The fission yeast rDNA-binding protein Reb1 regulates G1 phase under nutritional stress

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Summary

Yeast Reb1 and its mammalian ortholog TTF1 are conserved Myb-type DNA-binding proteins that bind to specific sites near the 3′-end of rRNA genes (rDNA). Here, they participate in the termination of transcription driven by RNA polymerase I and block DNA replication forks approaching in the opposite direction. We found that *Schizosaccharomyces pombe* Reb1 also upregulates transcription of the *ste9*$^{+}$ gene that is required for nitrogen-starvation-induced growth arrest with a G1 DNA content and sexual differentiation. Ste9 activates the anaphase-promoting complex or cyclosome ('APC/C') in G1, targeting B-cyclin for proteasomal degradation in response to nutritional stress. Reb1 binds in vivo and in vitro to a specific DNA sequence at the promoter of *ste9*$^{+}$, similar to the sequence recognized in the rDNA, and this binding is required for *ste9*$^{+}$ transcriptional activation and G1 arrest. This suggests that Reb1 acts as a link between rDNA metabolism and cell cycle control in response to nutritional stress. In agreement with this new role for Reb1 in the regulation of the G1–S transition, *reb1*Δ and *wee1*^{ts} mutations are synthetically lethal owing to the inability of these cells to lengthen G1 before entering S phase. Similarly, *reb1*Δ *cdc10*^{ts} cells are unable to arrest in G1 and die at the semi-permissive temperature.

Key words: G1 arrest, Cell cycle control, Nutritional stress, Sexual differentiation

Introduction

The G1–S transition of the cell cycle is a crucial stage for eukaryotic proliferating cells (Bahler, 2005). At late G1, cells make the decision either to commit to a new round of DNA replication and subsequent cell division, a process called 'Start' in yeast, or to arrest and leave proliferative growing. In the fission yeast *Schizosaccharomyces pombe*, nutritional limitation such as nitrogen source deprivation, leads to growth arrest with a G1 DNA content (usually referred to as G1 arrest), and cells undergo sexual differentiation by conjugation of two cells of different mating types. Meiosis of the resultant zygote produces four ascospores that will germinate and enter the mitotic cycle again if nutritional conditions permit. G1 arrest is required for conjugation and takes place by inactivation of Cdc2, the only cyclin-dependent kinase that drives the G1–S transition in *S. pombe*. Cdc2 is inactivated in G1 under nitrogen starvation both by degradation of cyclin Cdc13 and by binding of the stoichiometric inhibitor Rum1 to Cdc2–Cdc13 complexes (Labib et al., 1995; Yamaguchi et al., 1997). Cdc13 is degraded at G1 upon nitrogen deprivation by APC/C-mediated ubiquitylation and proteolysis by the proteasome. APC/C is inactive by itself and it is activated in G1 phase by the Fizzy-related protein Ste9 (also known as Srw1), which is therefore required for nitrogen-starvation-induced G1 arrest and sexual differentiation. Ste9 is regulated by posttranslational modification (Blanco et al., 2000; Yamaguchi et al., 2000) and mRNA decay (Álvarez et al., 2006), but little is known about the regulation of *ste9*$^{+}$ transcription in response to nutritional stress.

Here, we report that, in *S. pombe*, the Myb-type DNA-binding protein Reb1 regulates transcription of *ste9*$^{+}$ under nitrogen starvation. *S. pombe* Reb1 was originally found to be involved in the termination of rRNA transcription by binding to two identical 17-bp DNA sequences near the 3′ end of the rDNA coding region (Zhao et al., 1997). More recently, it was shown that binding of Reb1 to these two sequences also blocks DNA replication, giving rise to two natural rDNA replication fork barriers (RFBs) (Sánchez-Gorostiaga et al., 2004). These natural RFBs are highly conserved in eukaryotes, and they function in a unidirectional manner, stalling only replication forks moving in the direction opposite to that of transcription (López-Estraño et al., 1997; Mirkin and Mirkin, 2007). Therefore, these polar RFBs prevent head-on collision between the transcription and replication machineries.

The orthologous Reb1 protein from budding yeast is also involved in termination of rRNA transcription. In addition, *Saccharomyces cerevisiae* Reb1 regulates transcription of several RNA-polymerase-II-transcribed genes by binding to their promoter regions [Angermayr et al. (Angermayr et al., 2003) and references therein] and acts also as an enhancer for rRNA transcription (Morrow et al., 1993). In mammals, as in the fission yeast, the Reb1 ortholog protein TTF1 is one of the trans-acting factors for termination of rRNA transcription (Jansa et al., 2001) and is also involved in rDNA replication fork arrest (Gerber et al., 1997; López-Estraño et al., 1998). TTF1 regulates rRNA transcription by binding to one cognate site placed immediately upstream of the rDNA promoter (Langst et al., 1997; Strohner et al., 2004).

Here, we show that transcription of *S. pombe ste9*$^{+}$ is upregulated upon nitrogen deprivation. Reb1 binds in vivo and in vitro to a short DNA sequence upstream of *ste9*$^{+}$ and is required for *ste9*$^{+}$ upregulation and G1 arrest in response to nitrogen starvation. Consequently, the mating efficiency of cells lacking Reb1 is significantly reduced. Interestingly, Reb1 binds *in vivo* to the

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promoter of *ste9*$^{+}$ (termed 'Pste9') both in cells growing exponentially in complete medium as well as after nitrogen deprivation. However, our results indicate that other proteins associate with the Reb1–Pste9 complex in exponentially growing cells, when *ste9*$^{+}$ expression is low. Upon nitrogen deprivation, these proteins dissociate from the Reb1–Pste9 complex and *ste9*$^{+}$ is overexpressed.

Results

Cells lacking Reb1 are defective in nitrogen starvation-induced G1 arrest and mating

In the course of genetic crosses performed using *S. pombe reb1*Δ strains, we noticed that fertility of cells lacking Reb1 was significantly reduced. The mating efficiency of *reb1*Δ cells, expressed as the percentage of cells undergoing mating, was approximately fourfold lower compared with that of otherwise-wild-type isogenic cells after 24 hours of mating and approximately twofold lower after 48 hours (Fig. 1A, black and grey columns).

The orthologous Reb1 protein in the budding yeast *S. cerevisiae* regulates the expression of several genes transcribed by RNA polymerase II. Therefore, we reasoned that *S. pombe* Reb1 could be involved in the regulation of some gene(s) required for efficient mating. In an attempt to identify candidate genes, we looked for putative Reb1-binding sites by BLAST-searching the nucleotide sequence recognized by Reb1 at the rDNA in other locations of the *S. pombe* genome. The highest score obtained corresponded to a 27-bp sequence located at the promoter region of gene *ste9*$^{+}$ (*srw1*$^{+}$), covering nucleotide positions –198 through –172 relative to the 5′-end of the coding sequence (Fig. 1B).

Ste9 is a WD-repeat Fizzy-related protein that activates the APC, thereby promoting the ubiquitylation and degradation of mitotic cyclins specifically during the end of mitosis and G1 phase (Blanco et al., 2000; Kitamura et al., 1998; Yamaguchi et al., 1997; Yamaguchi et al., 2000). Thus, Ste9 is required for an efficient G1 arrest of cells undergoing sexual differentiation in response to nutritional stress, such as nitrogen deprivation. The finding of a putative Reb1-binding site at the promoter of *ste9*$^{+}$ raised the hypothesis that Reb1 could be a positive regulator of the expression of Ste9, such that it would collaborate in the arrest of cells in G1, which is required for conjugation and, ultimately, sexual differentiation. To address this hypothesis, we used flow cytometry

