The Mating Type Switch-Activating Protein Sap1 Is Required for Replication Fork Arrest at the rRNA Genes of Fission Yeast

Eva Mejía-Ramírez, Alicia Sánchez-Gorostiaga, Dora B. Kramer, Jorge B. Schwartzman, and Pablo Hernández*  
Departamento de Biología Celular y del Desarrollo, Centro de Investigaciones Biológicas (CSIC), Ramiro de Maeztu 9, 28040 Madrid, Spain  
Received 31 March 2005/Returned for modification 10 May 2005/Accepted 3 July 2005

Schizosaccharomyces pombe rRNA genes contain three replication fork barriers (RFB1-3) located in the nontranscribed spacer. RFB2 and RFB3 require binding of the transcription terminator factor Reb1p to two identical recognition sequences that colocalize with these barriers. RFB1, which is the strongest of the three barriers, functions in a Reb1p-independent manner, and cognate DNA-binding proteins for this barrier have not been identified yet. Here we functionally define RFB1 within a 78-bp sequence located near the 3′ end of the rRNA coding region. A protein that specifically binds to this sequence was purified by affinity chromatography and identified as Sap1p by mass spectrometry. Specific binding to RFB1 was confirmed by using Sap1p expressed in Escherichia coli. Sap1p is essential for viability and is required for efficient mating-type switching. Mutations in RFB1 that precluded formation of the Sap1p-RFB1 complex systematically abolished replication fork barrier function, indicating that Sap1p is required for replication fork blockage at RFB1.

DNA replication fork arrest induced by DNA lesions or as a consequence of errors during replication is an important cause of genome instability. Cells respond to this challenge by activating both the S-phase checkpoint pathway that slows down DNA replication and DNA repair mechanisms, which eliminate DNA lesions and reactivates the arrested replication forks. Aside from these accidental fork arrests, there are natural replication fork pausing sites that seem to play specific biological roles. The rRNA genes of all eukaryotic species studied so far contain several replication fork barriers (RFBs) (5, 9, 18–20, 26, 28, 29). RFBs are clustered and located in the nontranscribed spacer close to the 3′ end of the coding region. In most of the species studied, these RFBs function in a polar manner, arresting only replication forks moving in the direction opposite to transcription. As a consequence, rRNA genes are mainly replicated unidirectionally and cooriented with transcription. Therefore, one of the roles of rRNA gene RFBs may be to prevent the deleterious effects of head-on collisions between replication and transcription machineries (24, 27).

For Saccharomyces cerevisiae rRNA genes, Fob1p is required for the activity of RFBs and directly binds to the DNA at the barriers (12, 21). How Fob1p inhibits progression of the replisome, however, is still unknown. In this budding yeast, Fob1p-dependent RFBs are also required for HOT1 recombination, the expansion and contraction of rRNA gene repeats, and the formation of extrachromosomal rRNA gene circles (11, 13, 14), indicating that fork arrest at RFBs promotes homologous recombination within the rRNA gene locus (4).

Schizosaccharomyces pombe rRNA genes contain three independent closely spaced barriers, RFB1 to -3. RFB2 and RFB3 require binding of the transcription termination protein Reb1p to two 17-bp recognition sequences present close to the 3′ end of the 35S gene (26). RFB1 is the strongest of the three barriers and functions in a Reb1p-independent manner. Here we identify the mating type switch-activating protein Sap1 as the cognate DNA-binding protein for RFB1 and show that formation of the RFB1-Sap1p complex is required for replication fork stalling at this barrier.

MATERIALS AND METHODS

Cell strains and growth conditions. The S. pombe strain 35 (h· leu1-32) was used in most of the experiments. To test the requirement of the proteins Swi1 and Swi3 for RFB1 function, we used strains EN3182 (h· leu1-32 ura-D18 swi1::Kan') and EN3366 (h· leu1-32 ura-D18 swi3::Kan'), which were kindly provided by Paul Russell. Standard media and growth conditions were employed (22). Escherichia coli strain DH5α was used as the host for the construction of plasmids containing rRNA gene sequences, and strain TOP10 was used for the expression of Sap1p.

