* (Figure 6). The organization of this locus, where replication initiates 261 bp upstream from the transcription initiation point of the *hus5*⁺ gene, is homologous to that found in the human *PPV1* gene, in which a putative ORC maps ~200 bp upstream from the transcription factors bound at the promoter *in vivo* (Dimitrova *et al.*, 1996). Together, our results indicate that the organization of ORIs in *S.pombe* could be similar to that reported in animal cells, for which a number of ORIs have been mapped in close proximity to gene promoters (Kitsberg *et al.*, 1993; Giacca *et al.*, 1994; Zhao *et al.*, 1994; Delgado *et al.*, 1998; Antequera and Bird, 1999) and only very loosely conserved sequences have been found (Dobbs *et al.*, 1994).

The finding that ORIs co-localize with a large fraction of gene upstream regions implies a very high density of ORIs in the fission yeast genome. A previous study revealed the presence of three closely spaced ORIs within 5 kb at the *ura4*⁺ region (Dubey *et al.*, 1994). In agreement with our results, their localization onto the genome by sequence homology shows that they overlap the promoter regions of three adjacent genes (data not shown and Table I). This higher density of ORIs relative to *S.cerevisiae* probably determines a lower frequency of usage of each one during each cell cycle, which is consistent with the much weaker bubble arcs relative to the passive replication arcs in *S.pombe* (see e.g. Figures 2B and 3B and Caddle and Calos, 1994; Dubey *et al.*, 1994; Wohlgemuth *et al.*, 1994) than in *S.cerevisiae* (see e.g. Newlon *et al.*, 1993; Friedman *et al.*, 1996; Shirahige *et al.*, 1998).

Transcription and DNA replication in *S.pombe*

The results in Figures 5 and 7 show that transcription from the nearest promoter to an ORI is not required for replication initiation. This argues against a mechanistic connection between both processes and raises the question of why ORIs preferentially localize close to promoters. A possible explanation is that transcription factors could contribute to recruiting the replication machinery onto DNA, either directly or by facilitating its access to DNA by promoting an open chromatin conformation. According to this scenario, some of the factors bound to the promoter region could be dedicated to either transcription or replication, whereas others could

play a role in both processes. This is probably the case at *S.pombe ars1*, since deletion of 231 bp encompassing the transcription initiation point abolishes transcription but does not affect replication (Figure 7, Δ 231). On the other hand, deletion of 330 bp encompassing the replication initiation point simultaneously affects both transcription and replication (Figure 7, Δ 330). These results also imply that the weak initiation activity in the mutant Δ 330 cells must occur at a different site than in the wild type. This tolerance to sequence modification of *S.pombe* ORIs has also been observed in plasmid assays and suggests the presence of partially redundant elements (Clyne and Kelly, 1995; Dubey *et al.*, 1996; Kim and Huberman, 1998; Maundrell *et al.*, 1988). *In vivo* footprinting across the *ars1* region and mutational analysis of the relevant elements will be necessary to define the role of each of them in activating transcription or replication.

That ORIs lie close to promoters does not imply the inverse, namely, that all promoters have an ORI nearby (Figure 3). It is conceivable that specific arrangement of activators or differences in chromatin structure at different promoters could contribute differentially to facilitating replication initiation in each case. Given the compactness of the *S.pombe* genome, preferential activation of an ORI could prevent the activation of others in the vicinity. This has been shown at the *ura4⁺* region, where the three closely spaced ORIs interfere with each other in a hierarchical fashion (Dubey *et al.*, 1994). This phenomenon of interference also occurs in *S.cerevisiae* when two ARS elements are located close to one another in the chromosome (Brewer and Fangman, 1993; Marahrens and Stillman, 1994).