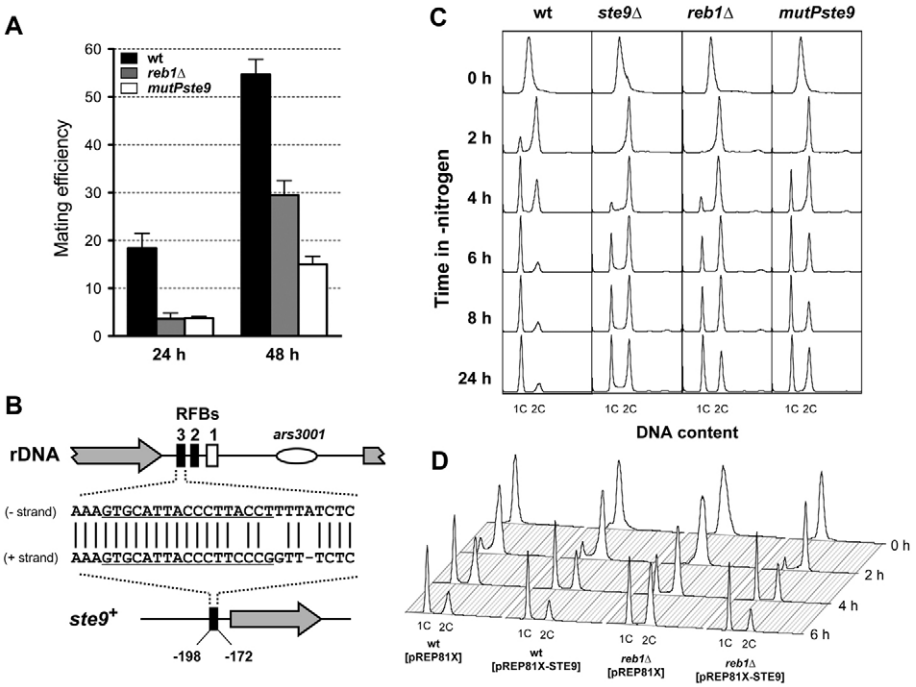

Fig. 1. Reb1 is required for efficient nitrogen-starvation-induced G1 arrest and mating. (**A**) Wild-type (wt; 117 h^{-} and 118 h^{+}), *reb1*Δ (D7 h^{-} and D9 h^{+}) and *mutPste9* (LR4Ph– and LR4Ph+) *S. pombe* strains were grown separately and equal numbers of cells were mixed and spotted on sporulating MEA plates. Wild-type mating: 117 h^{-} × 118 h^{+}; *reb1*Δ mating: D7 h^{-} × D9 h^{+}; *mutPste9* mating: LR4Ph– × LR4Ph+. After 24 and 48 hours at 25°C, mating efficiency was calculated as: [2×(number of zygotes and asci formed)]/[2×(number of zygotes and asci formed)+number of non-mating cells], expressed as a percentage. (**B**) The upper diagram represents the *S. pombe* rDNA nontranscribed spacer, containing replication fork barriers 1 through 3 (RFB1–RBF3). The ability to function of RFB2 and RFB3 (filled boxes) depends on Reb1 binding to two identical 17-bp sequences, whereas RFB1 (open box) is Reb1 independent and requires the protein Sap1. An alignment of the nucleotide sequence from RFB3 (the strongest of the two Reb1-dependent barriers) with the promoter region of the *ste9*$^{+}$ gene is shown in the middle. Underlined nucleotides correspond to the 17-bp Reb1-binding sequence (Zhao et al., 1997), sufficient for replication fork arrest activity (Sánchez-Gorostiaga et al., 2004). In the lower diagram, the location of the sequence upstream of *ste9*$^{+}$ similar to that of RFB3 is indicated. Numbers are relative to the translation start codon. (**C**) Fluorescence-activated cell sorting (FACS) analysis of cells cultured under conditions of nitrogen starvation. Wild-type (972), *reb1*Δ (D9P), *ste9*Δ (LR3) and *mutPste9* (LR4Ph–) prototrophic strains were grown in complete minimal medium (EMM), washed and cultured at 25°C in EMM without nitrogen (EMM–N) for the indicated times. (**D**) Deficiency of *reb1*Δ cells to arrest in G1 phase upon nitrogen deprivation is rescued by expression of *ste9*$^{+}$ under the *nmt1* promoter. Wild-type (MR021) and *reb1*Δ (MR025) cells transformed with empty pREP81X expression vector or expressing *ste9*$^{+}$ (pREP81X-Ste9) were grown to midlog phase in complete EMM with thiamine. Thereafter, cells were cultured at 25°C in EMM–N without thiamine for the indicated times, and the DNA content was determined by flow cytometry.

to analyze the response of *reb1*Δ cells to nitrogen starvation compared with those of *ste9*Δ and wild-type isogenic strains. In agreement with the hypothesis, *reb1*Δ cells were deficient in nitrogen-deprivation-induced G1 arrest, as for *ste9*Δ cells (Fig. 1C, three left-hand columns). To study the relevance of the putative 27-bp Reb1-binding sequence at the *ste9*$^{+}$ promoter for the appropriate cellular response to nutritional stress, we constructed a new strain identical to the wild-type strain where these 27 nucleotides were substituted by unrelated ones (A by C, G by T, and vice versa). As shown in Fig. 1C (right-hand column labeled *mutPste9*), this modification of the *ste9*$^{+}$ promoter also rendered cells deficient in G1 arrest. Moreover, the mating efficiency of *mutPste9* cells was also reduced (Fig. 1A, white columns).

Expression of Ste9 under the control of the *nmt1* promoter reverts *reb1*Δ phenotype

The results described above suggested that the deficiency of *reb1*Δ cells with respect to arrest in G1 could be due to a possible role of Reb1 in promoting Ste9 expression upon nitrogen starvation. If this hypothesis were correct, we would expect that expression of Ste9 under the *nmt1* promoter should facilitate cell cycle arrest in G1 phase in *reb1*Δ cells. To verify the latter, we analyzed *reb1*Δ cells transformed with the pREP81X expression vector that was either empty or containing the *ste9*$^{+}$ gene (pREP81X-Ste9, kindly provided by S. Moreno (Instituto de Biología Molecular y Celular del Cáncer, CSIC-USAL, Salamanca, Spain). While the empty plasmid had no effect on the deficiency of *reb1*Δ cells with respect to arrest in G1 (Fig. 1D, third histogram series), pREP81X-Ste9 efficiently reverted this defect, and a percentage of cells similar to that observed in the wild-type strain stalled with a 1C DNA content (Fig. 1D, right-hand histogram series).

reb1 and *wee1* mutations are synthetically lethal

Mitosis inhibitor protein kinase Wee1 negatively regulates the G2-to-M transition by inactivating Cdc2. Cells lacking Wee1 are viable, but mitosis takes place prematurely, and consequently approximately half-sized cells are generated after mitosis (Russell and Nurse, 1987). To survive, the G1 phase of these cells needs to be prolonged so that they can reach the minimum size required to pass Start. To examine whether Reb1 plays a role in this G1 lengthening, we used the thermosensitive *wee1-50* allele in combination with the *reb1*Δ mutation. While *reb1*Δ and *wee1-50* single mutants grew at all temperatures tested, these two mutations were synthetically lethal as growth temperature increased (Fig. 2A, upper panels). This means that Reb1 becomes essential in the absence of Wee1 function. Also, we analyzed the DNA content of cell cultures at different times after shifting them from the permissive temperature to 32°C or 35°C (Fig. 2B). A peak of 1C cells progressively appeared in the *wee1-50* single mutant and became more prominent at 35°C than at 32°C (Fig. 2B, arrowheads). This indicates that, in a number of cells, the G1 phase was lengthened enough for the cytokinesis to take place before they entered into the S phase. This 1C peak was absent in the double mutant, and an additional wider peak corresponding to higher DNA content appeared. These results indicate that *wee1-50* mutant cells lacking Reb1 were unable to lengthen G1 and

