Transcription termination factor reb1p causes two replication fork barriers at its cognate sites in fission yeast rDNA in vivo

Alicia Sánchez-Gorostiaga, Carlos López-Estraño¹, Dora B. Krimer, Jorge B. Schwartzman and Pablo Hernández*  

Departamento de Biología Celular y del Desarrollo, Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Ramiro de Maeztu 9, 28040 Madrid, Spain

¹Present address: Department of Pathology, Medical School, Northwestern University, 303 East Chicago Ave., 3-210 Ward Blgd., Chicago, IL 60610, USA

*Corresponding author: Pablo Hernández, Departamento de Biología Celular y del Desarrollo, Centro de Investigaciones Biológicas (CSIC), Ramiro de Maeztu 9, 28040 Madrid, Spain. Phone: +34-918373112, ext. 4240. Fax: +34-5360432. E-mail: p.hernandez@cib.csic.es.

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**ABSTRACT**

Polar replication fork barriers (RFBs) near the 3'-end of the rRNA transcriptional unit are a conserved feature of rDNA replication in eukaryotes. In the mouse, *in vivo* studies indicate that the *cis*-acting Sal boxes required for rRNA transcription termination are also involved in replication fork blockage. On the contrary, in the budding yeast *Saccharomyces cerevisiae*, the rRNA transcription termination factors are not required for RFBs. Here we characterized the rDNA RFBs in the fission yeast *Schizosaccharomyces pombe*. *S. pombe* rDNA contains three closely spaced polar replication barriers named RFB1-3, in the 3’ to 5’ order. The transcription termination protein reb1 and its two binding sites, present at the 3’ end of the coding region, were required for fork arrest at RFB2 and RFB3 *in vivo*. On the other hand, fork arrest at the strongest RFB1 barrier was independent of the above transcription termination factors. Therefore, RFB2 and RFB3 resemble the barriers present in the mouse rDNA, whereas RFB1 is similar to the budding yeast RFBs. These results suggest that during evolution, *cis*- and *trans*-acting factors required for rRNA transcription termination became also involved in replication fork blockage. *S. pombe* would be a transitional species where both mechanisms coexist.
INTRODUCTION

During eukaryotic rDNA replication the fork moving opposite to transcription is arrested at replication fork barriers (RFBs) close to the 3’ end of the coding region (3, 13, 24-27, 42, 43). RFBs must play a relevant biological role since they are highly conserved in eukaryotes. Due to the polar nature of RFBs, rDNA is mainly replicated in an unidirectional mode co-oriented with transcription. Thus, one possible role for the RFB may be to prevent the deleterious effects of head-on collision between replication and transcription machineries (32). Since the DNA sequence at the RFB is not sufficient per se to stall replication (4, 28), fork arrest must be induced by a protein factor(s) bound to the rDNA at the barrier.

In *S. cerevisiae* rDNA, protein Fob1 is required for RFB activity (19), although it is still unknown whether it arrests rDNA replication by binding to the RFB sites or through a different mechanism. Functional RFBs are required for *HOT1* recombination, contraction and expansion of the rDNA repeat number and the formation of extrachromosomal ribosomal circles (17-20), suggesting that RFB activity stimulates recombination occurring at the rDNA locus in this budding yeast (2). On the other hand, it has been recently shown that RFBs and *HOT1* recombination are independent activities although they share *cis*-acting sequences (41).

In mouse rDNA, replication forks stall at the rRNA transcriptional terminator elements known as Sal boxes (27), which are the specific binding sites for transcription termination factor mTTF-1 (12, 21). This protein is able to arrest replication forks in an *in vitro* replication assay (10, 35). These *in vivo* and *in vitro* results suggest that Sal boxes and mTTF-1 block replication forks with the opposite polarity as they direct transcription termination. A protein factor that specifically
binds to 27-bp repeated sequences located at the barrier has been proposed also to be involved in the RFBs of pea rDNA (28).

