The mating-type-switch activating protein Sap1 is required for replication fork arrest at the rDNA of fission yeast

Eva Mejía-Ramírez, Alicia Sánchez-Gorostiaga, Dora B. Krimer,

Jorge B. Schvartzman and Pablo Hernández*

Departamento de Biología Celular y del Desarrollo, Centro de Investigaciones Biológicas (CSIC), Ramiro de Maeztu 9, 28040 Madrid, SPAIN

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*Corresponding author: Tel. +34 918 373 112; Fax: +34 915 360 432

E-mail: p.hernandez@cib.csic.es

ABSTRACT

Schizosaccharomyces pombe rDNA contains three replication fork barriers (RFB1-3) located in the non-transcribed spacer. RFB2 and RFB3 require binding of the transcription terminator factor Reb1p to two identical recognition sequences that co-localize 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 defined RFB1 within a 78-bp sequence located near the 3'-end of the rDNA 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 *E. 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 barrier function, indicating that Sap1p is required for replication fork blockage at RFB1.

INTRODUCTION

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 reactivate the arrested replication forks. Aside from these *accidental* fork arrests, there are *natural* replication fork pausing sites that seem to play specific biological roles. Ribosomal DNA (rDNA) of all eukaryotic species studied so far contains several replication fork barriers (RFBs) (5, 9, 18-20, 26, 28, 29). RFBs are clustered and located in the non-transcribed 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, rDNA is mainly replicated unidirectionally and co-oriented with transcription. Therefore, one of the roles of rDNA RFBs may be to prevent the deleterious effects of head-on collisions between replication and transcription machineries (24, 27).

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

Schizosaccharomyces pombe rDNA contains three independent closely spaced barriers, RFB1-3. RFB2 and RFB3 require binding of the transcription termination protein Reb1 to

its 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.

MATERIAL 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 proteins Swi1 and Swi3 for RFB1 function, we used strains EN 3182 (*h⁻ leu1-32 ura-D18 swi1::Kan^r*) and EN3366 (*h⁻ leu1-32 ura-D18 swi3::Kan^r*), kindly provided by Paul Russell. Standard media and growth conditions were employed (22). *Escherichia coli* strain DH5α was used as the host for construction of plasmids containing rDNA sequences, and strain TOP10 for expression of Sap1p.

Construction of plasmids containing rDNA sequences. Plasmids were constructed by inserting the rDNA fragments into the polycloning site of vector pIRT2, close to the replication origin *ars1*. Transformation of *S. pombe* was performed by electroporation (25). Fragments analyzed in Fig. 1 were obtained by PCR from plasmid pIRT1.6(+) (26) and using the following primers listed in Table 1: SpRFB3-1/SpRFB4 for pΔ604-879, SpRFB3-2/SpRFB4 for pΔ677-879, SpRFB3-3/SpRFB4 for pΔ737-879, SpRFB3-2/SpRFB3-4up for pΔ677-825, SpRFB3-2/SpRFB3-3up for pΔ677-754 and SpRFB3-3/SpRFB3-4up for

pΔ737-825. PCR products were digested with *Pst*I and *Bam*HI and inserted into the polycloning site of pIRT2 (10). Fragments analyzed in Figs. 3 and 4 were obtained by annealing the following oligonucleotides (Table 1): SpIa/SpIb for fragment I, SpIIa/SpIIb for fragment II, SpIIIa/SpIIIb for fragment III, SpIVa/SpIVb for fragment IV, RFB1wt-a/RFB1wt-b for fragment wt, mut1a/mut1b for fragment mut1, mut2a/mut2b for fragment mut2, mut3a/mut3b for fragment mut3, mut4a/mut4b for fragment mut4, mut5a/mut5b for fragment mut5. The annealed oligonucleotides were inserted into pIRT2 digested with *Bam*HI and *Pst*I (fragments in Fig. 3) or with *Bam*HI (fragments in Fig. 4). All insertions were confirmed by sequencing.

Two-dimensional gel electrophoresis. DNA purification from asynchronous log-phase cultures and analysis of replication intermediates by two-dimensional gel electrophoresis were performed as previously described (26). Before analysis, plasmids were digested with *Pvu*II. The large *Pvu*II fragment from pIRT2 was used as 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 μl of extraction buffer (25 mM HEPES pH 7.6, 150 mM KCl, 0.1 mM EDTA, 0.1% TritonX-100, 25% glycerol, 4 μg/ml leupeptine, 2 mM DTT, 2 μg/ml pepstatine, 2 μg/ml aprotinine and 0.2 mM PMSF). After cell wall disruption with 425-600 μm glass beads (Sigma), the emulsion was diluted with 600 μl of extraction buffer, transferred to a new tube and clarified by centrifugation. The recovered supernatant was aliquoted and stored at -80ºC. 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 10x 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 mM DTT and 6 µl of radiolabeled probe (0.04-0.8 ng, 10000 cpm). The probe used in Fig. 1 was obtained by PCR from pIRT1.6(+) using primers SpRFB3-2/SpRFB3-3up (Table 1), followed by digestion with *Bam*HI. To obtain the probes of Figs. 3 and 4, the pIRT2 derivative plasmids containing each of the fragments were used in PCR reactions with primers pIRT2-ars1up/pIRT2-leu2do. The products were then digested with *Bam*HI-*Hind*III (Fig. 3) or with *Bam*HI (Fig. 4). All probes were labeled by filling the 5'-protruding ends with [α-^{32}P]dCTP and the Klenow fragment and purified through a G-25 Sephadex column (Roche). Binding reactions were incubated 20 min at room temperature and electrophorized at 4ºC on 6% polyacrylamide-0.5x TBE gels at 10 V/cm for 2.5 h.

