Transcription Termination Factor reb1p Causes Two Replication Fork Barriers at Its Cognate Sites in Fission Yeast Ribosomal DNA In Vivo

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During eukaryotic ribosomal DNA (rDNA) replication, the fork moving opposite to transcription is arrested at replication fork barriers (RFBs) near the 3′ end of the rRNA transcriptional unit in a conserved feature of ribosomal DNA (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, RFB2, and RFB3 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 involved in replication fork blockage also. S. pombe is suggested to be a transitional species in which both mechanisms coexist.

In mouse rDNA, replication forks stall at the cis-acting transcriptional terminator elements known as Sal boxes (27), which are the specific binding sites for transcription termination factor mTTF-1 (12, 21). This protein was 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 rDNA transcription termination factor Reb1p nor its rDNA binding sequence seems 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 in which the mechanisms operating in budding yeast and mammals coexist.

MATERIALS AND METHODS

Yeast strains and growth conditions. The S. pombe strains used were 972 h− (wt h−), 35 (h− leu1-32), 117 (h− leu1-32 ura4-d18 ade6-M210), 118 (h− leu1-32 ura4-d18 ade6-M216), 117×118 (h−/h− leu1-32/leu1-32 ura4-d18/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 reb1Δ). Media and growth conditions used were as previously described (31). All pIRT2-derived plasmids containing S. pombe rDNA fragments were transformed by electroporation (34). Escherichia 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 was cloned into this. The product was obtained by using oligonucleotides 5'-... (underlined nucleotides) and contained the pIRT2 polycloning site. To direct the ligation to the desired orientation, the CAC-3' site was located 60 bp from the binding site closer to the 5'-end of the 25S gene (Fig. 1). These primers annealed divergently next to each side of the reb1p binding sequence closer to ars1 in plasmids pRebs (underlined nucleotides) and contained a StuI site at its 5'-ends (boldface nucleotides). After digestion with StuI, the PCR product was ligase-mediated circularized. For the construction of pRebsSEP, first a StuI site was created by PCR between reb1p binding sequences of plasmid pRebs. This StuI site was located 60 bp from the binding site closer to ars1 and at 106 bp from the other binding site. Then, the 787-bp EcoRV-NruI fragment from the pBR322 tetracycline resistance gene was cloned into this StuI site.

Deletion of reb1* gene. Deletion of the reb1* gene was achieved by PCR-mediated replacement of the complete open reading frame by the kanMX6* gene (1). PCRs were performed with two 84-nucleotide-long primers (5'-... (underlined nucleotides) corresponding to sequences flanking the reb1* open reading frame. The remaining 24 nucleotides corresponded to sequences located at either side of the pBluescript SK(+) polycloning site, where kanMX6* was cloned (a gift from S. Moreno). Transformation of S. pombe 1177Y118 diploid strain with the PCR fragment and selection of G418-resistant diploids were performed as described previously (1). Genomic DNA from selected transformants was digested with EcoRV, electrophoresed, and hybridized with a probe specific for kanMX6* to confirm its integration. reb1* reb1Δ diploids were induced to form spores to allow further analysis by tetrad separation. The four spores from selected ascii were again checked for integration of the kanMX6* gene as described above (see Fig. 5C).

Two-dimensional gel electrophoresis. Genomic and plasmid DNAs for two-dimensional (2D) gel analysis were isolated from asynchronous log phase cultures by using the procedures described by Cadden and Calos (5) (for plasmid analysis) or by Huberman and coworkers (15) (for genomic DNA analysis). Electrophoresis conditions were as described in reference 9.

RESULTS

S. pombe rDNA contains three closely spaced RFBs. The S. pombe genome contains ~100 copies of rDNA genes organized in two arrays near both ends of chromosome III (29, 33). Two 17-bp binding sequences for the transcription termination factor rb1p, separated by 166 bp, are present in the nontranscribed spacer (NTS) close to the 3' end of the 25S gene (Fig. 1) (44). Replication of S. pombe rDNA was analyzed by 2D agarose gel electrophoresis. DNA from exponentially growing strain 972 h− 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 sites 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 (Fig. 1B), indicating that replication stalled at three alternative sites, herein called RB1, RB2, and RB3. 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 intensities of the spots of accumulated replication intermediates were clearly different. The strongest signal corresponded to the first pausing site that leftward moving forks encounter (RB1). The middle site, RB2, gave the weakest signal, and RB3 produced a signal of intermediate intensity. Replication analysis of fragment C (HindIII-Sall) showed no spots of accumulated intermediates (Fig. 1C), 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. 1D and E).