Contrary to what happens in the mouse, neither the rRNA transcription termination factor Reb1p nor its rDNA binding sequence seem to be involved in the RFB of S. cerevisiae (4, 41). These observations suggest that the molecular mechanism that regulates rDNA replication arrest diverged through evolution. In the present work, we found three independent closely spaced RFBs in the fission yeast rDNA. Two of these RFBs required both the transcription termination factor reb1p and its two binding sites near the 3’ end of the 25S gene, whereas the other RFB functioned in the absence of these cis- and trans-acting factors. Therefore, Schizosaccharomyces pombe could be a transitional species where the mechanisms operating in budding yeast and mammals coexist.

MATERIALS AND METHODS

Yeast strains and growth conditions. S. pombe strains used were 972h− (wt h−), 35 (h− leu1-32), 117 (h+ leu1-32 ura4-d18 ade6-M210), 118 (h+ leu1-32 ura4-d18 ade6-M210/ade6-M216), D7 (h+ leu1-32 ura4-d18 ade6-M210 reb1Δ::kanMX6) and D8 (h− leu1-32 ura4-d18 ade6-M210 reb1+). Media and growth conditions used were as previously described (31). All pIRT2 derived plasmids containing S. pombe rDNA fragments were transformed by electroporation (34). E. coli strain DH5α was used for recombinant DNA procedures.

Construction of plasmids containing rDNA sequences. Plasmid pBL1263, containing a complete repeat of S. pombe rDNA (23), was the source of
the sequences analyzed for RFB activity. Autonomous replicating plasmid pIRT2 (14) was used as vector. For convenience, the HindIII insert in pBL1263 was first cloned into the HindIII site of pUC18 generating plasmid pHH10.4. Plasmids pIRT1.6(+) and pIRT1.6(-) were obtained by inserting the blunt-ended 1.6-kb Xho-HindIII rDNA fragment from pHH10.4 into the SmaI site of pIRT2. The (+) plasmids contained the insert in the orientation expected to block fork progression. Construction of plasmid pRebs was carried out as follows. A PCR product was obtained using primer 1 (5’-CCCCTGCAGTTTTGAAGAGATAAAAGG-3’) and primer 2 (5’-CCCGGATCC TTTTACTAGGATTGTGC-3’) on pIRT1.6(+). Each primer contained 18 nucleotides (underlined) that annealed a few nucleotides upstream the 5’ reb1p binding sequence (primer 1) or downstream the 3’ reb1p binding sequence (primer 2), a PstI (primer 1) or BamHI (primer 2) restriction site and a CCC tail. This fragment was PstI+BamHI digested and inserted into the polycloning site of pIRT2 near ars1. Thus, the 229-bp fragment cloned in pRebs contained, in addition to both reb1p binding sites and the sequence between them, 15 bp upstream the 5’ binding sequence and 14 bp downstream the 3’ binding sequence. For construction of plasmids pRebBS(+) and pRebBS(-), a double stranded oligonucleotide containing the 17-bp reb1p binding site (5’-AGGTAAGGTAATGCAC-3’) (44) was inserted in both orientations into the PstI and BamHI sites of the pIRT2 polycloning site. To direct the ligation to the desired orientation, oligonucleotide contained PstI and BamHI sticky ends. Appropriate insertion was checked by sequencing. For construction of plasmid pRebs∆BS, a PCR product was obtained using oligonucleotides 5’-AAGGCCTA A A T C T C T A G T A A A A G G A T C - 3’ and 5’-AAGGCCTTTTCCCCTTCAAAAAG-3’. These primers annealed divergently next to
each side of the reb1p binding sequence closer to \textit{ars1} in pRebs (underlined nucleotides) and contained a \textit{StuI} site at their 5' ends (bold). After \textit{StuI} digestion, the PCR product was ligase-mediated circularized. For construction of pRebsSEP, first a \textit{StuI} site was created by PCR between both reb1p binding sequences of plasmid pRebs. This \textit{StuI} site was located at 60 bp from the binding site closer to \textit{ars1} and at 106 bp from the other binding site. Then, the 787-bp \textit{EcoRV-Nrul} fragment from the pBR322 tetracycline resistance gene was cloned into this \textit{StuI} site.