Expression of Sap1p in *Escherichia coli*. *sap1*$^+$ was obtained by PCR amplification from genomic DNA using primers SapEcoHis and SapHind (Table 1). The PCR product was digested with *Eco*RI and *Hind*III 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 expression of Sap1p was induced by addition of 0.02% arabinose to exponentially growing cells for 2 h. Total proteins from 2.5x10^8 cells were separated in 12% SDS-polyacrylamide gels, transferred to PVDF membranes and His_6-Sap1p was detected by using anti-His_6-peroxidase antibody (Roche).

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 primers SpRFB3-2bio/SpRFB3-3. The product was concentrated using Microcon-PCR columns (Millipore) and eluted with TEN100 (10mM Tris HCl pH 7.5, 1 mM EDTA, 100 mM NaCl). To purify Sap1p, 125 μg of Streptavidin Magnetic Particles (SMP, Roche) were washed with TEN100 and resuspended in 80 μl of TEN100 containing 13,5 μg of biotinylated-DNA. This mixture was incubated 15 min at room temperature with occasional stirring to promote the binding of the DNA to the SMP. Unbound DNA was removed by washing the particles with TEN1000 (10 mM Tris HCl pH 7.5, 1 mM EDTA, 1 M NaCl). DNA-SMP complexes were equilibrated with a solution identical to the binding reaction mixture without protein and 4.5 mg of protein extract in binding reaction mixture were added. After incubation at room temperature for 20 min, protein-DNA-SMP complexes were washed twice with binding reaction mixture. Finally, proteins were eluted from DNA by incubation in elution buffer (25 mM Hepes pH 7.6, 1 M KCl, 25mM EDTA, 0.5 mM DTT) during 15 min at room temperature. The samples obtained from seven purifications were pooled, dialyzed against storage buffer (0.5 M KCl, 1mM EDTA, 2mM DTT and 10% glycerol) and run on a 12% SDS-polyacrylamide gel. Three bands of about 30 kDa with very similar mobility were obtained. The protein of each band was analyzed by MALDI-TOF (performed at the CBMSO Proteomic Service, Universidad Autónoma de Madrid, Spain). The resulting data were searched using Mascot and Profound search routines. The protein corresponding to each band was identified as Sap1.

RESULTS AND DISCUSSION

RFB1 is a protein binding sequence. The *S. pombe* rDNA barrier RFB1 was previously mapped within a 383-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 non-transcribed spacer, considering +1 the first nucleotide after the end of the 25S gene (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 direction-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 *Pvu*II digested 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 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 for fork arrest (although insufficient), 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 demonstrated that the 78-bp 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 termination of replication takes place at the barrier at least in a fraction of the plasmid molecules (arrowhead in Fig. 1C, panel **e**, and after longer exposures in **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 regarded that a trans-acting factor(s) could directly bind to this sequence. To address this possibility an electrophoretic shift assay was performed with fragment **e** and a crude protein extract of *S. pombe* cells. The result confirmed that the 78-bp RFB1-containing fragment forms a stable DNA-protein complex, which was more abundant as the amount of protein extract increased (Fig. 1D, lanes 2-5). 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 MALDI-TOF (see Materials and Methods). To confirm the specific binding of Sap1p to RFB1, the coding gene *sap1*$^{+}$ was cloned in the *Escherichia coli* vector pBAD24 and its expression induced with arabinose (Fig. 1E, lanes 1 and 2). The addition of six histidines at the amino-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 electrophoretic shift assay 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). 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 DNaseI protection experiments, showed two protected DNA sequences that coincide with these repeats (Fig. 2A, underlined) (2). Alignment of RFB1 and SAS1 sequences shows that two of the SAS1 repeats are also present in RFB1 (Fig. 2A), one of them with one mismatch. Using recombinant Sap1p and randomized double-stranded oligonucleotides, Ghazvini and co-workers (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, a thymine being the fourth nucleotide of this spacer in most of the selected oligonucleotides (Fig. 2B). RFB1 fulfils these features except for one mismatch (Fig. 2B). Taken together these comparisons, both RFB1 and SAS1 contain three imperfect repeats, but repeat **b** is in opposite orientations in these two sites. In addition, repeats **a** and **b** in RFB1 are spaced by five nucleotides, as in the consensus sequence selected by Ghazvini and co-workers (7), whereas they are separated by twelve nucleotides in SAS1. Therefore, RFB1 and SAS1 sequences are similar, but they show differences that might be relevant for 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 sub-fragments covering the entire 78-bp RFB1-containing sequence to bind the purified Sap1p and to arrest replication forks in the *in vivo* plasmid replication assay described before (Fig. 3). These sub-fragments contained repeats **a** and **b** (fragment I), **a**, **b** and **c** (fragment II), **c**, (fragment III) or 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). As fragments I, III and IV resulted in spotless arcs, we concluded that all three repeats are required for an efficient RFB1 activity. Consistently, Sap1p bound with high affinity only to fragment II (Fig. 3C). This correlation between Sap1p binding and fork stalling strongly suggests that Sap1p is involved in fork blockage at *S. pombe* rDNA. Sap1p showed a weak affinity for fragment I (Figure 3C, lane 2), although it did not give rise to any detectable fork arrest (Fig. 3B, panel I).