A bubble arc was also visible in the 2D gel corresponding to fragment C (Fig. 1C), 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 autonomously replicating sequence (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 lying to the left of the HindIII site and the 3' end of the 25S gene. In pIRT1.6(+) the inserted sequence is replicated in the 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 PvuII and EcoRV, using a specific probe to detect the fragment containing the insert. Locating the insert close to ars1 ensured that the clockwise-moving fork would reach the RFBs before the fork moving counterclockwise entered the fragment analyzed. If the RFBs were active, simple-Y-shaped intermediates of this fragment would accumulate while the counterclockwise fork replicated 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). 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 HindIII-BamHI fragment of the NTS (not shown).

A longer exposure of the same gel as that shown in Fig. 2C allowed detection of a partial bubble arc (Fig. 2C'), 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'). 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). Replication termination structures were also visible (Fig. 3C and D), 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). The 2D gel of the PvuII-BseEII fragment containing the insert showed two signals of accumulated intermediates at the expected positions on the simple-Y arc (Fig. 4D), 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 pRebsΔBS and only the spot closer to the inflexion of the Y arc remained (Fig. 4E). These results indicated
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 XhoI-HindIII restriction fragment analyzed below, which includes both 17-bp reb1p binding sequences (striped boxes). (B) Diagram of plasmids pIRT1.6(+) and pIRT1.6(−) obtained by inserting the XhoI-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 panel C in which 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 panel C but from pIRT1.6(−), in which no accumulated intermediates were detected. The probe used in both autoradiograms was the PvuII-EcoRV fragment from vector pIRT2.
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 a 787-bp sequence between them (Fig. 4C), the two fork arrest positions also appeared to be 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) and had no effect in the opposite (−) orientation (Fig. 4I).

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 and D) 2D gels of the PvuII-BstEII and AatII-EcoRV fragments of p3′Rebs, respectively. Black arrowhead points to the spot of accumulated replication intermediates induced by RFB1. The open arrowhead indicates the arc of bubble-containing intermediates. The arrow points to the signal generated by replication termination structures due to fork meeting at the barrier. The probe used was the same as that for Fig. 2.
FIG. 4. Characterization of barriers RFB2 and RFB3. (A) Detail of the NTS indicating the location of the 229-bp sequence analyzed below. This sequence contains both 17-bp reb1p binding sites (striped boxes). (B) Diagram of plasmid pRebs obtained upon insertion of the above-mentioned 229-bp sequence between *ars1* and LEU2 of pIRT2. The modified insert in pRebsΔBS, where one of the reb1p binding sites was removed, is also shown. (C) Diagram of pRebs-derived pRebsSEP plasmid, in which both reb1p binding sites were separated further by inserting a 787-bp sequence between them (open box). (D through F) 2D gel analyses of the *Pvu*II-*Bst*EI fragment from pRebs, pRebsΔBS, 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 and I) 2D gels of the *Aat*II-*Eco*RV fragment of pRebBS(+) and pRebBS(−), respectively. The arrowhead in panel 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 that used for Fig. 2.
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 a heterozygous reb1* reb1Δ 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). Dissections of three representative tetrads of the G418-resistant reb1Δ diploids are 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 than 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 blotting (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 the 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. The probe used in these autoradiograms was the same as for Fig. 2.

**FIG. 5.** Deletion of the reb1* gene. (A) Diagrammatic representation of the reb1* gene deletion by PCR-mediated disruption. One allele of the reb1* reb1* diploid strain 117×118 was replaced by the G418-resistant gene kanMX6* (hatched box). Open small boxes within reb1* represent introns. The locations of two EcoRV 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 yeast extract with supplements without G418 (left panel). Colonies were then replicated onto a new plate containing 100 μg of G418/ml (right panel) to determine the segregation of the alleles. (C) Southern blot verifying the 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 the kanMX6* gene (see bottom map in panel A) and did not hybridize to the DNA from wild-type cells. As expected, the reb1* probe did not hybridize to the DNA from reb1Δ cells but detected the 3.9-kb fragment containing reb1* in the DNA from wild-type cells. (D and 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 to 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 for Fig. 2.
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, in a fashion similar to what has been proposed to occur in mouse (27, 35). Removal of the reb1p binding sequence or deletion of reb1p completely abolished replication blockage at RFB2 and RFB3 (Fig. 4E and 5E). Therefore, upon binding to its cognate sequence, reb1p inhibits both blockage at RFB2 and RFB3 (Fig. 4 and 5E). Therefore, reb1p binding sites are also involved in these two mechanisms, both of them blockage at RFB2 and RFB3 (Fig. 4E and 5E). Therefore, reb1p binding sites are also involved in two different and independent mechanisms that may be to avoid or regulate head-on collision between transcription and replication.

Besides the RFBs present in the rDNA, another barrier, named RTS1, has been described for *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 also plays a role in fork arrest at the *mat1* locus.

It is interesting that whereas REB1 is an essential gene in *S. cerevisiae* (16), deletion of reb1p in *S. pombe* has only a weak effect on cell growth (Fig. 5B and 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 (see reference 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) that 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 worthwhile to investigate if factors involved in *S. pombe* RFB1 play a role in this alternative pathway.

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