**Deletion of reb1\textsuperscript{+} gene.** Deletion of reb1\textsuperscript{+} gene was achieved by PCR-mediated replacement of the complete ORF by \textit{kanMX6\textsuperscript{+}} gene (1). PCR reactions were performed using two 84 nucleotide-long primers (5'-GATATTAGCG ATTGATAAGT TGAAGTGATT ACTCAATTAT AGTACTTCAA AAATATAATC CGCCAGGGTT TTCCCAGTCA CGAC-3' and 5'-ATTGTAAGGA CGTCAATTGG AGAATCCAGA AAGTACCACT TTAAAGTCAT CAATGGCTGA AGCGGATAAC AATTTCACAC AGGA-3'), where the first 60 nucleotides (underlined) corresponded to sequences flanking the reb1\textsuperscript{+} ORF. The remaining 24 nucleotides of each primer corresponded to sequences located at either side of the pBluescript SK\textsuperscript{+} polycloning site, where \textit{kanMX6\textsuperscript{+}} was cloned (a gift from S. Moreno). Transformation of \textit{S. pombe} 117x118 diploid strain with the PCR fragment and selection of G418-resistant diploids was performed as described (1). Genomic DNA from selected transformants was digested with \textit{EcoRV}, electrophoresed and hybridized with a probe specific for \textit{kanMX6\textsuperscript{+}} gene to confirm its integration. reb1\textsuperscript{+}/reb1\textsuperscript{Δ} diploids were induced to form spores to further analyze them by tetrad separation. The four spores from selected asci were again checked for integration.
of the *kanMX6* gene as described above (Fig. 5C).

**Two-dimensional gel electrophoresis.** Genomic and plasmid DNA for 2D gel analysis was isolated from asynchronous log-phase cultures using the procedures described by Caddle and Calos (5) (for plasmid analysis) or by Huberman and co-workers (15) (for genomic rDNA analysis). Electrophoresis conditions were as in (9).

**RESULTS**

*S. pombe rDNA contains three closely spaced RFBs.* The *S. pombe* genome contains ~100 copies of rRNA genes organized in two arrays near both ends of chromosome III (29, 33). Two 17-bp binding sequences for the transcription termination factor reb1p separated by 166 bp, are present in the NTS close to the 3' end of the 25S gene (Fig. 1, striped boxes) (44). Replication of *S. pombe* rDNA was analyzed by two-dimensional (2D) agarose gel electrophoresis. DNA from exponentially growing 972h− strain cells was digested with the restriction enzymes indicated, separated in 2D gels, transferred and hybridized with probes specific for a series of overlapping fragments covering the rDNA repeat (Fig. 1, fragments A through E). Analysis of fragment A showed a spot corresponding to an accumulated Y-shaped replication intermediate (Fig. 1A, arrow), confirming previous observations of a replication barrier in *S. pombe* rDNA (26, 37). However, the elongated appearance of this signal suggested that forks stalled at several rather than at a single site. 2D gel analysis of the overlapping fragment B supported this possibility. Three independent spots were identified on the descending portion of the simple Y arc (arrowheads in Fig. 1, panel B), indicating
that replication stalled at three alternative sites, herein called RFB1, RFB2 and RFB3. Equivalent spots on the ascending portion of the arc were absent, indicating that, as in other species, these three RFBs were polar, arresting only forks moving against the direction of transcription (leftwards in the map of Fig. 1). The intensity of the spots of accumulated replication intermediates was clearly different. The strongest signal corresponded to the first pausing site that leftward moving forks encounter (RFB1). The middle, RFB2, gave the weakest signal and RFB3 produced a signal of intermediate intensity. Replication analysis of fragment C (HindIII-SalI) showed no spots of accumulated intermediates (Fig. 1, panel C), indicating that DNA sequences involved in fork stalling were located to the left of the HindIII site. Two additional fragments, corresponding to the coding region, revealed no replication impediment as uniform simple Y arcs were observed (Fig. 1, panels D and E).