Construction of plasmids containing rRNA gene sequences. Plasmids were constructed by inserting rRNA gene fragments into the polycloning site of vector pIRT2, close to the replication origin ars1. The transformation of S. pombe was performed by electroporation (25). The fragments shown in Fig. 1 were obtained by PCR from plasmid pIRT1::(+) (26), using the following primers (listed in Table 1): SpRFB3-1 and SpRFB4 for pΔ604-879, SpRFB3-2 and SpRFB4 for pΔ77-879, SpRFB3-3 and SpRFB4 for pΔ77-879, SpRFB3-2 and SpRFB3-4 for pΔ77-825, SpRFB3-2 and SpRFB3-3up for pΔ677-754 and SpRFB3-3 and SpRFB3-4up for pΔ737-825. The PCR products were digested with PstI and BamHI and inserted into the polycloning site of vector pIRT2 (10). The fragments shown in Fig. 3 and 4 were obtained by annealing the following oligonucleotides (Table 1): SpRFB1 for fragment I, SpRFA and SpRFB for fragment II, SpRFA and SpRFB for fragment III, SpRFA and SpRFB for fragment IV, RFB1wt-a and RFB1wt-b for fragment wt, mut1a and mut1b for fragment mut1, mut2a and mut2b for fragment mut2, mut3a and mut3b for fragment mut3, mut4a and mut4b for fragment mut4, and mut5a and mut5b for fragment mut5. The annealed oligonucleotides were inserted into pIRT2 digested with BamHI and PstI (fragments in Fig. 3) or with BamHI (fragments in Fig. 4). All insertions were confirmed by sequencing.

Two-dimensional gel electrophoresis. DNA purification from asynchronous log-phase cultures and analyses of replication intermediates by two-dimensional (2D) gel electrophoresis were performed as previously described (26). Before analysis, plasmids were digested with Pvull. The large Pvull fragment from pIRT2 was used as a probe.
Protein extracts. *S. pombe* whole-cell extracts were prepared from asynchronous mid-log-phase cultures (100 ml). Cells were harvested and resuspended in 40 ml of extraction buffer (25 mM HEPES, pH 7.6, 150 mM KCl, 0.1 mM EDTA, 0.1% Triton X-100, 25% glycerol, 4 μg/ml leupeptin, 2 mM dithiothreitol [DTT], 2 μg/ml pepstatin, 2 μg/ml aprotinin, and 0.2 mM phenylmethylsulfonyl fluoride). After cell wall disruption with 425- to 600-μm glass beads (Sigma), the emulsion was diluted with 600 ml of extraction buffer, transferred to a new tube, and clarified by centrifugation. The recovered supernatant was divided into aliquots and stored at −80°C. The protein concentration was estimated by the Bradford method.

*E. coli* protein extracts were prepared as previously described (8).

**Electrophoretic mobility shift assay (EMSA).** Binding reaction mixtures (20 μl) included 8 μl of extraction buffer containing the indicated amounts of protein extract or purified Sap1p, 2 μl of 10× binding buffer (250 mM HEPES, pH 7.6, 350 mM KCl, 100 mM EDTA), 1 μl of 2-μg/μl poly(dI-dC), 2 μl of 1-μg/μl salmon sperm DNA, 1 μl of 10 nM DTT, and 6 μl of radiolabeled probe (0.04 to 0.8 ng; 10,000 cpmp). The probe used for Fig. 1 was obtained by PCR from pIRT1.6, using primers SpRFB3-2 and SpRFB3-3up (Table 1), followed by digestion with BamHI. To obtain the probes used for Fig. 3 and 4, the pIRT2- derivative plasmids containing each of the fragments were used in PCRs with primers pIRT2-ars1up and pIRT2-leu2do. The products were then digested with BamHI-HindIII (Fig. 3) or with BamHI (Fig. 4). All probes were labeled by filling the 5′ protruding ends with [α-32P]dCTP and the Klenow fragment and were purified through a G-25 Sephadex column (Roche). Binding reaction mixtures were incubated for 20 min at room temperature and electrophoresed at 4°C in 6% polyacrylamide–0.5 Tris-borate-EDTA gels at 10 V/cm for 2.5 h.