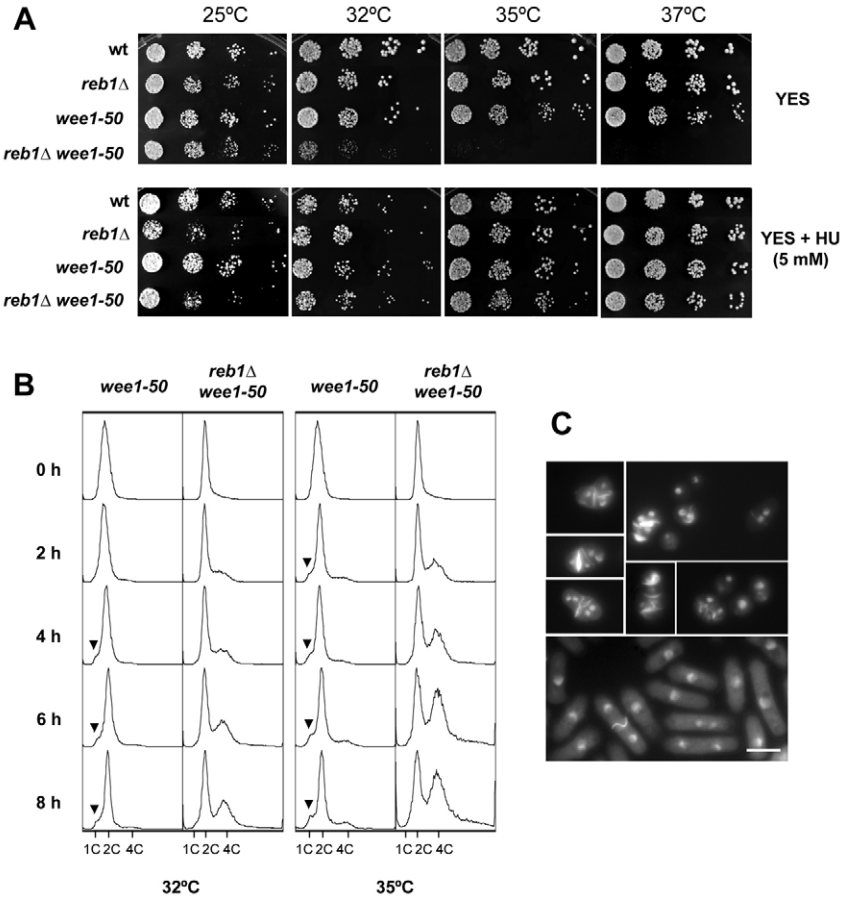

Fig. 2. *reb1Δ* and *wee1-50* mutations are synthetically lethal and deficient in G1 phase lengthening. (**A**) Serial dilutions of exponential cultures of strains wt (972), *reb1*Δ (D9P), *wee1-50* (MR011) and *reb1*Δ *wee1-50* (MR014) growing at 25°C were spotted on YES or YES+HU plates and grown at the indicated temperatures. (**B**) DNA content of the same strains used in A grown in YES at 25°C to midlog phase and shifted to 32°C or 35°C for the indicated times. (**C**) Phenotype of h^{-} *reb1*Δ *wee1-50* cells cultured at 35°C for 6 hours and stained with DAPI and calcofluor. Wild-type cells are shown at the bottom (scale bar: 5 μm).

consequently entered prematurely into S phase. As they complete replication, these cells enter mitosis also prematurely owing to the *wee1-50* mutation. Reiteration of these premature G1–S and G2–M transitions leads to cell lethality. Consistent with this interpretation, double-mutant cells cultured for 6 hours at the restrictive temperature showed up to four nuclei and multiple septa, indicating that cells undergo mitosis before cytokinesis was completed (Fig. 2C). It is likely that completion of cytokinesis was compromised owing to the small size and round shape of these *reb1*Δ *wee1-50* cells. As a consequence, cells with a DNA content higher that 2C were generated (Fig. 2B).

Finally, to test whether premature DNA replication after mitosis was causing synthetic lethality of *reb1*Δ *wee1-50* cells, we used a low concentration of the inhibitor of DNA synthesis hydroxyurea. This treatment allowed the growth of the double mutant at all temperatures tested (Fig. 2A, lower panels).

cdc10-129 cells lacking Reb1 are defective in G1 arrest and are nonviable at a semipermissive temperature

The expression of several genes coding for proteins required for the initiation of DNA replication is regulated by the transcription factor MBF (also known as DSC1) (Bahler, 2005). Cdc10 protein is an essential component of the MBF complex and, as a consequence, its inactivation leads to cell cycle arrest in G1 phase. We found that *reb1*Δ cells bearing the thermosensitive *cdc10-129* allele were nonviable at the semipermissive temperature of 32°C, whereas single mutants formed colonies (Fig. 3A). At 30°C, the double mutant also exhibited reduced growth. As shown in the flow cytometry analysis of Fig. 3B (upper histograms), 4, 6 and 8 hours after the *cdc10-129* single mutant was shifted from 25°C to 30°C, a small percentage of cells with 1C DNA content was generated, indicating that, at this temperature, G1 phase lengthened enough for some cells to undergo cytokinesis before initiating S phase. The peak of 1C cells was absent in the *reb1*Δ *cdc10-129* double mutant. This effect was more evident when cells were shifted to a higher temperature. After 4 hours at 32°C, a high percentage of *cdc10-129* cells showed a 1C DNA content, whereas only a very small 1C peak was visible in the double mutant at all times analyzed (Fig. 3B, middle histograms). This indicates that Reb1 is required for an efficient G1 arrest when the activity of Cdc10 is diminished, as previously observed for the *ste9*Δ *cdc10-129* mutant (Kitamura et al., 1998).

Cdc10-129 is a leaky allele, and therefore *cdc10-129* cells escape from G1 arrest when they are maintained long enough at the shifted temperature. As a consequence, the 1C peak observed in *cdc10-129* cells 4 hours after the shift to 32°C diminished at 6 and 8 hours, whereas the peak corresponding to 2C cells increased. This was not observed in the double mutant as no significant accumulation of cells with a 1C DNA content took place in this strain. Our interpretation is that leaking of *cdc10-129* cells from G1 arrest is exacerbated in the absence of Reb1, which becomes deleterious. We have not observed a significant number of *cut* cells in this double mutant.

The DNA content profiles of *cdc10-129* and *reb1*Δ *cdc10-129* strains at 37°C were also quite different (Fig. 3B, lower histograms). Two hours after the shift, only a low fraction of the double-mutant cells arrested at G1 compared with *cdc10-129* cells. After 4 hours, the temperature shift time routinely used in cell synchronization experiments, most of the *cdc10-129* cells were in G1 phase, whereas the profile observed in the double mutant consisted of either a wide peak of cells with a more heterogeneous DNA content or two almost-fused peaks. As observed at 32°C, leaking from G1 arrest was also detected when *cdc10-129* cells were maintained at 37°C for longer than 4 hours. Double-mutant cells, however, showed a heterogeneous distribution of DNA content after 8 hours at 37°C.

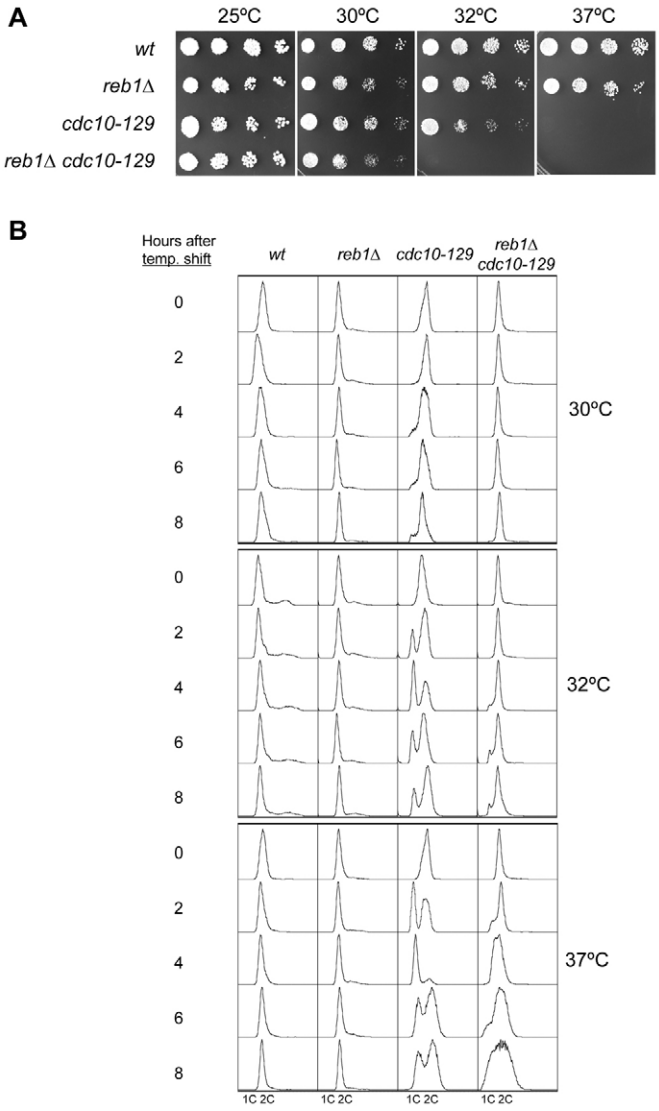

Fig. 3. *reb1Δ cdc10-129* double mutants are nonviable at semipermissive temperature and defective in G1 arrest. (**A**) Serial dilutions of exponential cultures of strains wild-type (wt, 972), *reb1*Δ (D9P), *cdc10-129* (MR030) and *reb1*Δ *cdc10-129* (MR018) grown in YES at 25°C were spotted on YES plates and grown at the indicated temperatures. (**B**) Flow cytometry analysis of the same strains used in A grown in YES to midlog phase at 25°C and shifted to 30°C, 32°C or 37°C for the indicated times.

Reb1 is required for *ste9*$^{+}$ upregulation induced by nitrogen deprivation

The presence of a putative Reb1-binding sequence upstream of *ste9*$^{+}$, together with the requirement for Reb1 for nitrogen-deprivation-induced G1 arrest, suggested that Reb1 might regulate *ste9*$^{+}$ expression when nitrogen was limiting. To test the latter, wild-type and *reb1*Δ log-phase cells were transferred to nitrogen-free medium and the expression of *ste9*$^{+}$ was monitored at different times by northern blotting.