A bubble arc was also visible in the 2D gel corresponding to fragment C (Fig. 1, panel C, open arrowhead), indicating that a replication origin located within this fragment fires in a fraction of rDNA repeats. This observation is in agreement with the previous identification of an ARS (ars3001) in the NTS of S. pombe rDNA (37).

All three S. pombe RFBs are active in autonomously replicating plasmids with the same polarity and relative efficiency as in the chromosome. As described above, S. pombe RFBs are located in the NTS, 5’ to the HindIII site. Therefore, the 1.6-kb XhoI-HindIII restriction fragment of S. pombe rDNA (Fig. 2A) was cloned in both orientations close to the ars1 replication origin of vector pIRT2 (Fig. 2B). This fragment contained the NTS portion laying to the left
of the *Hind*III site and the 3' end of the 25S gene. In pIRT1.6(+) the inserted sequence is replicated in the same direction in which the barriers are active in the chromosome, whereas in pIRT1.6(-) the insert is replicated in the opposite direction. Replication of these plasmids in strain 35 was analyzed by 2D gel electrophoresis after double digestion with *Pvu*II and *Eco*RV, using a specific probe to detect the fragment containing the insert. Location of the insert close to *ars1* ensured that the clockwise moving fork reaches the RFBs before the fork moving counterclockwise enters the fragment analyzed. If the RFBs were active, simple-Y shaped intermediates of this fragment will accumulate meanwhile the counterclockwise fork replicates the other fragment. The results obtained are shown in Fig. 2C and D. All three RFBs observed in the chromosomal context were also detected in pIRT1.6(+), visualized as three spots of accumulated intermediates on the Y arc (Fig. 2C, arrowheads). The relative intensities of these spots fitted well with those observed in the chromosome. No arrest sites were detected in pIRT1.6(-) (Fig. 2D), indicating that all three RFBs retained the same polarity as in the chromosomal context. No additional barriers were detected within the rightward *Hind*III-*Bam*HI fragment of the NTS (not shown).

A longer exposure of the same gel shown in Fig. 2C allowed detection of a partial bubble arc (Fig. 2C’, open arrowhead), generated upon bidirectional replication from *ars1*. In addition, a straight line emerged from the spots of accumulated intermediates extending upward and to the left in a diagonal fashion (Fig. 2C’, black arrow). This signal corresponded to double-Y intermediates generated when the counterclockwise advancing fork entered the fragment until it encountered the clockwise fork arrested at the barriers. These observations showed that in a significant fraction of the plasmid molecules replication
termination occurred at the barriers.

Location of RFB1. The spots of accumulated intermediates showed in Fig. 2 appeared on the simple-Y arc to the right of the inflexion point. This indicated that RFBs were located closer to the HindIII site. Therefore, to locate the DNA sequence required for RFB1, we analyzed a fragment spanning 383 bp next to the HindIII site (Fig. 3A). This fragment was cloned into pIRT2 and two restriction fragments of the resulting plasmid (p3’Rebs, Fig. 3B) were analyzed. 2D gels showed a single strong spot of a specific Y-shaped intermediate corresponding to RFB1 (Fig. 3C and D, black arrowhead). Replication termination structures were also visible (Fig. 3C and D, arrows), indicating termination at the barrier.