To determine the significance of the repeats in RFB1 function, we substituted each repeat with a different sequence such that the least conservative changes were made: adenines were replaced by cytosines, guanines by thymines, and vice versa (Fig. 4A, mut2, mut3 and mut4). All these mutations caused both the loss of RFB1 function and the absence of Sap1p binding (mut2, mut3 and mut4 in Fig. 4B and C). Mutation of repeat **c** in mut4, however, allowed a weak Sap1p binding. This agrees with the result obtained for fragment I, which also lacks repeat **c** (Fig. 3C, lane 2). Five nucleotides at both sides of the repeat cluster were also mutated and, as expected, they affected neither barrier activity nor

Sap1p binding (mut1 and mut5 in Fig. 4B and C). These results indicate that all the repeats within the cluster are required for both Sap1p binding and fork arrest and that 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 rDNA 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 rDNA repeats and the nucleolar architecture.

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

As shown in Fig 1C (panels e-swi1 and e-swi3), replication fork arrest induced by the 78-bp sequence of RFB1 was abolished in *swi1Δ* or *swi3Δ* mutant strains, indicating that these two proteins, which operate together as a heterodimer (23), were required for RFB1 function. This is in agreement with the recent finding that 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 Swi1-Swi3 heterodimer may be part of this complex,

even though it does not physically interact with Sap1p (17). Alternatively, 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 as Y-shaped structures long enough to be detected in 2D gels. This is supported by the observation that simple-Y replication intermediates accumulated at the two natural pausing sites flanking the *mat1 locus* are not detected in *swi1* and *swi3* mutants (6).

Concluding remarks. In summary, the factors currently known to be involved in *S. pombe* rDNA barriers are shown in Fig. 5. Two cognate DNA-binding proteins recognize the three barriers present in the rDNA non-transcribed spacer. Transcription termination factor Reb1p binds to two specific 17-bp sequences and blocks the replication fork moving counter-transcription that originated at *ars3001* (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 *reb1*Δ mutant cells (26). Therefore, it is unlikely that 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.

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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. **Bada, 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. **Benguría, A., P. Hernández, D. B. Krimer, and J. B. Schvartzman.** 2003. Sir2p suppresses recombination of replication forks stalled at the replication fork barrier of ribosomal DNA in *Saccharomyces cerevisiae*. Nucleic Acids Res. **31:**893-898.

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 dictate the relative orientation of the DNA-binding domain. Mol. Cell. Biol. **15:**4939-4946.

8. **Giraldo, R., J. M. Andreu, and R. Diaz-Orejas.** 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. Schvartzman.** 1993. Conserved features in the mode of replication of eukaryotic ribosomal RNA genes. EMBO J. **12:**1475-1485.

10. **Hindley, J., G. Phear, M. Stein, and D. Beach.** 1987. Suc11encodes 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. **Kaeberlein, 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. Nomura, and T. Horiuchi.** 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. Horiuchi.** 1996. A yeast gene product, Fob1 protein, required for both replication fork blocking and recombinational hotspot activities. Genes Cells **1:**465-474.

15. **Krings, G., and D. Bastia.** 2004. swi1- and swi3-dependent and independent replication fork arrest at the ribosomal DNA of Schizosaccharomyces pombe. Proc. Natl. Acad. Sci. USA **101:**14085-14090.

16. **Lahondès, R., V. Ribes, and B. Arcangioli.** 2003. Fission yeast Sap1 protein is essential for chromosome stability. Eukaryotic 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:**9813-22.

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. Schvartzman, 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. Schvartzman.** 2002. DNA knotting caused by head-on collision of transcription and replication. J. Mol. Biol.**:**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. Schvartzman, 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. Cel. Biol. **17:**6147-6156.