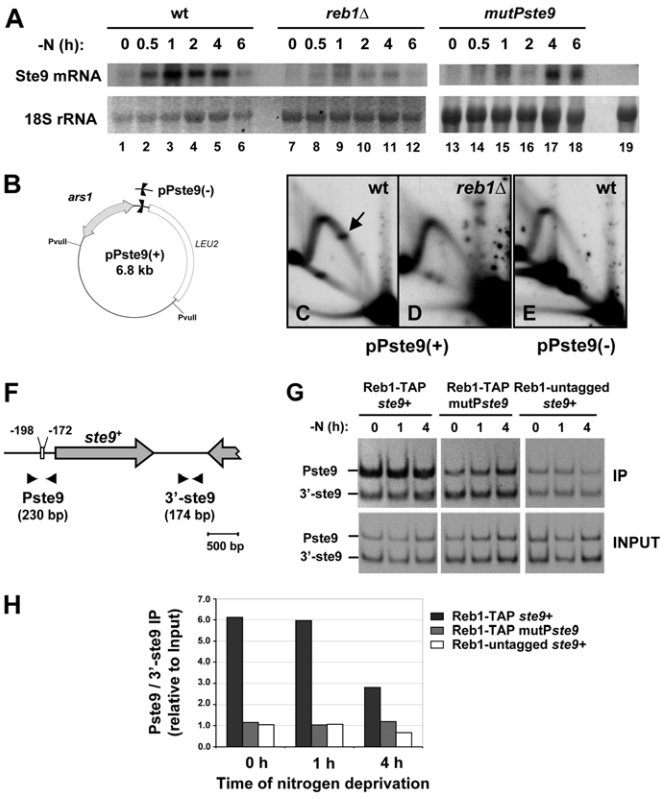

Fig. 4. Reb1 is required for *ste9*$^{+}$ transcriptional activation induced by nitrogen deprivation and binds in vivo to the *ste9*$^{+}$ promoter. (**A**) Wild-type (wt; 972), *reb1*Δ (D9P) and *mutPste9* (LR4Ph–) prototrophic cells were grown in EMM to midlog phase, washed and cultured in EMM–N at 25°C for the indicated times. RNA was extracted and mRNA encoding Ste9 was analyzed by northern blotting using a radiolabeled specific probe. A *ste9*Δ*::ura4* strain (LR3) grown for 1 hour without nitrogen was used as a negative control (lane 19). 18S rRNA was used as loading control. (**B**) Binding of Reb1 to the promoter of *ste9*$^{+}$ arrests the replication fork in a polar manner. Shown is a map of the autonomously replicating plasmids analyzed by 2-D electrophoresis in C–E. A 230-bp fragment from the promoter of *ste9*$^{+}$ (indicated as Pste9 in F) was inserted in both orientations at the indicated location of vector pIRT2, resulting in plasmids pPste9(+) and pPste9(–). (**C**–**E**) Replication analyses of the large *Pvu*II restriction fragment of the plasmids shown in B. The arrow in panel C points to the signal corresponding to the specific Y-shaped replication intermediate accumulated as a consequence of fork stalling at the Pste9+ insert. Note that this fragment corresponding to the promoter of *ste9*$^{+}$ induced fork arrest in a wild-type strain (118) (panel C) but not in an isogenic *reb1*Δ strain (D9) (panel D). This Reb1-mediated fork arrest was unidirectional as the replication fork did not suffer impediment when Pste9 was cloned in the (–) orientation (panel E). (**F**) Location of the primer pairs used for PCR amplification of immunoprecipitated chromatin. The size of each amplified product is indicated in parentheses. (**G**) Strains ZG023 (*reb1-2xTAP:KanMX6 ste9*$^{+}$), ZGLR002 (*reb1-2xTAP:KanMX6 mutPste9*) and 972 (wt) were grown to midlog phase in complete EMM, washed and cultured in EMM–N at 25°C. Before nitrogen deprivation (0 hours) and after the indicated times of starvation, cells were crosslinked with formaldehyde, chromatin was sheared, a sample was taken as control ('INPUT') and Reb1–TAP was immunoprecipitated (IP) using IgG–sepharose resin. DNAs from IP and INPUT chromatin were amplified by duplex PCR using both primer sets indicated in F to determine simultaneously the enrichment of the *ste9*$^{+}$ promoter (Pste9 product) and a region downstream of *ste9*$^{+}$ (3′-ste9 product). (**H**) Quantification of the relative enrichment of *ste9*$^{+}$ promoter, calculated by dividing the relative intensity of Pste9 and 3′-ste9 PCR products from IP chromatin and from INPUT chromatin. Band intensity was determined using ImageJ software.

Wild-type cells growing exponentially in nitrogen-containing medium showed a low level of mRNA encoding Ste9 (Fig. 4A, lane 1). The expression of Ste9, however, was rapidly upregulated after nitrogen deprivation, and the level of Ste9 mRNA reached a maximum after ~1 hour of nutritional stress (Fig. 4A, lanes 2–5). After 6 hours of starvation, Ste9 mRNA declined back to a level similar to that found in cells growing in complete medium (Fig. 4A, lane 6). This wave of Ste9 overexpression induced by nitrogen starvation was not observed in *reb1*Δ cells (Fig. 4A, lanes 7–12), in agreement with their failure to arrest in G1. These results indicate that Reb1 is required for the overexpression of Ste9 leading to G1 arrest, a prerequisite for conjugation and sexual differentiation.

To study the relevance of the putative 27-bp Reb1-binding sequence present at the *ste9*$^{+}$ promoter for the overexpression of Ste9, the isogenic strain examined in Fig. 1A and Fig. 1C (mutPste9) was also analyzed by northern blotting (Fig. 4A, lanes 13–18). The rapid overexpression of Ste9 observed in wild-type cells upon starvation did not take place when this 27-bp sequence was mutated (lanes 13–16). Only after 4 hours of starvation did Ste9 mRNA increase somewhat (lanes 17 and 18).

Reb1 binds to the promoter sequence of *ste9*$^{+}$ and causes replication fork stalling

As mentioned in the Introduction, Reb1 has two DNA-binding sequences specific for the non-transcribed spacer of each rDNA repeat, close to the 3′ end of the coding region. We have previously shown that Reb1 binding to these two sites induces replication fork arrest in a polar manner, so that only forks moving in the anti-transcription direction are stalled (Sánchez-Gorostiaga et al., 2004). Owing to the similarity of these two rDNA sequences and the 27-bp sequence present at the *ste9*$^{+}$ promoter, we expected that, if Reb1 binds to this sequence in vivo, it might also cause replication fork stalling upstream of *ste9*$^{+}$. We used two-dimensional (2-D) agarose gel electrophoresis to analyze whether the DNA upstream of *ste9*$^{+}$ is able to arrest replication forks when inserted into an autonomously replicating plasmid and whether this fork arrest is abolished in cells lacking Reb1. The use of this in vivo replication plasmid assay permitted us to verify whether fork arrest occurs in a polar manner, as in the case of Reb1-induced rDNA fork barriers RFB2 and RFB3. We have previously demonstrated that these two rDNA barriers show a behavior, when inserted into a replicating plasmid, identical to that displayed in their chromosomal context (Sánchez-Gorostiaga et al., 2004).

As diagrammed in Fig. 4B, a 230-bp fragment containing the putative Reb1-binding site (Pste9) from the promoter of *ste9*$^{+}$ was inserted in both orientations next to the replication origin *ars1* of plasmid pIRT2. The resultant plasmids, pPste9(+) and pPste9(–), were introduced into wild-type and *reb1Δ* strains, isolated from log-phase cells and digested with the restriction enzyme *Pvu*II. Finally, replication of the large *Pvu*II fragment containing the insert was analyzed on 2-D gels. Plasmid pPste9(+) replicating in wild-type cells showed a strong hybridization signal at the expected position on the arc of Y-shaped replication intermediates (Fig. 4C, arrow), indicating that fork arrest at Pste9 took place. By contrast, this signal, corresponding to arrested forks, completely vanished in *reb1*Δ cells (Fig. 4D), indicating that Reb1 binds to Pste9 sequences in vivo and induces replication fork stalling. Moreover, as in the rDNA, this activity was polar as we did not detect the signal corresponding to arrested forks