The 17-bp binding sequence for the rRNA transcription terminator factor reb1p is required and sufficient to induce replication fork arrest at RFB2 and RFB3. As mentioned before, S. pombe rDNA contains two identical 17-bp binding sequences for the transcription termination protein reb1p (30, 44). Both termination signals are included in the fragment where RFBs were mapped (Fig. 2). To address if these binding sequences are also DNA cis-acting elements for the remaining barriers RFB2 and RFB3, we tested the capacity of a 229-bp fragment, containing both reb1p binding sites (Fig. 4A), to arrest replication forks. This fragment was cloned in the proper orientation into pIRT2 (pRebs, Fig. 4B). 2D gel of the PvuII-BstEII fragment containing the insert showed two signals of accumulated intermediates at the expected positions on the simple-Y arc (Fig. 4D, arrowheads), indicating that this fragment contains the cis-acting signals required for RFB2 and RFB3. We removed one of the 17-bp binding sequences (the one
closer to $ars1$) from this plasmid (the modified insert is shown in Fig. 4B). As a consequence of this deletion, one of the spots was missing in the 2D gel of the resulting plasmid pRebs$\Delta$BS and only the spot closer to the inflexion of the Y arc remained (Fig. 4E). These results indicated that reb1p binding sites are essential cis-acting signals for RFB2 and RFB3 and that they function independently. When both binding sites were placed further apart by inserting in between a 787-bp sequence (Fig. 4C), the two fork arrest positions appeared also separated from each other as indicated by the new relative locations of the spots in the corresponding 2D gel (compare panels F and D of Fig. 4). In addition, we found that the 17-bp sequence was not only necessary, but also sufficient for fork arrest. A synthetic sequence identical to the reb1p binding site inserted into pIRT2 (Fig. 4G) induced fork arrest in the (+) orientation (Fig. 4H, arrowhead) and had no effect in the opposite (-) orientation (Fig. 4I).

**Deletion of reb1$^+$ abolishes fork arrest at RFB2 and RFB3.** Since the reb1p binding sequence was necessary and sufficient to induce polar replication fork arrest, we regarded this transcription termination protein as a candidate to be involved in RFB2 and RFB3, even though the S. cerevisiae homologue Reb1p is not involved in rDNA RFBs (41).

To analyze the function of S. pombe reb1p in RFB activity, we constructed an heterozygous reb1$^+$/reb1$\Delta$ diploid strain. The null allele was obtained by PCR-mediated gene replacement of reb1$^+$ by the selectable gene marker kanMX6$^+$, which confers resistance to the antibiotic G418 (Fig. 5A). Dissection of three representative tetrads of the G418 resistant reb1$^+$/reb1$\Delta$ diploids is shown in Fig.
5B. In the absence of G418 all four spores were viable, indicating that reb1$^+$ is not an essential gene in *S. pombe* (Fig. 5B, left panel). However, two spores of each tetrad gave colonies slightly smaller compared with the other two. In all cases the smaller colonies were those generated by reb1Δ::kanMX6$^+$ spores, as they grew in the presence of G418 (Fig. 5B, right panel). Replacement of reb1$^+$ by kanMX6$^+$ was confirmed by Southern blot (Fig. 5C) and PCR analysis (not shown). Therefore, deletion of reb1$^+$ gene was not lethal, but mutant cells grew somewhat more slowly than wild-type cells. This small effect of reb1$^+$ deletion on cell growth was confirmed by dilution assays (data not shown).

To address involvement of reb1p in the barriers, replication of pIRT1.6(+) containing all three RFBs was analyzed in isogenic reb1$^+$ and reb1Δ haploid strains. As shown in Fig. 5D and E, in the reb1Δ strain the spots corresponding to RFB2 and RFB3 disappeared and only the one generated by RFB1 remained. Similar results were obtained upon analysis of plasmids pRebs and pRebBS(+) (data not shown). This observation demonstrates that the transcription termination factor reb1p is required for RFB2 and RFB3.