LEGENDS TO FIGURES

FIGURE 1. Barrier RFB1 maps to a 78-bp DNA fragment that is specifically recognized by a binding protein. **A**. Diagram of the non-transcribed spacer of the *S. pombe* rDNA, where the locations of the three barriers (RFB1-3) and the replication origin *ars3001* are indicated. Below the map, lines **a** to **f** correspond to the deleted fragments inserted in pIRT2 and assayed in C. The names of the resultant plasmids and the results obtained are shown to the right. **B**. Map of the vector pIRT2 indicating the site where the fragments shown in A were cloned (insert). **C**. RFB1 activity of fragments **a** to **f** in the wild type strain 35 (panels a to f) and RFB1 activity of fragment **e** in *swi1* (EN3182) or *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, 166x excess of unlabeled fragment **e** was added to the binding reaction. **E**. Expression of Sap1p in *E. coli* TOP10 cells was induced by addition of 0.02% arabinose to the culture during 2 h. Proteins from $2.5x10^8$ cells were separated in 12% SDS-polyacrylamide gels (lanes 1 and 2), transferred to PVDF membranes and His_6-Sap1p detected with anti-His_6-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 non-induced (-) or induced (+) cells and the same fragment as in D.

FIGURE 2. Sequence comparisons of RFB1 with previously reported Sap1p recognition sequences. Upper and lower case letters in the consensus oligo indicate, respectively, higher or lower nucleotide frequency according to Ghazvini *et al* (7) (see text for details).

FIGURE 3. The three repeats in RFB1 are required together for both replication fork stalling and Sap1p binding. **A**. Map of the 78-bp RFB1-containing sequence indicating the location of repeats a, b and c (bold face). Lines I through IV correspond to the overlapping sub-fragments analyzed in B and C. **B**. 2D gels of plasmids bearing sub-fragments I-IV. The arrow points to the accumulated replication intermediate. **C**. EMSA with purified Sap1p (0.57 μg) and the overlapping sub-fragments.

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

FIGURE 5. State-of-the-art of replication fork blockage in *S. pombe* rDNA (see text for details).

Table 1. List of oligonucleotides used

Name	Sequence (5'-3')
SpRFB4	CCC**GGATCC**TATGACATTACGTTAAAC
SpRFB3-1	CCC**CTGCAG**AGGTAGGTCGTGAATCGT
SpRFB3-2	CCC**CTGCAG**AATAGGAGAGGGATTTAA
SpRFB3-2bio	Biotin-CCCCTGCAGAATAGGAGAGGGATTTAA
SpRFB3-3	CCC**CTGCAG**TTCAAGTTCCATTTGTAA
SpRFB3-4	CCC**CTGCAG**GAGAACATTTTTGGACAT
SpRFB3-3up	CCC**GGATCC**TTACAAATGGAACTTGAA
SpRFB3-4up	CCC**GGATCC**ATGTCCAAAAATGTTCTC
SpIa	**GATCC**CCTTGCACTGCGTTAAATCCCTCTCCTATT**CTGCA**
SpIb	**G**AATAGGAGAGGGATTTAACGCAGTGCAAGG**G**
SpIIa	**GATCC**ACCACCAAGATAGCTCCTTGCACTGCGTTA**CTGCA**
SpIIb	**G**TAACGCAGTGCAAGGAGCTATCTTGGTGGT**G**
SpIIIa	**GATCC**ATTTGAAAAGGGGGAACCACCAAGATAGCT**CTGCA**
SpIIIb	**G**AGCTATCTTGGTGGTTCCCCCTTTTCAAAT**G**
SpIVa	**GATCC**TTACAAATGGAACTTGAAATTTGAAAAGGGGGA**CTGCA**
SpIVb	**G**TCCCCCTTTTCAAATTTCAAGTTCCATTTGTAA**G**
RFB1wt-a	**GATCC**AGGGATTTAACGCAGTGCAAGGAGCTATCTTGGTGGT**G**
RFB1wt-b	**GATCC**ACCACCAAGATAGCTCCTTGCACTGCGTTAAATCCCT**G**
Mut1a	**GATCC**CTTTCTTTAACGCAGTGCAAGGAGCTATCTTGGTGGT**G**
Mut1b	**GATCC**ACCACCAAGATAGCTCCTTGCACTGCGTTAAAGAAAG**G**
Mut2a	**GATCC**AGGGATTGCCATCAGTGCAAGGAGCTATCTTGGTGGT**G**
Mut2b	**GATCC**ACCACCAAGATAGCTCCTTGCACTGATGGCAATCCCT**G**
Mut3a	**GATCC**AGGGATTTAACGCAGTGACCTTAGCTATCTTGGTGGT**G**
Mut3b	**GATCC**ACCACCAAGATAGCTAAGGTCACTGCGTTAAATCCCT**G**
Mut4a	**GATCC**AGGGATTTAACGCAGTGCAAGGCTAGCGCTTGGTGGT**G**
Mut4b	**GATCC**ACCACCAAGCGCTAGCCTTGCACTGCGTTAAATCCCT**G**
Mut5a	**GATCC**AGGGATTTAACGCAGTGCAAGGAGCTATCTTGTGTTG**G**
Mut5b	**GATCC**CAACACAAGATAGCTCCTTGCACTGCGTTAAATCCCT**G**
pIRT2-ars1up	CTGATGGAGGACTCGATTTAATG
pIRT2-leu2do	TCCCATAATGGTGAAAGTTCC
SapEcoHis	CCC**GAATTC**ACCATGCATCATCATCATCATCATATGGAAGCTCCCAAGAT GGAACTGAAGAGC
SapHind	CCC**AAGCTT**GTTGGGATTAATGGTCACCA