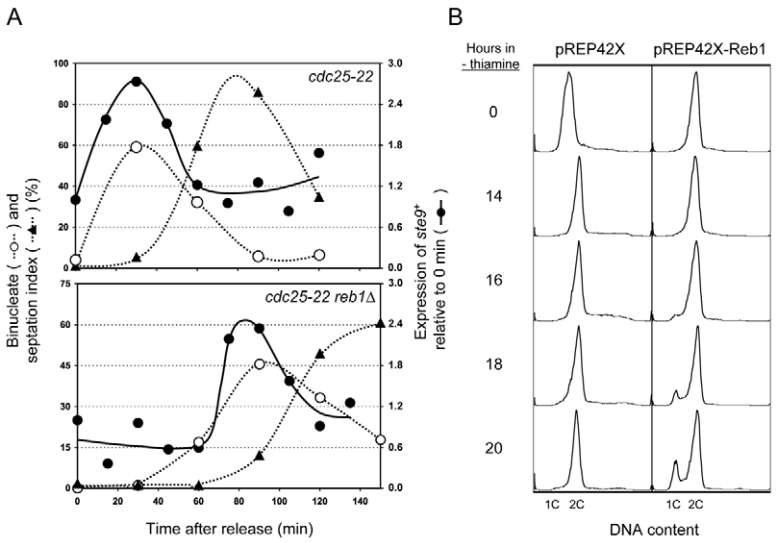

Fig. 5. (**A**) Periodic expression of *ste9*$^{+}$ occurs in the absence of Reb1. Cells from *cdc25-22* and *cdc25-22 reb1*Δ (MR006) strains were synchronized at late G2 phase by incubation at 36°C for 4 hours and released from the blockage at 25°C for the indicated times. The relative level of *ste9*$^{+}$ mRNA was measured by RT-qPCR using *cdc2* mRNA as an internal reference gene (filled circles, right-hand ordinates). To monitor the release from the arrest, the percentage of binucleate cells (open circles, left-hand ordinates) and binucleate cells containing septa (triangles, left-hand ordinates; 'septation index') were scored by DAPI and calcofluor staining. (**B**) Overexpression of *reb1*$^{+}$ causes the accumulation of cells with a 1C DNA content. Shown is a flow cytometry analysis of MR003 cells transformed with pREP42X or pREP42X-Reb1 and cultured for the indicated times in thiamine-free medium.

when Pste9 was inserted in the (–) orientation in pPste9(–) (Fig. 4E).

Reb1 is bound to the promoter of *ste9*$^{+}$ in exponentially growing cells and remains bound upon nitrogen deprivation

We also analyzed binding of Reb1 to the promoter of *ste9*$^{+}$ in vivo by chromatin immunoprecipitation (ChIP) assays. We fused Reb1 to the tandem affinity purification (TAP) tag and used IgG–sepharose to immunoprecipitate chromatin crosslinked to Reb1–TAP. Chromatin was prepared from three otherwise-isogenic strains: a Reb1–TAP strain bearing wild-type *ste9*$^{+}$ gene, a Reb1–TAP strain bearing the *mutPste9* gene described above and, as a control, a strain with the wild-type untagged *reb1*$^{+}$ and *ste9*$^{+}$ genes. Chromatin was prepared from exponentially growing cells and after 1 and 4 hours of nitrogen starvation. As illustrated in Fig. 4F, amplification of the immunoprecipitated chromatin was performed by duplex PCR using two pairs of oligonucleotides targeted to the promoter of *ste9*$^{+}$ and to a region downstream of *ste9*$^{+}$. The co-amplified products, separated by 2.2 kb, were 230 (Pste9) and 174 bp (3′-ste9) long, respectively.

The promoter region of *ste9*$^{+}$ was significantly enriched (about sixfold) in the immunoprecipitated chromatin from exponentially growing Reb1–TAP *ste9*$^{+}$ cells (Fig. 4G, left gel, 0 hours in '–N', quantified in Fig. 4H). Moreover, the enrichment of Pste9 was maintained after 1 and 4 hours of nitrogen starvation (Fig. 4G, left gels; Fig. 4H, dark-gray bars). No enrichment was observed in the control Reb1-untagged *ste9*$^{+}$ strain (Fig. 4G, right gels; Fig. 4H, white bars). By contrast, the *ste9*$^{+}$ promoter was not enriched when the 27-bp putative Reb1-binding sequence was mutated in the Reb1–TAP mutPste9 strain, neither before nor after nitrogen deprivation (Fig. 4G, middle gels; Fig. 4H, pale-gray bars). These results indicate that the Reb1 protein binds to the promoter of *ste9*$^{+}$ and that the 27-bp sequence upstream of Ste9 is required for this binding. They also indicate that Reb1 binding to the promoter of *ste9*$^{+}$ was not triggered by nitrogen starvation as Reb1 was bound to the promoter before transferring the cells to nitrogen-free medium.

It has been reported that *ste9*$^{+}$ expression peaks in G1 phase, in agreement with its role in the regulation of this phase of the cell cycle (Peng et al., 2005; Rustici et al., 2004; Tournier and Millar, 2000). Our observation that Reb1 is bound to the *ste9*$^{+}$ promoter in exponentially growing cells raised the possibility that Reb1 could be involved in the periodic expression of *ste9*$^{+}$ during a normal mitotic cell cycle. To address this issue, we used a *cdc25-22* block at the restrictive temperature to synchronize wild-type and *reb1*Δ strains in late G2 phase and analyzed the *ste9*$^{+}$ mRNA by retrotranscription real-time quantitative PCR (RT-qPCR) at different times after their release at the permissive temperature. The percentage of binucleate cells and the septation index were also measured by DAPI and calcofluor staining to monitor synchronization (Fig. 5A). As deduced from the time of appearance of binucleate cells after the release, *reb1*Δ cells showed a delay of ~50 minutes until they completed mitosis (Fig. 5A, bottom panel), compared with wild-type cells (Fig. 5A, upper panel). As in wild-type cells, however, expression of *ste9*$^{+}$ was upregulated in *reb1*Δ cells while they entered G1 phase and declined later, just before the wave of septation. These results indicate that Reb1 is not required for the periodic expression of *ste9*$^{+}$ in a normal cell cycle.

The effect of *reb1*$^{+}$ overexpression was also studied. *reb1*$^{+}$*-HA* was cloned in the pREP24X expression vector under the control of the thiamine-repressible promoter Pnmt1, and the resultant plasmid pREP24X-Reb1 was used to transform strain MR003 (*reb1*Δ::*kanMX6 ura4-D18*). The highest expression level of *reb1*$^{+}$ was observed 16 hours after cell incubation in the absence of thiamine (data not shown). At the same time-point, expression of *ste9*$^{+}$ was 3.24-fold (s.d.=0.65) the level observed before overexpression of *reb1*$^{+}$, determined by RT-qPCR. As shown in the flow cytometry data of Fig. 5B, overexpression of *reb1*$^{+}$ caused the progressive accumulation of cells with a 1C DNA content, which represented ~20% of the culture after 20 hours of activation. Significant diploidization, resulting in cells with DNA contents higher than 2C, was not observed.

Additional proteins interact with the Reb1p–Pste9 complex in a Reb1-dependent manner and dissociate upon nitrogen deprivation

Altogether, our results show that Reb1 is required for the overexpression of *ste9*$^{+}$ that leads to G1 arrest under nitrogen starvation. For this upregulation, Reb1 needs to be bound to the 27-bp upstream-activating sequence at the promoter region of *ste9*$^{+}$. However, as shown above, Reb1 is already bound to the