**DISCUSSION**

In *S. pombe*, the rRNA transcription termination factor reb1p has been recently identified, showing sequence similarity with *S. cerevisiae* Reb1p and mouse m-TTF1 (44). These three termination factors share myb-like DNA binding domains. *S. pombe* reb1p has two identical 17-bp binding sites that block read-through transcription *in vitro* (44). In addition, reb1p also causes *in vitro* 3'-end RNA formation at two sites of *S. pombe* rDNA that correspond to the transcription termination sites determined *in vivo* (38, 44).
In this work we have identified three RFBs in the *S. pombe* rDNA. Fork arrest at two of these RFBs (RFB2 and RFB3) is produced upon binding of the transcription termination protein reb1, similar to what has been proposed to occur in mouse (27, 35). Removal of the reb1p binding sequence or deletion of *reb1*\(^{+}\) completely abolished replication blockage at RFB2 and RFB3 (Figs. 4E and 5E). Therefore, upon binding to its cognate sequence, reb1p inhibits both rRNA transcription and rDNA replication occurring with opposite directions, thus preventing head-on collision of both machineries. It can not be ruled out, that other factor(s) besides reb1p are also required for fork arrest at RFB2 and RFB3. On the other hand, fork arrest at RFB1 is independent of this transcription termination factor, since it remains active in a *reb1*\(^{\Delta}\) mutant strain (Fig. 5E). Moreover, we have found a reb1p-unrelated protein that specifically binds to a short sequence within the RFB1-containing fragment analyzed in Fig. 3 (manuscript in preparation). Thus, *S. pombe* RFB1 seems to be similar to the two barriers found in budding yeast, as both of them are normal in strains with a temperature-sensitive allele of *REB1* growing at restrictive temperature (41).

Therefore, two different and independent mechanisms operate in *S. pombe* rDNA to arrest replication forks. Remarkably, although different trans- and cis-acting factors are involved in these two mechanisms, both of them block replication progression in a polar fashion. This indicates that polarity of rDNA barriers is an essential feature to accomplish their biological role, which may be to avoid or regulate head-collision between transcription and replication.

Besides the RFBs present in the rDNA, another barrier, named RTS1, has been described in *S. pombe*. RTS1 is involved in the mating-type switching by determining the direction of replication at the *mat1* locus (6, 7). As in the case of
rDNA RFBs, RTS1 is a polar barrier that contains a cluster of three full-length and one truncated ~60-bp imperfect direct repeats (7). Interestingly, each of these repeats includes a sequence that shows homology to the 17-bp reb1p binding sequence required for RFB2 and RFB3. Moreover, according to our findings, the orientation of these homologous sequences is in agreement with the reported polarity of RTS1 (7). This observation raises the possibility that reb1p plays also a role in fork arrest at the mat1 locus.

It is interesting to note that whereas REB1 is an essential gene in S. cerevisiae (16), deletion of reb1+ in S. pombe has only a weak effect on cell growth (Fig. 5B, D). Besides its function in RNA polymerase I transcription termination (22, 36), S. cerevisiae Reb1p regulates the expression of several unrelated RNA polymerase II transcribed genes (40 and references therein). In addition, Reb1p binding sites are also present in the subtelomeric X and Y’ regions in budding yeast (8). Thus, the essential nature of Reb1p in S. cerevisiae could be due to the function(s) this protein performs at these additional sites, but not at the rDNA locus. In agreement with this hypothesis, it was recently shown that deletion of the Reb1p binding site in all rDNA chromosomal repeats of S. cerevisiae has no effect on cell growth or rRNA synthesis (39). Altogether these results suggest that, at least in these two species, in the absence of the cis- or trans-acting factors currently known to be involved in rRNA transcription termination, rRNA transcripts are terminated by an alternative unknown pathway and processed properly to form functional ribosomes. It would be worthy to investigate if factors involved on S. pombe RFB1 play a role in this alternative pathway.
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REFERENCES