**Expression of Sap1p in *Escherichia coli***. The *sap1* gene was obtained by PCR amplification from genomic DNA using the primers SapEcoHis and SapHind (Table 1). The PCR product was digested with EcoRI and HindIII to generate sticky ends and inserted into the expression vector pBAD24 (Invitrogen) digested with the same enzymes. The plasmid obtained was used to transform the *E. coli* strain TOP10 (Invitrogen), and the expression of Sap1p was induced by the addition of 0.02% arabinose to the culture for 2 h. Proteins from 2.5 × 10^8 cells were separated in 12% SDS-polyacrylamide gels (lanes 1 and 2) and transferred to polyvinylidene difluoride membranes, and His6-Sap1p was detected with anti-His6-peroxidase antibody (Roche) (lanes 3 and 4). Lanes 5 and 6 correspond to mobility shift assays using 4.7 μg of a protein extract from noninduced (−) or induced (+) cells and the same fragment as that used for panel D.

---

**FIG. 1.** Barrier RFB1 maps to a 78-bp DNA fragment that is specifically recognized by a binding protein. (A) Diagram of the nontranscribed spacer of the *S. pombe* rRNA gene, with the locations of the three barriers (RFB1-3) and the replication origin ars3001 indicated. Below the map, lines a to f correspond to the deleted fragments inserted in pIRT2 and assayed as shown in panel C. The names of the resultant plasmids and the results obtained are shown to the right. (B) Map of vector pIRT2 indicating the site where the fragments shown in panel A were cloned (insert). (C) RFB1 activity of fragments a to f in wild-type strain (panels a to f) and RFB1 activity of fragment e in swi1 (EN3182) and swi3 (EN3366) mutant strains (panels e-swi1 and e-swi3). Arrows point to the signals corresponding to Y-shaped accumulated replication intermediates with the fork arrested at RFB1. (D) EMSA using labeled fragment e and the indicated amounts of protein extract. In lane 6, a 166-fold excess of unlabeled fragment e was added to the binding reaction. (E) Expression of Sap1p in *E. coli* TOP10 cells was induced by the addition of 0.02% arabinose to the culture for 2 h. Proteins from 2.5 × 10^8 cells were separated in 12% SDS-polyacrylamide gels (lanes 1 and 2) and transferred to polyvinylidene difluoride membranes, and His6-Sap1p was detected with anti-His6-peroxidase antibody (Roche) (lanes 3 and 4). Lanes 5 and 6 correspond to mobility shift assays using 4.7 μg of a protein extract from noninduced (−) or induced (+) cells and the same fragment as that used for panel D.
TABLE 1. List of oligonucleotides used for this study

| Name | Sequence (5'-3') |
|------|-----------------|
| SpRFB4 | CCTTGACCTATGACATTACGTTAAC |
| SpRFB3-1 | CCTTGACAGGTTAGTGGCTGAATCGT |
| SpRFB3-2 | CCTTGACAGTTAGGAGGGATTTAA |
| SpRFB3-2bio | CCTTGACAGTTAGGAGGGATTTAA |
| SpRFB3-3 | CCTTGACAGTTAGGAGGACCATTTGGGACAT |
| SpRFB3-4 | CCTTGACGAGGAACATTTTTGGACAT |
| SpRFB3-3up | CCTGATGGAGGACTCGATTTAATG |
| SpRFB3-4up | CCTGATGGAGGACTCGATTTAATG |
| SpIa | GATCCCTCTTTGACTCTGGTTAATATCTCCTATATTCGCA |
| SpIb | GTAATGGAAGAGGATTTAACGAGTGCAGGAGG |
| SpIIa | GTAATGGAAGAGGATTTAACGAGTGCAGGAGG |
| SpIIb | GTAATGGAAGAGGATTTAACGAGTGCAGGAGG |
| Mut1a | GAATGGAAGAGGATTTAACGAGTGCAGGAGG |
| Mut1b | GAATGGAAGAGGATTTAACGAGTGCAGGAGG |
| Mut2a | GAATGGAAGAGGATTTAACGAGTGCAGGAGG |
| Mut2b | GAATGGAAGAGGATTTAACGAGTGCAGGAGG |
| Mut3a | GAATGGAAGAGGATTTAACGAGTGCAGGAGG |
| Mut3b | GAATGGAAGAGGATTTAACGAGTGCAGGAGG |
| Mut4a | GAATGGAAGAGGATTTAACGAGTGCAGGAGG |
| Mut4b | GAATGGAAGAGGATTTAACGAGTGCAGGAGG |
| Mut5a | GAATGGAAGAGGATTTAACGAGTGCAGGAGG |
| Mut5b | GAATGGAAGAGGATTTAACGAGTGCAGGAGG |
| Mut6a | CTGCCAGAAGGATTTAACGAGTGCAGGAGG |
| Mut6b | CTGCCAGAAGGATTTAACGAGTGCAGGAGG |
| Mut7a | CTGCCAGAAGGATTTAACGAGTGCAGGAGG |
| Mut7b | CTGCCAGAAGGATTTAACGAGTGCAGGAGG |
| Mut8a | CTGCCAGAAGGATTTAACGAGTGCAGGAGG |
| Mut8b | CTGCCAGAAGGATTTAACGAGTGCAGGAGG |
| Mut9a | CTGCCAGAAGGATTTAACGAGTGCAGGAGG |
| Mut9b | CTGCCAGAAGGATTTAACGAGTGCAGGAGG |
| Mut10a | CTGCCAGAAGGATTTAACGAGTGCAGGAGG |
| Mut10b | CTGCCAGAAGGATTTAACGAGTGCAGGAGG |
| Mut11a | CTGCCAGAAGGATTTAACGAGTGCAGGAGG |
| Mut11b | CTGCCAGAAGGATTTAACGAGTGCAGGAGG |
| Mut12a | CTGCCAGAAGGATTTAACGAGTGCAGGAGG |
| Mut12b | CTGCCAGAAGGATTTAACGAGTGCAGGAGG |
| Mut13a | CTGCCAGAAGGATTTAACGAGTGCAGGAGG |
| Mut13b | CTGCCAGAAGGATTTAACGAGTGCAGGAGG |
| Mut14a | CTGCCAGAAGGATTTAACGAGTGCAGGAGG |
| Mut14b | CTGCCAGAAGGATTTAACGAGTGCAGGAGG |
| Mut15a | CTGCCAGAAGGATTTAACGAGTGCAGGAGG |
| Mut15b | CTGCCAGAAGGATTTAACGAGTGCAGGAGG |
| pIRT2-ars1up | CTGATGGAGGACTCGATTTAATG |
| pIRT2-leu2do | TCCCTATAATGGTAGAAGTTC |
| SapEcoHis | CCTGATGGAGGACTCGATTTAATG |
| SapHind | CCTGATGGAGGACTCGATTTAATG |