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

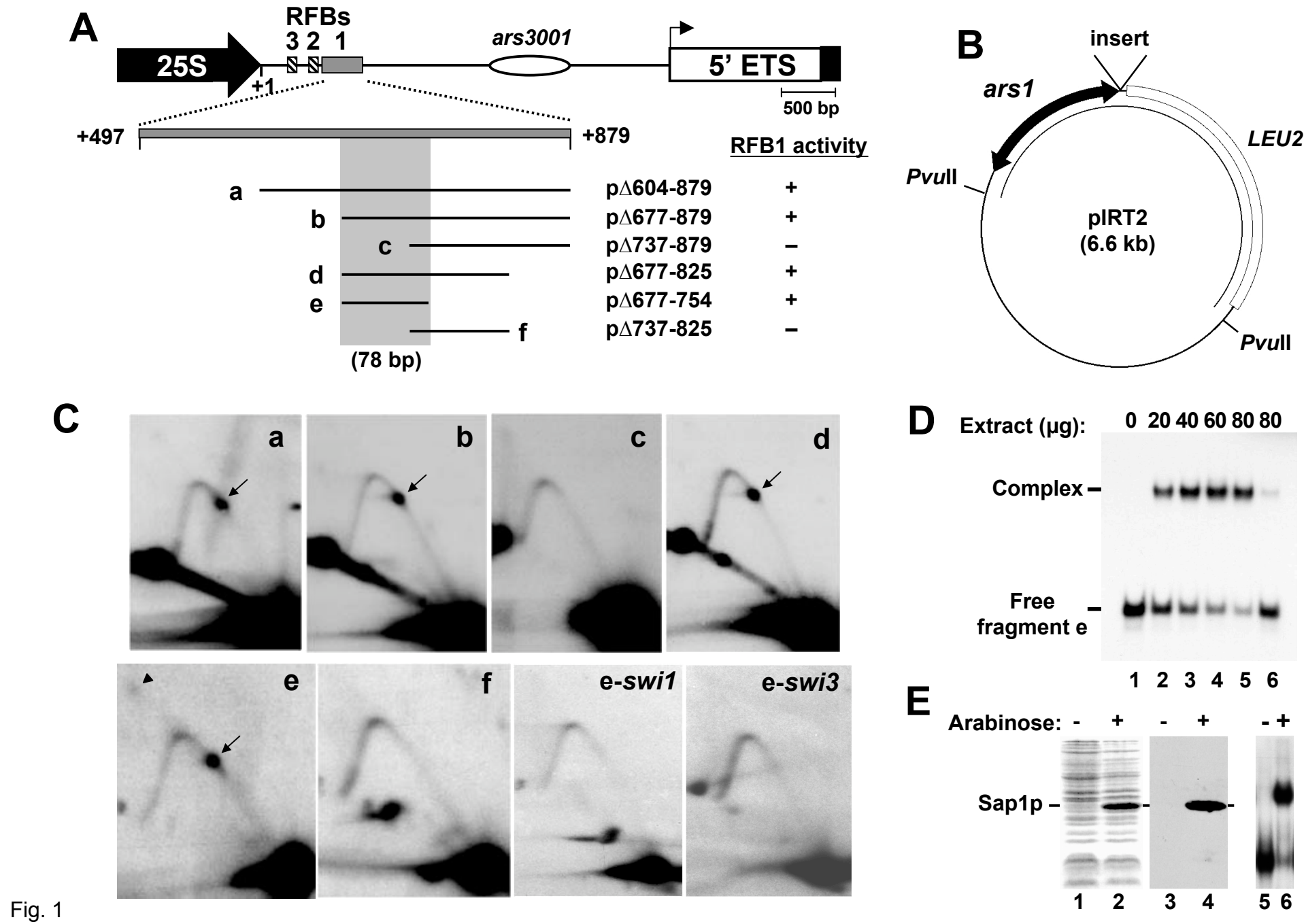

A
RFBs
3 2 1
25S
+1
ars3001
5' ETS
500 bp
+497
+879
(78 bp)
RFB1 activity
a
b
c
d
e
f
pΔ604-879 +
pΔ677-879 +
pΔ737-879 –
pΔ677-825 +
pΔ677-754 +
pΔ737-825 –
B
insert
ars1
LEU2
PvuII
pIRT2
(6.6 kb)
PvuII
C
a
b
c
d
e
f
e-swi1
e-swi3
D
Extract (μg): 0 20 40 60 80 80
Complex
Free fragment e
1 2 3 4 5 6
E
Arabinose: - + - + - +
Sap1p
1 2 3 4 5 6