FIGURE LEGENDS

FIG. 1
Analysis of *S. pombe* chromosomal rDNA replication by 2D agarose gel electrophoresis. The upper diagram represents an rDNA repeat unit, where the regions coding for the mature rRNAs (black boxes), the non-transcribed spacer (NTS) and the 17-bp reb1p binding sequences (striped boxes) are indicated. Restriction sites included are: B, *Bam*HI; BII, *Bgl*II; H, *Hind*III; K, *Kpn*I; S, *Sal*I; X, *Xho*I. Horizontal lines labeled A through E represent the restriction fragments analyzed in the panels below. Arrow in (A) points to the elongated signal of accumulated replication intermediates. Arrowheads in (B) indicate three spots of accumulated intermediates labeled 1, 2 and 3, that correspond to barriers RFB1, RFB2 and RFB3, respectively. Open arrowhead in (C) points to the signal corresponding to replication intermediates containing a growing bubble. The probe used for 2D gels in (A), (B) and (C) was the 3.0-kb *Hind*III-*Kpn*I fragment spanning most of the NTS and the 5’ end of the transcription unit. The probe used for 2D gels in (D) and (E) was the 3.5-kb *Sal*I-*Bgl*II fragment within the coding region.

FIG. 2
All three *S. pombe* RFBs are active in autonomously replicating plasmids with the same polarity as in their chromosomal context. (A) Detail of the rDNA NTS. The horizontal line represents the 1.6-kb *Xho*I-*Hind*III restriction fragment analyzed below, that includes both 17-bp reb1p binding sequences (striped boxes). (B) Diagram of plasmids pIRT1.6(+) and pIRT1.6(-) obtained by inserting the *Xho*I-
HindIII fragment (internal arc) in both orientations into pIRT2. The ars1 containing fragment (large striped box) and the site where replication initiates (black rhomb) (11) are included. (C) 2D gel analyzing replication of the PvuII-EcoRV fragment of pIRT1.6(+) containing the rDNA insert. As in Fig. 1B, arrowheads labeled 1, 2 and 3 point to the spots of accumulated replication intermediates induced by RFB1, RFB2 and RFB3, respectively. (C') A longer exposure of the autoradiogram shown in (C), where the signals corresponding to bubble-containing intermediates (open arrowhead) and double-Ys (arrow) are visible. (D) 2D gel analyzing replication of the same fragment as in (C) but from pIRT1.6(-), where no accumulated intermediates were detected. The probe used in both autoradiograms was the PvuII-EcoRV fragment from vector pIRT2.

**FIG. 3**

Location of barrier RFB1. (A) NTS of *S. pombe* rDNA where the location of the 383-bp sequence analyzed below is indicated. (B) Diagram of plasmid p3'Rebs obtained upon insertion of the aforementioned 383-bp sequence (internal arc) between ars1 and LEU2 of pIRT2. (C) (D) 2D gels of the PvuII-BstEII and AattII-EcoRV fragments of p3'Rebs, respectively. Black arrowhead points to the spot of accumulated replication intermediates induced by RFB1. Open arrowhead indicates the arc of bubble-containing intermediates. Arrow points to the signal generated by replication termination structures due to fork meeting at the barrier. Probe used was the same as in Fig. 2.
FIG. 4
Characterization of barriers RFB2 and RFB3. (A) Detail of the NTS where the location of the 229-bp sequence analyzed below is indicated. This sequence contains both 17-bp reb1p binding sites (striped boxes). (B) Diagram of plasmid pRebs obtained upon insertion of the mentioned 229-bp sequence between $ars1$ and $LEU2$ of pIRT2. The modified insert in pRebs∆BS is also shown, where one of the reb1p binding sites was removed. (C) Diagram of pRebs-derived pRebsSEP plasmid, where both reb1p binding sites were separated further by inserting in between a 787-bp sequence (open box). (D) (E) (F) 2D gels analysis of the $Pvu$II-$Bst$EII fragment from pRebs, pRebs∆BS and pRebsSEP, respectively. Arrowheads point to the spots of accumulated replication intermediates induced by barriers RFB2 and RFB3. (G) Diagram of plasmids pRebBS(+) and pRebBS(-), where the 17-bp reb1p binding sequence was inserted in both orientations into pIRT2. Only the (+) orientation of the sequence is indicated above the diagram. (H) (I) 2D gels of the $Aat$I-$Eco$RV fragment of pRebBS(+) and pRebBS(-), respectively. Arrowhead in (H) points to the spot of the accumulated replication intermediate induced by the reb1p binding sequence only in the (+) orientation. The probe used in these autoradiograms was the same as in Fig. 2.