* Restriction enzyme sites used for cloning or DNA labeling are shown in bold.

Purification of Sap1p. The 78-bp fragment containing RFB1 and analyzed in the mobility shift assay (Fig. 1) was used to purify Sap1p by affinity chromatography. A biotinylated DNA fragment was generated from pIRT1.6 (by PCR using the primers SpRFB3-2bio and SpRFB3-3). The product was concentrated using Microcon-PCR columns (Millipore) and eluted with TEN100 (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl). To purify Sap1p, 125 μg of streptavidin magnetic particles (SMP; Roche) was washed with TEN100 and TEN1000 (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl) to promote binding of the DNA to the SMP. Unbound DNA was removed by washing the particles with TEN1000 and resuspended in 80 μl of TEN100 containing 135 μg of biotinylated DNA. This mixture was incubated for 15 min at room temperature with occasional stirring to avoid binding of the DNA to the SMP. Biotinylated DNA-SMP complexes were washed twice with binding reaction buffer and resuspended in TEN100 containing 135 μg of biotinylated DNA. The resulting data were searched using Mascot and Profound search routines. The protein corresponding to each band was identified as Sap1. Thus, the 5′-end-labeled DNA fragment was directionally ligated to delimit the minimal cis-acting sequence of RFB1 (Fig. 1A).

We made systematic external deletions of the 383-bp region to delimit the minimal cis-acting sequence of RFB1 (Fig. 1A). The fragments obtained by PCR were directionally ligated close to the ars1 replication origin of vector pIRT2 so that the clockwise-moving fork meets the insert in the same direction in which the barrier is active in its chromosomal context (Fig. 1A and B). Plasmids were digested with PvuII, and replication intermediates were analyzed by 2D gel electrophoresis. The probe used detected the insert-containing fragment (Fig. 1B).