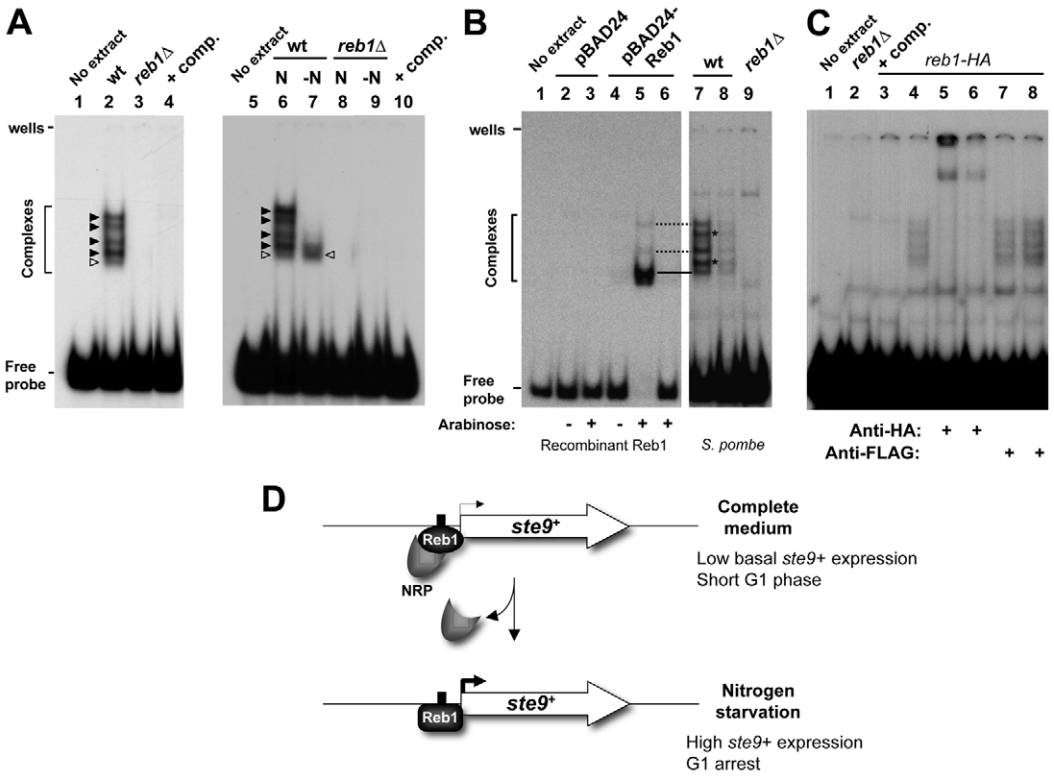

Fig. 6. Several Reb1-dependent DNA–protein complexes are formed at the promoter of *ste9*+, and most of them dissociate upon nitrogen starvation. (**A**) Electrophoretic mobility-shift assay (EMSA) analysis using protein extracts from wild-type (wt; 117) and *reb1*Δ (D7) strains. The nucleotide sequence from –198 to –160 positions of the *ste9*+ promoter was used as a probe for all assays shown in this figure. Cells were grown exponentially in rich medium (lanes 1–4) or EMM (lanes 5–10). The latter cultures were grown for 1 additional hour in the same medium (lanes 6, 8 and 10) or in the same medium without nitrogen source (lanes 7 and 9). In lanes 4 and 10, a 200-fold excess of unlabelled probe was added to the binding reaction as a specific competitor, and proteins from wild-type cells were used. (**B**) EMSA analysis of Reb1 expressed in *E. coli*. Proteins from TOP10 *E. coli* cells transformed with the empty expression vector pBAD24 (lanes 2 and 3), or pBAD24-Reb1 (lanes 4–6), containing the *reb1*+ gene, were analyzed. To induce the *araBAD* promoter, cells were incubated for 2 hours in medium containing L-arabinose (lanes 3, 5 and 6). To verify the specificity of binding, an excess of unlabeled probe was added to the reaction in lane 6. To compare the mobility of the complexes, EMSA analysis of protein extracts from wild-type (wt; lanes 7 and 8) and *reb1*Δ (lane 9) *S. pombe* cells were run in the same gel. In lane 8, a twofold excess of unlabeled probe was added to the binding reaction. (**C**) EMSA supershift using protein extracts from strain MR020 (*reb1-3HA*) (lanes 3–8). The reaction mixture was incubated with a monoclonal antibody against HA (lanes 5 and 6) or against FLAG (lanes 7 and 8). This incubation was performed before (lanes 5 and 7) or after (lanes 6 and 8) addition of the probe. In lanes 3 and 4, binding reactions took place without antibody, and, in lane 3, an excess of unlabeled probe was present. (**D**) Model for the upregulation of *ste9*+ transcription upon nitrogen starvation. See the Discussion section for further details.

promoter of *ste9*+ in exponentially growing cells. Therefore, binding of Reb1 to the promoter is required but not sufficient for upregulation of Ste9. What does it ultimately take for *ste9*+ to be overexpressed upon starvation? We hypothesized that additional protein(s) might be involved in the trans-activation of *ste9*+ by interacting with the Reb1–Pste9 complex. To detect these possible DNA–proteins complexes, we carried out electrophoretic mobility-shift assays (EMSAs), using as target the DNA sequence upstream of *ste9*+ from position –198 through –160 containing the 27-bp Reb1-binding site. We first analyzed complexes formed using protein extracts from wild-type and *reb1*Δ cells growing exponentially in rich medium (Fig. 6A, lanes 2–4). Using a wild-type extract, five retarded complexes were visible (arrowheads). Interestingly, formation of all these complexes required Reb1 as none of them appeared when *reb1*Δ extracts were used (Fig. 6A, lane 3). Next, we analyzed complexes formed using proteins from cells growing exponentially in minimal medium containing a nitrogen source (Fig. 6A, lanes 6 and 8) or after 1 hour of nitrogen starvation (Fig. 6A, lanes 7 and 9). As in rich medium, the same complexes described above were detected using wild-type protein extracts (Fig. 6A, lane 6). However, after 1 hour of starvation, none of these complexes was formed except that showing the highest electrophoretic mobility (Fig. 6A, lane 7, hollow arrowhead). Using *reb1*Δ extracts, no complexes were detected regardless of whether cells were grown with or without a nitrogen source (Fig. 6A, lanes 8 and 9).

One interpretation of these results is that, in exponentially growing cells, when expression of Ste9 is low (Fig. 4A, lane 1), other proteins interact with this region of the *ste9*+ promoter (Fig. 6A, lanes 2 and 6) in a Reb1-dependent manner (Fig. 6A, lanes 3 and 8). After 1 hour of nitrogen starvation, when expression of Ste9 is high (Fig. 4A, lane 3), the affinity of these proteins for the promoter is lost and Reb1 is mostly the only protein that remains bound (Fig. 6A, lane 7). This suggests that Reb1 acts as a transcription factor that is capable of inducing expression of Ste9. However, this activity would be inhibited by other negative trans-acting factors in exponentially growing cells in the absence of nutritional stress. Upon nitrogen deprivation, the affinity of these negative trans-acting factors is lost, and then Reb1 mediates Ste9 overexpression, leading to G1 arrest.

This interpretation predicts that some of the DNA–protein complexes formed with *S. pombe* extracts should not form using Reb1 expressed in a heterologous system as the *S. pombe* negative trans-acting factors would be absent. Another prediction is that Reb1 should be present in all the DNA–protein complexes observed.

To verify the first prediction, *reb1*$^+$ cDNA was obtained by RT-PCR and cloned into the *Escherichia coli* expression vector pBAD24, under the control of the arabinose-inducible *araBAD* promoter (Mejía-Ramírez et al., 2005). EMSAs were performed using the same DNA probe as in Fig. 6A and proteins from the *E. coli* strain TOP10 transformed with either the empty vector pBAD24 (Fig. 6B, lanes 2 and 3) or the recombinant plasmid pBAD24-Reb1 (Fig. 6B, lanes 4–6). Only proteins from bacteria transformed with pBAD24-Reb1 and incubated in the presence of arabinose gave rise to the formation of retarded complexes. The specificity of the complexes was verified by the addition of an excess of unlabelled probe to the binding reaction (Fig. 6B, lane 6). The recombinant Reb1 protein expressed in *E. coli* formed one major complex, and its electrophoretic mobility was similar to that of the *S. pombe* complex that showed the highest mobility (Fig. 6B, lane 7, unbroken line), indicating that Reb1 is the only protein present in this *S. pombe* complex. Two faint additional retarded bands showing a higher retardation were observed using Reb1 expressed in *E. coli*. They might correspond to multimeric forms of Reb1 bound to the probe, as has been recently observed using as a probe the DNA sequence of the rDNA replication fork barrier RFB3 (Biswas and Bastia, 2008). The mobility of two of the complexes formed by the *S. pombe* extract coincided with these two faint bands (Fig. 6B, lane 6, broken lines), and therefore they might also be generated by Reb1 multimers. However, other DNA–protein complexes that formed using the *S. pombe* extracts did not form using Reb1 expressed in *E. coli* (Fig. 6B, lane 7, asterisks). This indicates that protein(s) different from Reb1 are present in these complexes.

To verify that Reb1 was present in all the DNA–protein complexes formed, supershift EMSAs were performed using extracts from a Reb1–HA *S. pombe* strain. Addition of monoclonal antibody against HA to the reaction mixture, before (Fig. 6C, lane 5) or after (Fig. 6C, lane 6) the probe, supershifted all the DNA–protein complexes formed in the absence of the antibody (Fig. 6C, lane 4), whereas a monoclonal antibody against FLAG, used as a control, had no effect (Fig. 6C, lanes 7 and 8).