FIG. 5
Deletion of $reb1^+$ gene. (A) Diagrammatic representation of $reb1^+$ gene deletion by PCR-mediated disruption. One allele of the $reb1^+/reb1^+$ diploid strain 117x118 was substituted by the G418-resistant gene $kanMX6^+$ (hatched box). Open small boxes within $reb1^+$ represent introns. The locations of two $Eco$RV restriction sites flanking
reb1+ and an additional one within kanMX6+ are indicated. (B) Tetrad analysis of three selected heterozygous diploids. Spores from three asci were separated and grown in YES medium without G418 (left panel). Colonies were then replicated to a new plate containing 100 µg/ml G418 (right panel) to determine segregation of the alleles. (C) Southern blot verifying deletion of reb1+ gene by replacement with kanMX6+. EcoRV digested DNA from reb1Δ and wild type haploid cells was hybridized with a probe specific for kanMX6+ (left panel) or reb1+ (right panel). The kanMX6+ probe hybridized to the expected 2.1-kb restriction fragment from reb1Δ DNA, containing most of kanMX6+ gene (see bottom map in A) and did not hybridized to the DNA from wild type cells. As expected, the reb1+ probe did not hybridized to the DNA from reb1Δ cells but detected the 3.9-kb fragment containing reb1+ in the DNA from wild type cells. (D) (E) 2D gels of plasmid pIRT1.6(+) replicating in wild type (wt) and reb1Δ cells, respectively. The restriction fragment analyzed was the same as in Fig. 2C. Arrowheads labeled 1-3 point to the spots of accumulated replication intermediates induced by the three barriers in wt cells. Note that in reb1Δ cells only the spot generated by RFB1 remained. The probe used in these autoradiograms was the same as in Fig. 2.
FIG. 1

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FIG. 2

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A.  

\[
\begin{array}{c}
XhoI & \quad \text{HindIII} & \quad \text{BamHI} \\
\setlength\fboxsep{0pt} & \quad \fbox{25S} & \quad \text{NTS} & \quad 5'ETS \\
\end{array}
\]

B.  

\[
\begin{array}{c}
\text{ars1} & \quad PvuII \\
\setlength\fboxsep{0pt} & \quad \fbox{25S} & \quad \text{LEU} & \quad \text{ars1} & \quad PvuII \\
\end{array}
\]

pIR1T.6 (+)  
(8.2 kb)

pIR1T.6 (−)  
(8.2 kb)

C  

C'  

D

pIR1T.6 (+)  

pIR1T.6 (−)
FIG. 4  Sánchez-Gorostiaga et al.

A.  

- XhoI  
- HindIII  
- BamHI  
- KpnI  

25S  

229 bp  

NTS  

5'ETS  

B.  

- XhoI  
- Hin  
- dIII  
- Bam  
- HI  
- Kpn  

BstEII  

ars1  

LEU2  

BstEII  

BstEII  

pRebs (6,8 kb)  

PvuII  

C.  

- XhoI  
- Hin  
- dIII  
- Bam  
- HI  
- Kpn  

BstEII  

ars1  

LEU2  

BstEII  

BstEII  

pRebsSEP (7,6 kb)  

PvuII  

D.  

E.  

F.  

pRebs  

pRebs∆BS  

pRebsSEP  

G.  

- XhoI  
- Hin  
- dIII  
- Bam  
- HI  
- Kpn  

BstEII  

ars1  

EcoRV  

LEU2  

AatII  

pRebBS (+/-)  

(6.6 kb)  

pRebsBS (+)  

pRebsBS (-)  

H.  

I.
A. 

B. - G418  + G418

C. 

D. 

E. 

Fig. 5 Sánchez-Gorostiaga et al.