Three 5′ external deletions were analyzed (Fig. 1A, fragments a, b, and c). Fragments a and b conserved the capacity to arrest replication forks, as confirmed by the strong spot that appeared at the expected position on the arc of the Y-shaped replication intermediates (arrows in Fig. 1C, panels a and b). Thus, the 5′ half of the 383-bp region was dispensable for RFB1 function. On the other hand, no arrest sites were detected for fragment c, as indicated by the spotless simple Y arc (Fig. 1C, panel c). Since it could not be ruled out that sequences in fragment c were required (although insufficient) for fork arrest, two 3′ deletions of fragment b were also analyzed (Fig. 1A, fragments d and e). Both of these fragments gave a positive result in the assay (Fig. 1C, panels d and e), whereas no arrest sites were identified within the internal fragment f (panel f). Altogether, these results demonstrate that the 78-bp region that contains the sequences required to stall replication forks in a polar manner (26). This fragment lies between nucleotides +497 and +879 of the nontranscribed spacer, with the first nucleotide after the end of the 25S gene considered +1 (Fig. 1A).
fragment e contains the sequences required to block replication at RFB1. Double-Y intermediates were also visible in the 2D gels of the active fragments, indicating that the termination of replication takes place at the barrier in at least a fraction of the plasmid molecules (arrowhead in Fig. 1C, panel e, and after longer exposures of panels a, b, and d).

The nucleotide sequence of fragment e does not show any feature that might induce a secondary structure potentially able to stall replication forks. As in the case of RFB2 and RFB3, we considered that a trans-acting factor(s) could directly bind to this sequence. To address this possibility, an EMSA was performed with fragment e and a crude protein extract of *S. pombe* cells. The result confirmed that the 78-bp RFB1-containing fragment formed a stable DNA-protein complex, which was more abundant as the amount of protein extract increased (Fig. 1D, lanes 2 to 5). The specificity of this complex was verified by the addition of an excess of unlabeled fragment e to the reaction (Fig. 1D, lane 6).

The mating type switch-activating protein Sap1 binds specifically to RFB1. The protein that binds RFB1 was purified by means of affinity chromatography and identified as the mating type switch-activating protein Sap1 by matrix-assisted laser desorption ionization–time of flight (see Materials and Methods). To confirm the specific binding of Sap1p to RFB1, the coding gene *sap1* was cloned into the *Escherichia coli* vector pBAD24, and its expression was induced with arabinose (Fig. 1E, lanes 1 and 2). The addition of six histidines at the amino-terminal end of Sap1p allowed its detection by Western blotting (Fig. 1E, lanes 3 and 4). A protein extract from *E. coli* cells expressing Sap1p bound to RFB1, as indicated by the retarded complex obtained in the EMSA shown in Fig. 1E (lanes 5 and 6).

Sap1 is a 29-kDa essential DNA-binding protein that binds the SAS1 region at the *S. pombe* mating-type locus (2). Sap1p forms dimers in solution through a long coiled-coil domain at its C terminus (3) and controls the efficiency of mating-type switching (1). An analysis of the SAS1 DNA sequence showed that it is composed of three inverted and imperfect repeats separated by 12 nucleotides (Fig. 2A, arrows). Moreover, characterization of the Sap1p binding sites at SAS1, using DNase I protection experiments, showed two protected DNA sequences that coincide with these repeats (Fig. 2A, underlined sequences) (2). An alignment of RFB1 and SAS1 sequences showed that two of the SAS1 repeats are also present in RFB1 (Fig. 2A), one of them with one mismatch. Using recombinant

![FIG. 2](image-url)

**FIG. 2.** Sequence comparisons of RFB1 and previously reported Sap1p recognition sequences. (A) RFB1 and SAS1 sequences; (B) RFB1 and consensus sequences. Upper- and lowercase letters in the consensus oligonucleotide indicate higher and lower nucleotide frequencies, respectively, according to the work of Ghazvini et al. (7) (see text for details).

![FIG. 3](image-url)

**FIG. 3.** The three repeats in RFB1 are required together for both replication fork stalling and Sap1p binding. (A) Map of 78-bp RFB1-containing sequence indicating the locations of repeats a, b, and c. Lines I through IV correspond to the overlapping subfragments analyzed in panels B and C. (B) 2D gels of plasmids bearing subfragments I to IV. The arrow points to the accumulated replication intermediates. (C) EMSA with purified Sap1p (0.57 μg) and the overlapping subfragments.
Sap1p and randomized double-stranded oligonucleotides, Ghazvini and coworkers (7) proposed a consensus sequence as the most favorable DNA-binding site for Sap1p. This high-affinity binding site is a 5-bp direct repeat separated by five nucleotides, with a thymine being the fourth nucleotide of this spacer in most of the selected oligonucleotides (Fig. 2B). RFB1 fulfills these features, except for one mismatch (Fig. 2B). Taken together, these comparisons show that both RFB1 and SAS1 contain three imperfect repeats, but repeat b is in opposite orientations at these two sites. In addition, repeats a and b in RFB1 are spaced apart by 5 nucleotides, as in the consensus sequence selected by Ghazvini and coworkers (7), whereas they are separated by 12 nucleotides in SAS1. Therefore, the RFB1 and SAS1 sequences are similar, but they show differences that might be relevant to the function of Sap1 at these two loci. Our results are the first evidence of a binding site for the essential protein Sap1 outside the mating-type locus.