Fig. 1

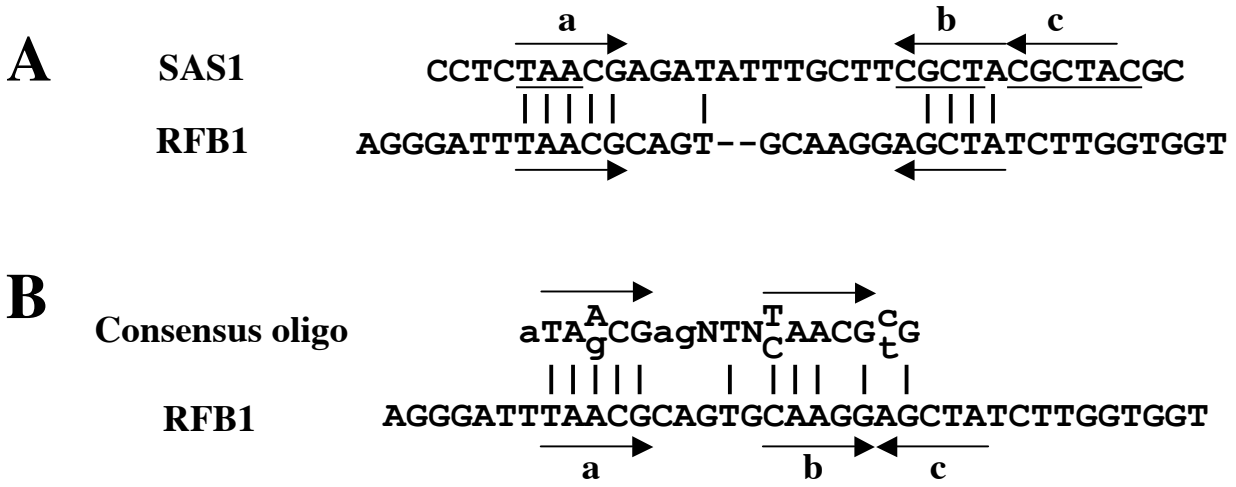
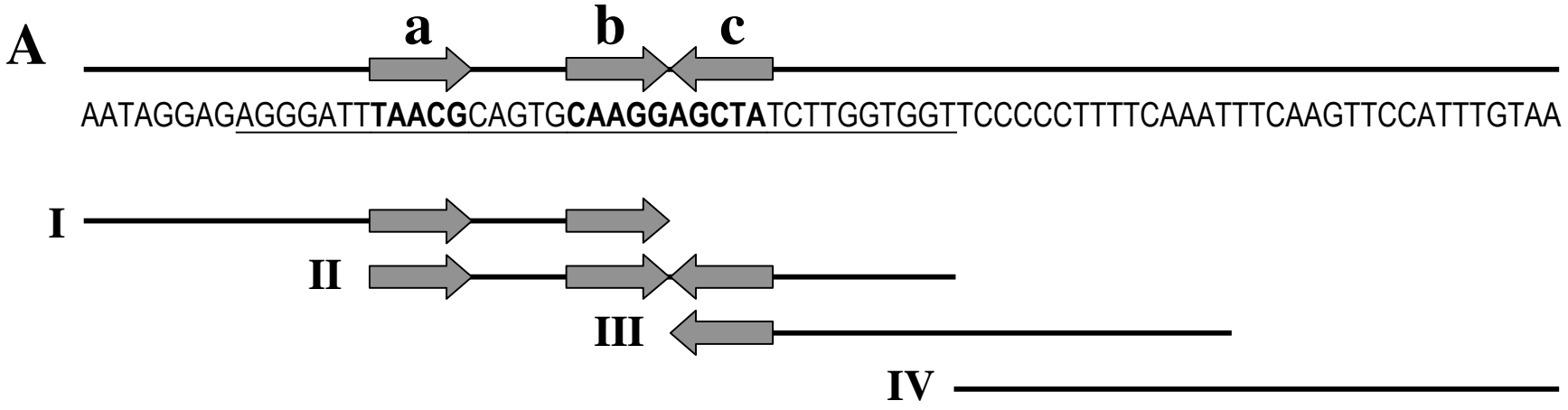

A
SAS1
RFB1
a
b
c
CCTCTAACGAGATATTTGCTTCGCTACGCTACGC
AGGGATTTAACGCAGT--GCAAGGAGCTATCTTGGTGGT
B
Consensus oligo
RFB1
aTA[A/g]CGagNTN[T/C]AACG[C/t]G
AGGGATTTAACGCAGTGCAAGGAGCTATCTTGGTGGT
a
b
c