Materials and methods

Yeast strains and media

Culture conditions, RNA isolation and strain manipulations were as described (Moreno *et al.*, 1991). *Schizosaccharomyces pombe* h⁻*ura4*-d18 *leu1*-32 cells were transformed by the lithium acetate protocol (Norbury and Moreno, 1997). Deletion and replacement mutants were constructed by insertion of the *ura4⁺* marker. The resulting strains were transformed with a DNA fragment lacking the sequences deleted in each case and selected in the presence of 5-fluoroorotic acid. All mutations were confirmed by PCR amplification followed by DNA sequencing. For [³H]BrdU incorporation assays, cells were grown for 2.5 h in Edinburgh Minimal Medium (EMM) (Moreno *et al.*, 1991) at 32°C containing the amount of BrdU indicated (Figure 1B), and the radioactivity incorporated was measured on total isolated DNA by scintillation counting.

Cosmid accession

The cloned fragments in Figure 2 are contained in cosmids with the following DDBJ/EMBL/GenBank accession Nos: J, SPAC18G6; C27, SPAC16E8; B3, SPAC3F10; C11, SPCC285; C10, SPAC6G9. The DDBJ/EMBL/GenBank accession Nos of cosmids containing genes in Figure 3 are: *rum1⁺*, SPBC32F12; *rip1⁺*, SPAC6B12 (open reading frame accession No.: L37885); *cde25⁺*, SPAC24H6; *mnt1⁺*, SPCC1223; *tug1⁺*, SPBC32F12; *rhp6⁺*, SPAC18B11; *pcr1⁺*, SPAC21E11 and *thr1⁺*, SPAC9E9. The DDBJ/EMBL GenBank accession Nos of cosmids containing genes in Figure 4 are: *sui1⁺*, SPBC23G67; *gpd1⁺*, SPBC32F12; *mra1⁺*, SPAC18G6; *spm1⁺*, SPBC119; *scd1⁺*, SPAC16E8; *rhp7⁺*, SPCC330; *hes1⁺*, SPBC354; TFIIB, SPAC16E8.

Alkaline sucrose gradients and cloning of short nascent DNA strands

Asynchronous exponential *S.pombe* cells were pulsed with 100 μ M BrdU for 15 min and nascent DNA strands were isolated by alkaline sucrose centrifugation as described (Zhao *et al.*, 1994). Nascent strands up to 2.0 kb long from the gradient top fraction were subjected to two rounds of immunoprecipitation with an anti-BrdU monoclonal antibody

(Vassilev *et al.*, 1990). Immunoprecipitated single-stranded DNA replication intermediates were tailed with poly(C) at the 3' end with terminal transferase. Complementary strands were synthesized by extending a poly(G) 8mer primer annealed to the poly(C) tail with T4 DNA polymerase. A second tail of poly(C) was added to this newly synthesized strand and the final product was amplified by 35 cycles of PCR (94°C for 1 min, 48°C for 1 min, 72°C for 2 min) using the oligonucleotide 5'-AACTGCAGGGGGGGGGG as primer. PCR products were cloned and sequenced.

DNA isolation and two-dimensional gel electrophoresis

DNA was prepared following the method described by Huberman *et al.* (1987) and two-dimensional neutral gel electrophoresis was performed as described by Brewer and Fangman (1987).

Replication mapping assay

The mapping assay was performed as described by Bielinsky and Gerbi (1999) with minor modifications. Total *S.pombe* DNA was cleaved with *Bam*HI, heat denatured and 5–10 μ g were incubated overnight with 6–8 U of λ -exonuclease to remove all DNA fragments without an RNA primer at their 5' ends. Primers 5'-AGCAGTTAGCG-AAAATTAAT and 5'-GTTTTCTATTTGTACGTCC were labelled at a specific radioactivity of 10⁸ c.p.m./ μ g and annealed to replication intermediates. Primer extension reactions contained 200–500 ng of replication intermediates, 300 μ M nucleoside triphosphates, 30 pmol of γ -³²P-labelled primer and 2 U of Vent (exo-) DNA polymerase (New England Biolabs). Thirty cycles of extension were performed at 95°C for 1 min, 56°C for 1 min and 72°C for 1.5 min.

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