Replication blockage at RFB1 is linked to Sap1p binding. Sap1p might play a role in RFB1 activity by recognizing the three repeats depicted above. To address this possibility, we tested the capability of four overlapping subfragments covering the entire 78-bp RFB1-containing sequence to bind the purified Sap1p protein and to arrest replication forks in the in vivo plasmid replication assay described above (Fig. 3). These subfragments contained repeats a and b (fragment I), a, b, and c (fragment II), c (fragment III), and the 3′ end lacking the repeats (fragment IV) (Fig. 3A).

Fragment II, bearing all three repeats, stalled replication forks, as indicated by the spot on top of the simple Y arc (Fig. 3B, arrow). Since fragments I, III, and IV resulted in spotless arcs, we concluded that all three repeats are required for efficient RFB1 activity. Consistently, Sap1p bound with a high affinity only to fragment II (Fig. 3C, lane 2), although it did not give rise to any detectable fork arrest (Fig. 3B, panel I).

FIG. 4. Sap1p binding to RFB1 repeats is required for replication fork arrest. (A) The sequence underlined in Fig. 3A was used as the wild-type sequence to introduce the indicated mutations (boxes) assayed in panels B and C. (B) Replication intermediates of plasmids bearing wild-type or mutated sequences, analyzed in 2D gels. Arrows point to the accumulated replication intermediates containing arrested forks. (C) EMSA with purified Sap1p (0.57 μg) and wild-type or mutated sequences. In lane 1, the binding reaction contained no protein.

FIG. 5. State of the art of replication fork blockage in S. pombe rRNA genes (see the text for details).
To determine the significance of the repeats in RFB1 function, we replaced each repeat with a different sequence such that the least conservative changes were made, i.e., adenines were replaced with cytosines, guanines were replaced with thymines, and vice versa (Fig. 4A, mut2, mut3, and mut4). All of these mutations caused both the loss of RFB1 function and the absence of Sap1p binding (mut2, mut3, and mut4 in Fig. 4B and C). The mutation of repeat c in mut4, however, allowed weak Sap1p binding. This agrees with the result obtained for fragment I, which also lacked repeat c (Fig. 3C, lane 2). Five nucleotides at both sides of the repeat cluster were also mutated, and as expected, they affected neither the barrier activity nor Sap1p binding (mut1 and mut5 in Fig. 4B and C). These results indicate that all of the repeats within the cluster are required for both Sap1p binding and fork arrest and that the surrounding sequences are dispensable.

As mentioned before, Sap1p is essential for cell growth and was originally described as required for efficient mating-type switching. The essential nature of Sap1p, however, is not attributed to its role in switching but is likely due to a more general function in chromosome organization (16). It is also unlikely that the function of Sap1p in arresting replication forks at the rRNA genes makes this protein essential. This is supported by the observation that budding yeast fob1 mutants lacking functional barriers are viable (14). However, our findings raise the possibility that Sap1p could play an essential role in the organization of rRNA gene repeats and the nucleolar architecture.

Interestingly, in contrast to our results with the rRNA genes, the binding of Sap1p to the mat1 locus does not act as a replication fork obstacle (6). We believe that this could rely on the different disposition of the recognition sequences in RFB1 compared to that of sequences in SAS1 (Fig. 2), which may determine different binding modes of Sap1p dimers.

As shown in Fig. 1C (panels e-sw1 and e-sw3), replication fork arrest induced by the 78-bp sequence of RFB1 was abolished in the sw1ΔΔ and sw3ΔΔ mutant strains, indicating that these two proteins, which operate together as a heterodimer (23), are required for RFB1 function. This is in agreement with the recent finding that the accumulation of forks at all three RFBs depends on the presence of Swi1 and Swi3, although neither of these two proteins directly associates with the DNA at the barriers (15, 17). Here we identified Sap1p as the cognate RFB1 binding protein, which in turn may serve as a platform for the formation of a more sophisticated complex.