Fig. 2


A
a
b
c
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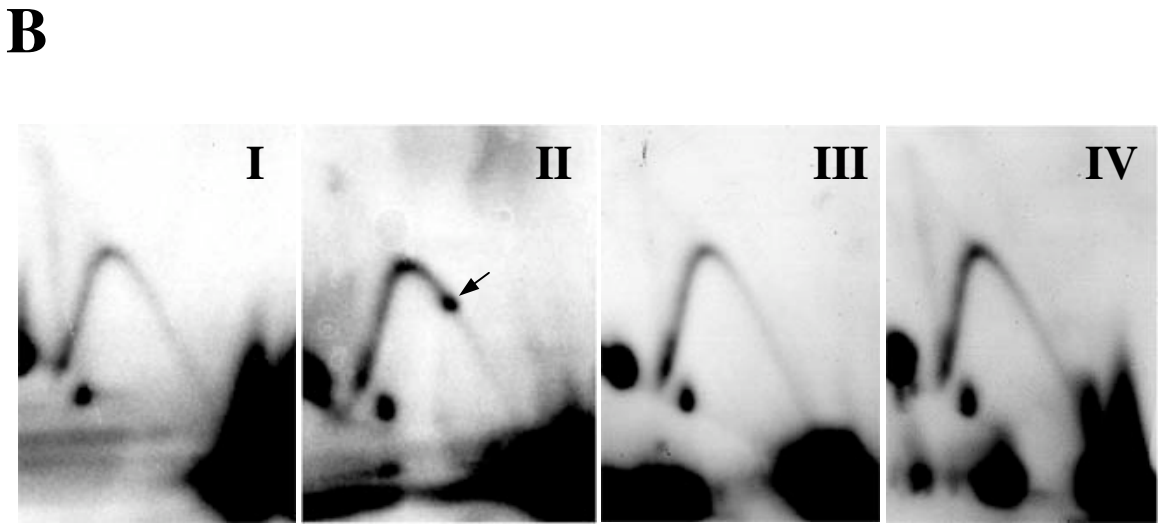
I
II
III
IV


B
I
II
III
IV

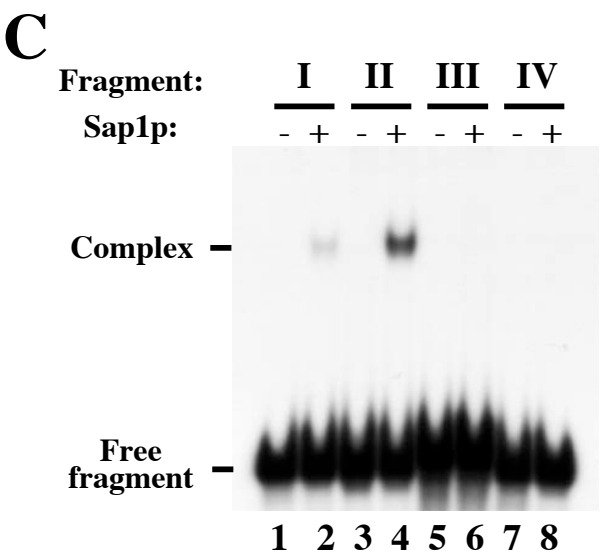
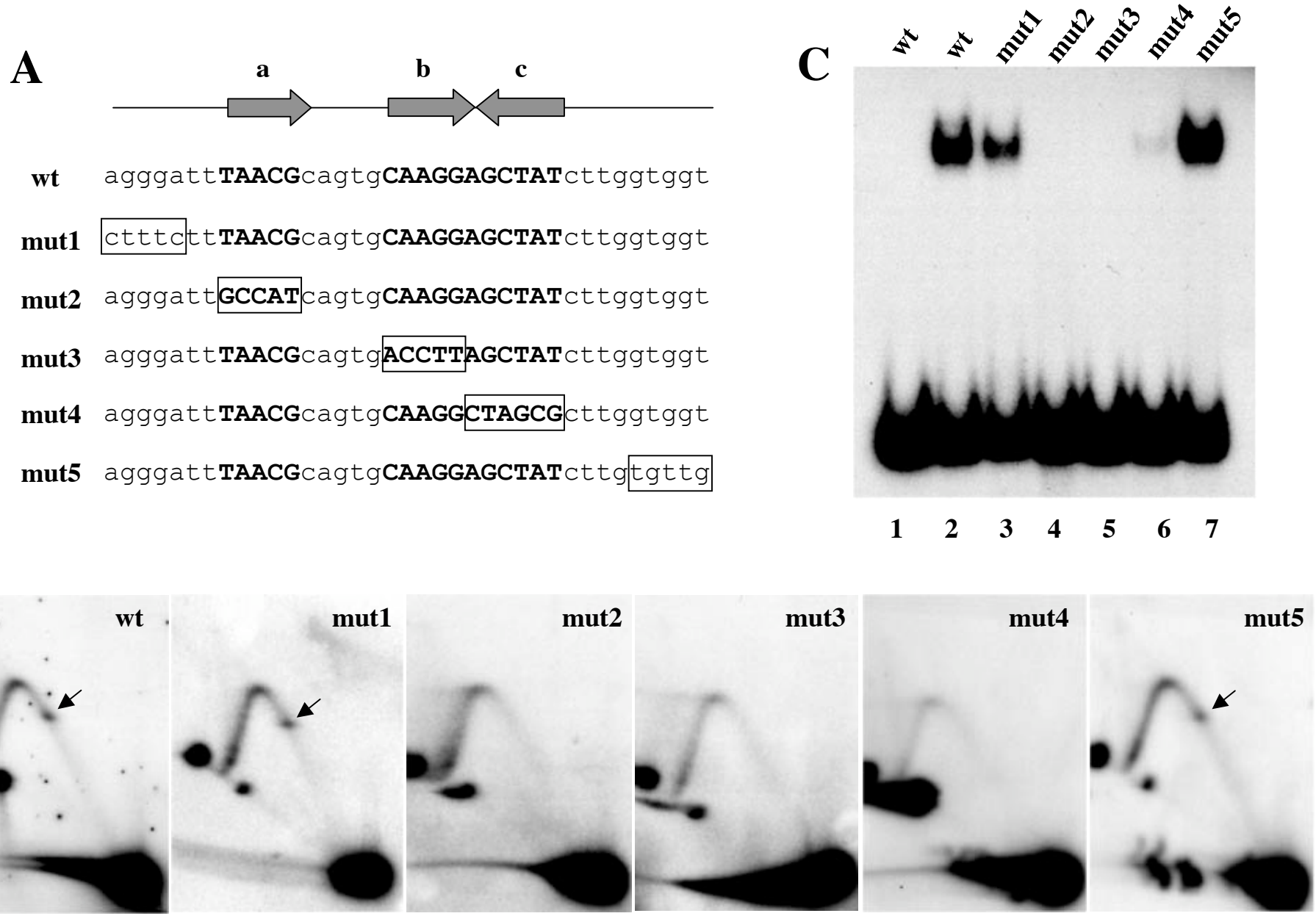