Discussion

Conjugation and sexual differentiation in fission yeast require cell arrest in the G1 phase, which can be induced by nutritional stress such as deprivation of the nitrogen source in the medium. Ste9p is a G1-specific APC/C activator that promotes degradation of B-cyclin at this cell cycle phase, playing a crucial role in nutritional stress-induced G1 arrest. As a consequence, *ste9* mutant cells are unable to exit the vegetative cell cycle from G1 under nitrogen deprivation and are deficient in conjugation and sexual differentiation. The activity of Ste9 is regulated at different levels. During S and G2, Ste9 is targeted for proteolysis by Cdc2–Cdc13-mediated phosphorylation (Blanco et al., 2000; Yamaguchi et al., 2000). Also, Ste9 mRNA decay has been recently shown to downregulate Ste9 during G2, which permits accumulation of Cdc13 and the activation of Cdc2 for the timely entrance of cells into mitosis (Álvarez et al., 2006). Finally, in agreement with its role, *ste9*$^+$ is expressed periodically during G1 (Peng et al., 2005; Rustici et al., 2004; Tournier and Millar, 2000).

In the present study, we found that the sequence specific DNA-binding protein Reb1 is a transcriptional activator of Ste9 that binds in vivo to a specific sequence at its promoter. Null-*reb1* mutant cells do not overexpress *ste9*$^+$ in response to nitrogen deprivation and are unable to arrest in G1 phase. Expression of *ste9*$^+$ under the control of the *nmt1* promoter reversed this G1 arrest deficiency of *reb1*Δ cells. Moreover, mutation of the nucleotide sequence recognized by Reb1 at the promoter of *ste9*$^+$ in otherwise-wild-type cells precludes its binding in vivo, significantly reduces transcriptional upregulation of *ste9*$^+$ in the absence of nitrogen source and renders cells deficient in G1 arrest and mating.

Interestingly, although binding of Reb1 to the *ste9*$^+$ promoter (Pste9) is required for the overexpression of Ste9 induced by nitrogen starvation, ChIP assays showed that Reb1 is already bound to the promoter before cells are deprived of nitrogen (Fig. 4G and H), when expression of *ste9*$^+$ is very low (Fig. 4A, lane 1). Thus, Reb1–Pste9 binding is not triggered by this nutritional stress, although this binding is required for the proper cell response to the stress. These findings made us consider the possibility that other protein factor(s) are also involved. EMSA experiments indicated that other proteins do associate to the Reb1–Pste9 complex in cells growing in nitrogen-containing medium, when expression of *ste9*$^+$ is low, whereas Reb1–Pste9 is nearly the only remaining complex after 1 hour of nitrogen deprivation (Fig. 6A), when overexpression of *ste9*$^+$ reaches the highest level (Fig. 4A, lane 3).

As shown in the model presented in Fig. 6D, these findings suggest that the associated proteins prevent Reb1-mediated transcriptional activation. Soon after nitrogen deprivation, the negative regulation proteins (NRPs) dissociate from the *ste9*$^+$ promoter, and Reb1, which remains bound, is now able to stimulate overexpression. The interaction of the negative transcriptional regulation proteins with the *ste9*$^+$ promoter region must be mediated by Reb1 as, in *reb1Δ* cells, no Pste9–protein complexes formed regardless of whether nitrogen was present in the medium (Fig. 6A), and Reb1 was present in all DNA–protein complexes formed (Fig. 6C). The quick release of negative transcriptional regulation proteins from the promoter upon nutritional stress suggests that it might be prompted by posttranslational modification of some of these proteins or Reb1 leading to loss of protein–protein interactions (Fig. 5B).

We observed that overexpression of *reb1*$^+$ leads to an increase of *ste9*$^+$ expression and to the accumulation of cells with a 1C DNA content. This is probably due to the sequestration of the inhibitory proteins by the overexpressed Reb1, leaving free Reb1 to promote transcriptional activation of *ste9*$^+$.

It was recently shown that Reb1p expressed in *E. coli* binds in vitro as a dimer to the rDNA sequence corresponding to replication fork barrier RFB3 (Biswas and Bastia, 2008). The Reb1 dimerization domain is located within the 146 N-terminal amino acids, whereas the two Myb (SANT) DNA-binding domains are located near the C-terminus. This raises the possibility that the negative transcriptional regulation proteins associate with the Reb1–Pste9 complex through the N-terminus of Reb1 during vegetative growth. Upon nitrogen starvation, modification of this Reb1 protein–protein interacting domain, as part of the cell response to nutritional stress, would lead to the dissociation of the repressing factors.

The mammalian ortholog of Reb1, TTF1, is involved in the regulation of rRNA transcription by binding to a short rDNA sequence adjacent to the promoter. Interestingly, TTF1 also contains an N-terminal oligomerization domain that acts by masking the

DNA-binding capacity of two Myb domains located near the C-terminus (Sander et al., 1996). Supporting the interpretation of our results in *S. pombe*, this N-terminal region of TTF1 interacts directly with the chromatin-remodeling complex NoRC. This enables TTF1 to bind to the rDNA promoter and to repress rRNA transcription (Nemeth et al., 2004; Strohner et al., 2004).

Ste9 is expressed periodically during the G1 phase in cells growing exponentially (Peng et al., 2005; Rustici et al., 2004; Tournier and Millar, 2000). It was suggested that the MBF complex (Mlu cell-cycle-box binding factor) controls this periodic expression in hydroxyurea-synchronized *S. pombe* cells (Tournier and Millar, 2000). MBF regulates the expression of genes encoding proteins required for S phase (Bahler, 2005). Binding of MBF to the promoter of *ste9*$^+$ has not been proved yet, although two putative binding sequences are present at ~670 bp upstream of the ATG (Tournier and Millar, 2000) (475 bp upstream of the Reb1-binding sequence). We found that upregulation of *ste9*$^+$ in G1 phase during a normal mitotic cell cycle occurs in the absence of Reb1. This indicates that Reb1 does not control periodic expression of *ste9*$^+$ under conditions of normal growth but it is responsible for the upregulation of *ste9*$^+$ under conditions of nutritional stress, collaborating with sexual differentiation, and under conditions that require G1 lengthening for survival, such as in *wee1*-deficient cells.

We found that a sequence containing the Reb1-binding site from the promoter of *ste9*$^+$ caused arrest of replication forks in an orientation- and Reb1-dependent manner (Fig. 4C–E). As mentioned before, binding of Reb1 to two sites present near the 3′ end of the coding region of each rDNA repeat induces stalling of replication forks moving in the direction opposite to rRNA transcription, whereas forks moving in the other direction pass by unhindered (Sánchez-Gorostiaga et al., 2004). There is evidence indicating that these highly conserved natural RFBs prevent the deleterious effects of head-on collisions between the transcription and replication machineries (López-Estraño et al., 1998; Olavarrieta et al., 2002; Takeuchi et al., 2003). It is unlikely that the RFB at *ste9*$^+$ plays a similar role as it is located at the promoter region, and the Reb1-binding sequence is oriented so that only replication forks moving in the same direction as transcription of *ste9*$^+$ would be arrested. We speculate that the direction of replication, determined by the Reb1-dependent *Pste9* RFB, might be an important factor for the proper coordination between the transcriptional status of *ste9*$^+$ and the cell cycle. Experiments designed to address this hypothesis are under way. Interestingly, *S. pombe* RTS1, another natural RFB located in the mating-type locus, determines the appropriate direction of replication of this region required for efficient mating-type switching (Dalgaard and Klar, 2001). Rtf1 is a DNA-binding protein responsible for the function of RTS1 that shows a high similarity to Reb1 (Eydmann et al., 2008).

Materials and Methods

Yeast strains and growth conditions

All the *S. pombe* strains used in this work are listed in supplementary material Table S1. Cells were grown in rich medium (YES) or in Edinburgh minimal medium (EMM) with supplements as required (Moreno et al., 1991). For nitrogen starvation, cells were grown in EMM to midlog phase, washed and cultured in NH_4Cl-free EMM (EMM–N) at 25°C for the indicated times. Activation or repression of the *nmt1* promoter was achieved by growing cells in EMM or EMM containing 15 μM thiamine, respectively. For mating efficiency determination, cells to be mated were separately grown to midlog phase in YES medium, and equal numbers of cells were pelleted, washed, mixed and spotted on sporulating MEA plates. After 24 and 48 hours of growth at 25°C, cells were examined under the microscope and mating efficiency was calculated as: [2×(number of zygotes and asci formed)]/[2×(number of zygotes and asci formed)+number of nonmating cells], expressed as a percentage.