The Swi-Swi3 heterodimer may be part of this complex, even though it does not physically interact with Sap1p (17). Alternatively, the Swi1-Swi3 complex may not be involved in the arrest of replication forks. Instead, it could stabilize the stalled forks, as proposed for those accidentally arrested (23), so that these forks are not processed and remain Y-shaped structures long enough to be detected in 2D gels. This is supported by the observation that the simple Y replication intermediates that accumulate at the two natural pausing sites flanking the mat1 locus are not detected in sw1Δ and sw3Δ mutants (6).

**Concluding remarks.** In summary, the factors currently known to be involved in S. pombe rRNA gene barriers are shown in Fig. 5. Two cognate DNA-binding proteins recognize the three barriers present in the rRNA gene nontranscribed spacer. The transcription termination factor Reb1p binds to two specific 17-bp sequences and blocks the replication fork moving countertranscriptionally that originated at am3001 (26). These two fork arrest sites correspond to barriers RFB2 and RFB3. RFB1 functions upon binding of a Sap1p dimer. This barrier is the strongest and the first that the replication fork encounters. Our data demonstrated that these three barriers are independent of each other. RFB2 and RFB3 sequences are fully functional in the absence of the sequence required for RFB1 and vice versa. Moreover, RFB1 is fully active in reb1Δ mutant cells (26). Therefore, it is unlikely that an interaction between Reb1p and Sap1p is needed for fork arrest at any of the barriers. Finally, the switching proteins Swi1 and Swi3 are required for all three barriers, although as discussed above, the molecular basis for this requirement remains unknown.

**ACKNOWLEDGMENTS**

This work was partially supported by grants BFU2004-00125, SAF2001-17440, and BMC2002-05496 and a fellowship to E.M.-R. from the Spanish Ministerio de Educación y Ciencia and by a fellowship from the Comunidad de Madrid-Fondo Social Europeo to A.S.-G.

We are grateful to R. Giraldo for technical advice and R. Torres, R. Rodríguez, and L. Rodríguez for stimulating discussions and help throughout the course of this study.