C
Fragment:
I
II
III
IV
Sap1p:
- + - + - + - +
Complex
Free fragment
1 2 3 4 5 6 7 8

Fig. 3


A
a
b
c
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mut1 ctttcttTAACGcagtgCAAGGAGCTATcttggtggt
mut2 agggattGCCATcagtgCAAGGAGCTATcttggtggt
mut3 agggattTAACGcagtgACCTTAGCTATcttggtggt
mut4 agggattTAACGcagtgCAAGGCTAGCGcttggtggt
mut5 agggattTAACGcagtgCAAGGAGCTATcttgtgttg
C
wt wt mut1 mut2 mut3 mut4 mut5
1 2 3 4 5 6 7
B
wt
mut1
mut2
mut3
mut4
mut5

Fig. 4

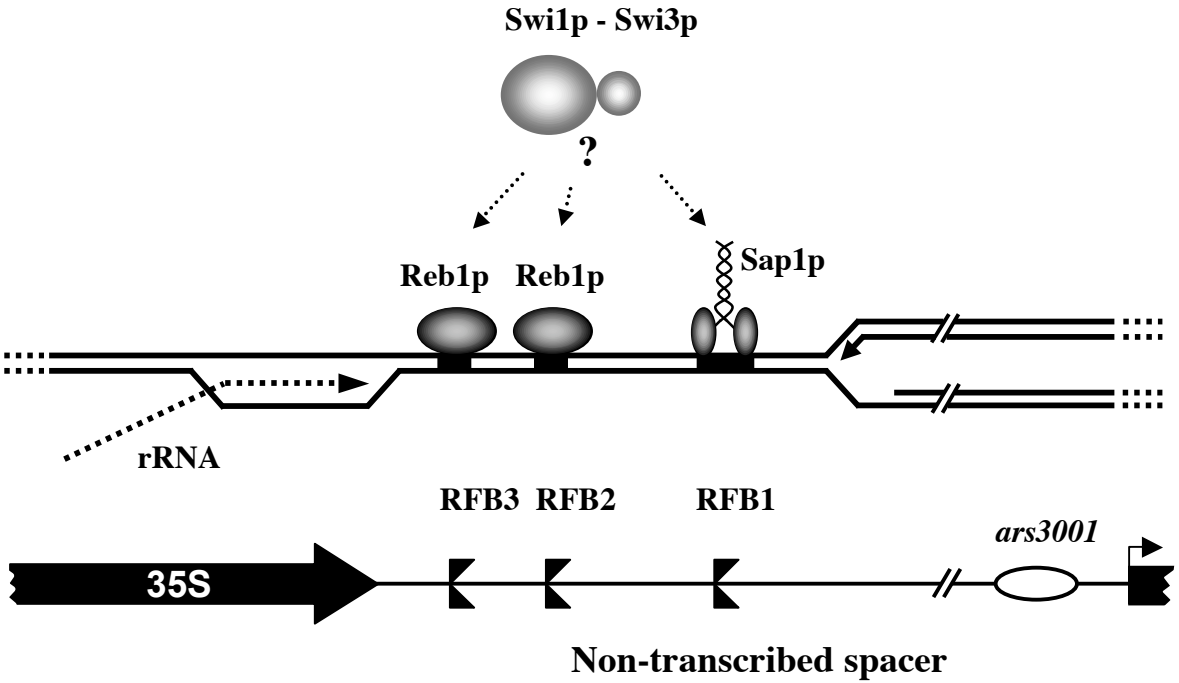
Swi1p - Swi3p
?
Reb1p
Reb1p
Sap1p
rRNA
RFB3
RFB2
RFB1
ars3001
35S
Non-transcribed spacer

Fig. 5