Construction of strains

Mutation of the Reb1 DNA-binding sequence at the promoter of *ste9*$^+$, named *mutPste9*, was achieved as follows. First, the complete *ste9*$^+$ ORF of strain S1335 (*h*$^-$ *ura4-d18*) was replaced by *ura4*$^+$ by a one-step PCR-based substitution method, obtaining strain LR3. Next, the *ste9*$^+$ gene was inserted back into strain LR3 by replacing *ura4*$^+$ with a PCR product containing *ste9*$^+$ and the mutation *mutPste9* at its promoter. This PCR product was obtained using oligonucleotides Mut-ste9-Fw (5′-TTCGCTTAAAGTCCAACCCTCGCTACACGCTGTTCAGCCATTTAATTAT-GCCC**GGTACC**GCAAAGGAAATTGGGAGCAGTAGACAATGGTGATTGTGA-AGGATGC-3′) and Mut-ste9-Rv (5′-GATGATGTAGAAGTAGATGG-3′) on DNA from a wild-type *ste9*$^+$ strain. Oligonucleotide Mut-ste9-Fw contained a 27-bp mismatch (underlined) corresponding to the *mutPste* mutation. Recombinant Ura$^-$ colonies were selected in EMM plates supplemented with uracil and containing 5-fluoro-orotic acid (5-FOA). The construction was confirmed by Southern blot (a *Kpn*I restriction site, shown in bold face, was included within the mismatch of Mut-ste9-Fw oligo to facilitate this task), PCR and sequencing. Finally, the resultant strain, LR4, was made a prototroph by crossing with the *ura4*$^+$ strain 975 *h*$^+$, obtaining strains LR4Ph– and LR4Ph+.

C-terminal tagging of Reb1 with TAP was performed by a one-step PCR-based insertion method as described previously (Tasto et al., 2001). The PCR-amplified sequence was obtained from plasmid pKLG1810 (pFA6a-kanMX6-CTAP2, kindly provided by K. Gould (Tasto et al., 2001), using 100-nucleotide-long primers TagRebFwd and TagRebRev (sequence available upon request). The same primers were used for C-terminal tagging of Reb1 with HA, using plasmid pFA6a-3HA-hphMX6 as a substrate (kindly provided by T. Toda) (Sato et al., 2005). Strain selection was confirmed by PCR, sequencing and western blotting. Tagging did not affect Reb1 function as cells responded to nitrogen starvation in the same way as wild-type cells and rDNA replication fork barriers were fully functional.

Overexpression of Reb1 was achieved by cloning *reb1-HA* into the pREP42X vector. The insert was obtained by PCR from strain MR020 and cloned at the *Xho*I site of pREP42X. The expressed Reb1–HA protein was functional as it reverted the *reb1*Δ phenotype of the MR003 host strain.

Chromatin immunoprecipitation

ChIP assays were performed as described previously (Pidoux et al., 2004), with few modifications. The immunoprecipitated (IP) and 'INPUT' DNA were amplified by duplex PCR using two pairs of primers in the same reaction, one to amplify the promoter region of *ste9*$^+$ (Pste9) (ChSte9Fw: 5′-CTTTCGCTTAAAGTCCAACC-3′, and ChSte9Rev: 5′-GACTTCACCGATTAAATGTC-3′) and the other to amplify a region 3′ of *ste9*$^+$ (3′-ste9), used as an internal control (CtrSteFw: 5′-GCTT-CGTTATTCACGTAATCAA-3′, and CtrSteRev: 5′-TTCAATTAACGTGCGT-CCGT-3′). PCR of serial dilutions of DNA samples were performed to ensure analysis within the linear range of the PCR. Amplified products were separated by polyacrylamide gel electrophoresis and quantified using ImageJ software. Enrichment of the Pste9 region in the immunoprecipitated chromatin, indicative of Reb1 binding, was estimated as $(Pste9/3'\text{-}ste9)^{IP}/(Pste9/3'\text{-}ste9)^{INPUT}$.

RNA preparation and northern blots

Total RNA was isolated from ~5×10^7 cells using the RNeasy Mini Kit (Qiagen) following the supplier's instructions. RNA samples (8 μg) were electrophoresed through a formaldehyde–agarose gel, transferred to membranes and hybridized with the *ste9*$^+$ ORF amplified by PCR.

Protein extracts

S. pombe and *E. coli* extracts were prepared as described previously (Mejía-Ramírez et al., 2005), except that *S. pombe* extraction buffer contained 25 mM HEPES, pH 7.6, 150 mM KCl, 0.1 mM EDTA, 0.1% Triton X-100, 1 mM urea, 25% glycerol, 0.2 mM PMSF and 1.5× protease inhibitors mix (Roche).

Electrophoretic mobility shift assay (EMSA)

Binding reaction mixtures (20 μl) included 44 μg of *S. pombe* protein extract or 15 μg of proteins from *E. coli*, 25 mM HEPES, pH 7.6, 34 mM KCl, 10 mM EDTA, 0.5 mM DTT, 0.1 μg /μl poly(dI-dC), 0.1 μg/μl salmon sperm DNA and 10^4 c.p.m. of radiolabeled probe. The probe was obtained by PCR from a pIRT2-derivative plasmid where oligonucleotides ste9a (5′-GATCCAAAGTGCATTACCCT-TCCCGGTTTCTCAGTAGACAATGGG-3′) and ste9b (5′-GATCCCCATTG-TCTACTGAGAAACCGGGAAGGGTAATGCACTTTG-3′) were annealed and the resultant double-stranded oligo with *Bam*HI sticky ends (underlined) was inserted at the *Bam*HI site. Primers used for PCR amplification flanked the insert. The PCR product was digested with *Bam*HI, and the fragment containing the sequence from the promoter of *ste9*$^+$ was separated by electrophoresis and purified. Labeling was performed by filling the 5′ protruding ends with [α-^{32}P]dCTP and Klenow and purified through a G-25 Sephadex column (Roche). Binding reaction mixtures were incubated for 20 minutes at room temperature and electrophoresed at 4°C in 6% polyacrylamide–0.5× Tris–borate–EDTA gels at 10 V/cm for 2.5 hours. For supershift assays, 0.1 μg of anti-HA (3F10, Roche) or anti-FLAG M2 (F3165, Sigma) monoclonal antibody was added to the reaction mixture and incubated for 30 minutes at 4°C before addition of the probe or after the DNA–protein binding reaction.

Retrotranscription real-time quantitative PCR (RT-qPCR)

Total RNA was extracted using the RNeasy Mini Kit (Qiagen). On-column digestion with DNase was performed using the RNase-free DNase Set (Qiagen). cDNA was obtained using the Superscript II reversed transcriptase and random primers (Invitrogen). RT-qPCR reactions (20 µl) were performed in triplicate, containing IQ Sybr Green SuperMix (Bio-Rad), 1 µl of cDNA (at 1:1, 1:10 or 1:100 dilution) and 0.4 µM of each primer, with *cdc2* as an internal control. The reactions were analysed using an IQ5 System (Bio-Rad). In each sample, the amount of *ste9*$^+$ transcript relative to *cdc2*$^+$ was calculated as $(1–E)^{\Delta Ct}$, where ΔC_t is the difference between the threshold cycle for the *cdc2*$^+$ mRNA and for the *ste9*$^+$ mRNA, and E is the efficiency for the pair of *ste9*$^+$ primers. For each experiment, the data were normalized to the initial transcription level. The primers used were 5′-CCTACTTCATCGAATTCGTC-3′ and 5′-GTAGAAGCATCCCTAGAAGG-3′ for *ste9*$^+$, and 5′-ACCAGCTAGT-GAACGGTGTA-3′ and 5′-ACAATACTTCAGGAGCACGA-3′ for *cdc2*$^+$.

Two-dimensional agarose gel electrophoresis

DNA purification from asynchronous log-phase cultures and analyses of replication intermediates by 2-D gel electrophoresis were performed as described previously (Sánchez-Gorostiaga et al., 2004). The plasmids analyzed, pPste9(+) and pPste9(–), were constructed as follows. A 230-bp fragment containing the Reb1-binding sequence upstream *ste9*$^+$ was obtained by PCR using oligonucleotides ChSte9Fw and ChSte9Rev (see above). This fragment was first inserted into the TA-cloning vector pGEM-T Easy (Promega), extracted by digestion of the resultant plasmid with *Bam*HI and cloned in both orientations at the *Bam*HI site of the *S. pombe* vector pIRT2. The probe used for hybridization of 2-D gels was the *Pvu*II restriction fragment from pIRT2, containing *ars1* and *LEU2*.

Flow cytometry

Approximately 10^7 cells were spun down, fixed in 70% cold ethanol and washed in 50 mM sodium citrate. Cells were treated with 200 µg/ml RNaseA for 2 hours at 37°C and stained with 1 µM Sytox Green (Invitrogen – Molecular Probes) in the same buffer. After sonication, a Becton-Dickinson FACS Vantage apparatus was used for flow cytometry.

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/124/1/25/DC1

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