**REFERENCES**

1. Arcangioli, B., T. Copeland, and A. Klar. 1994. Sap1, a protein that binds to sequences required for mating-type switching, is essential for viability in Schizosaccharomyces pombe. Mol. Cell. Biol. 14:2058–2065.
2. Arcangioli, B., and A. J. S. Klar. 1991. A novel switch-activating site (SAS1) and its cognate binding factor (SAP1) required for efficient mat1 switching in Schizosaccharomyces pombe. EMBO J. 10:3025–3032.
3. Badia, M., D. Walther, B. Arcangioli, S. Doniach, and M. Delarue. 2000. Solution structural studies and low-resolution model of the Schizosaccharomyces pombe sap1 protein. J. Mol. Biol. 300:563–574.
4. Benguria, A., P. Hernández, D. B. Krimer, and J. B. Schwartzman. 2003. Sir2p suppresses recombinations of replication forks stalled at the replication fork barrier of ribosomal DNA in Saccharomyces cerevisiae. Nucleic Acids Res. 31:889–898.
5. Brewer, B. J., and W. L. Fangman. 1988. A replication fork barrier at the 3′ end of yeast ribosomal RNA genes. Cell 55:637–643.
6. Dalgaard, J. Z., and A. J. S. Klar. 2000. swi1 and swi3 perform imprinting, pausing, and termination of DNA replication in S. pombe. Cell 102:745–751.
7. Ghazvini, M., V. Ribes, and B. Arcangioli. 1995. The essential DNA-binding protein Sap1 of Schizosaccharomyces pombe contains two independent oligomerization interfaces that determine the relative orientation of the DNA-binding domain. Mol. Cell. Biol. 15:4939–4946.
8. Giraldo, R., J. M. Andreu, and R. Diaz-Oregias. 1998. Protein domains and conformational changes in the activation of RepA, a DNA replication initiator. EMBO J. 17:4511–4526.
9. Hernández, P., L. Martín-Parras, M. L. Martínez-Robles, and J. B. Schwartzman. 1993. Conserved features in the mode of replication of eukaryotic ribosomal RNA genes. Mol. Cell. Biol. 13:1475–1485.
10. Hindley, J., G. Phear, M. Stein, and D. Baddiley. 1987. sucl encodes a predicted 13-kilodalton protein that is essential for cell viability and is directly involved in the division cycle of Schizosaccharomyces pombe. Mol. Cell. Biol. 7:504–511.
11. Kaerberlein, M., M. McVey, and L. Guarente. 1999. The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms. Genes Dev. 13:2570–2580.
12. Kobayashi, T. 2003. The replication fork barrier site forms a unique structure with Fob1p and inhibits the replication fork. Mol. Cell. Biol. 23:9178–9188.
13. Kobayashi, T., D. J. Heck, M. Nonuma, and T. Horiiuchi. 1998. Expansion and contraction of ribosomal DNA repeats in Saccharomyces cerevisiae: requirement of replication fork blocking (Fob1) protein and the role of RNA polymerase I. Genes Dev. 12:3821–3830.
14. Kobayashi, T., and T. Horiiuchi. 1996. A yeast gene product, Fob1 protein, required for both replication fork blocking and recombinational hotspot activities. Genes Cells 1:465–474.
15. Kring, G., and D. Bastia. 2004. sw1- and sw3-dependent and independent replication fork arrest at the ribosomal DNA of Schizosaccharomyces pombe. Proc. Natl. Acad. Sci. USA 101:4885–14090.
16. Lahonde, R., V. Ribes, and B. Arcangioli. 2003. Fission yeast Sap1 protein is essential for chromosome stability. Eukaryot. Cell 2:910–921.
17. Lee, B. S., S. I. Grewal, and A. J. Klar. 2004. Biochemical interactions between proteins and mat1 cis-acting sequences required for imprinting in fission yeast. Mol. Cell. Biol. 24:59813–59822.
18. Linskens, M. H. K., and J. A. Huberman. 1988. Organization of replication of ribosomal DNA in Saccharomyces cerevisiae. Mol. Cell. Biol. 8:4927–4935.
19. Little, R. D., T. H. K. Platt, and C. L. Schildkraut. 1993. Initiation and termination of DNA replication in human rRNA genes. Mol. Cell. Biol. 13:6600–6613.
20. López-Estraño, C., J. B. Schwartzman, D. B. Krimer, and P. Hernández. 1998. Co-localization of polar replication fork barriers and rRNA transcription terminators in mouse rDNA. J. Mol. Biol. 277:249–256.
21. Mohanty, B. K., and D. Bastia. 2004. Binding of the replication terminator protein Fob1p to the Ter sites of yeast causes polar fork arrest. J. Biol. Chem. 279:1932–1941.
22. Moreno, S., A. Klar, and P. Nurse. 1991. Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. Methods Enzymol. 194:795–823.
23. Noguchi, E., C. Noguchi, W. H. McDonald, J. R. Yates, and P. Russell. 2004. Swi1 and Swi3 are components of a replication fork protection complex in fission yeast. Mol. Cell. Biol. 24:8342–8355.
24. Olavarrieta, L., P. Hernández, D. B. Krimer, and J. B. Schwartzman. 2002. DNA knotting caused by head-on collision of transcription and replication. J. Mol. Biol. 322:1–6.
25. Prentice, H. L. 1992. High efficiency transformation of Schizosaccharomyces pombe by electroporation. Nucleic Acids Res. 20:621.
26. Sánchez-Gorostiaga, A., C. López-Estraño, D. B. Krimer, J. B. Schwartzman, and P. Hernández. 2004. Transcription termination factor Reb1p causes two replication fork barriers at its cognate sites in fission yeast ribosomal DNA in vivo. Mol. Cell. Biol. 24:398–406.
27. Takeuchi, Y., T. Horiuchi, and T. Kobayashi. 2003. Transcription-dependent recombination and the role of fork collision in yeast rDNA. Genes Dev. 17:1497–1506.
28. Wiesendanger, B., R. Lucchini, T. Koller, and J. M. Sogo. 1994. Replication fork barriers in the Xenopus rDNA. Nucleic Acids Res. 22:5038–5046.
29. Zhang, Z., D. M. Macalpine, and G. M. Kapler. 1997. Developmental regulation of DNA replication: replication fork barriers and programmed gene amplification in Tetrahymena thermophila. Mol. Cell. Biol. 17:6147–6156.