Local chromatin structure at the ribosomal DNA causes replication fork pausing and genome instability in the absence of the S. cerevisiae DNA helicase Rrm3p

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Lack of the yeast Rrm3p DNA helicase causes replication defects at multiple sites within ribosomal DNA (rDNA), including at the replication fork barrier (RFB). These defects were unaltered in rrm3 sir2 cells. When the RFB binding Fob1p was deleted, rrm3-generated defects at the RFB were eliminated, but defects at other rDNA sites were not affected. Thus, specific protein–DNA complexes make replication Rrm3p-dependent. Because rrm3-induced increases in recombination and cell cycle length were only partially suppressed in rrm3 fob1 cells, which still required checkpoint and fork restart activities for viability, non-RFB rrm3-induced defects contribute to rDNA fragility and genome instability.

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Completion of DNA replication is critical for cell viability, yet many factors such as DNA damage, transcribing RNA polymerases, and protein complexes can impede the progress of replication forks. The yeast Rrm3p, a 5′-to-3′ DNA helicase (Ivessa et al. 2002), is needed for efficient fork progression at ~1400 discrete sites throughout the genome. In its absence, replication forks pause at multiple sites in the ribosomal DNA (rDNA; Ivessa et al. 2000) as well as at telomeres (Ivessa et al. 2002), tRNA genes, centromeres, inactive replication origins, and the silent mating type loci (Ivessa et al. 2003). These paused replication forks have a propensity to break, which probably accounts for the increased recombination (Keil and McWilliams 1993; Ivessa et al. 2000, 2002, 2003) and elevated Ty1 transposition (Scholes et al. 2001) seen in rrm3 cells. Because of their widespread replication defects, rrm3 cells require the intra-S-phase checkpoint and fork repair activities for viability (Ivessa et al. 2003; Schmidt and Kolodner 2004; Torres et al. 2004).

Yeast rDNA exists as a single locus of ~150 tandem repeats. The tandem nature of the rDNA array makes possible several modes of intrachromosomal recombination, including the Rad52p-dependent liberation of rDNA circles (Kim and Wang 1989; Park et al. 1999). Each rDNA repeat contains the Polymerase (Pol) I transcribed 35S rRNA gene and the Pol III transcribed 5S rRNA gene (Fig. 1A). The activity of Sir2p, a histone deacetylase that is rDNA-associated, makes rDNA chromatin structure more compact, which reduces Pol II transcription and rDNA recombination, including the generation of rDNA circles (for review, see Ivessa and Zakian 2002; Rusche et al. 2003).

Each rDNA repeat also contains a potential origin of DNA replication (ARS), although only ~20% of the ARSs are active in a given S phase (Fig. 1B; Brewer and Fangman 1988; Linskens and Huberman 1988). Like rDNA recombination and Pol II transcription, origin activation is suppressed by Sir2p (Pasero et al. 2002). Replication of repeats with active origins is in the same direction. (Brewer and Fangman 1988; Linskens and Huberman 1988). However, when the leftward-moving fork encounters the replication fork barrier (RFB), a cis-acting sequence near the 3′-end of the 35S rDNA unit, it arrests. Fork arrest at the RFB is dependent on the RFB binding Fob1p (Kobayashi and Horiiuchi 1996; Huang and Moazed 2003). At the end of rDNA replication, rightward-moving forks converge on forks arrested at the RFB, completing rDNA replication. Because of the RFB, rDNA is the only region of the yeast genome where replication is largely unidirectional. Fob1p is also required for most rDNA recombination, including generation of rDNA circles (Defossez et al. 1999; Johzuka and Horiiuchi 2002).

rDNA replication is markedly altered in rrm3 cells (Ivessa et al. 2000). The abundance of forks converged at the RFB is elevated 10-fold without an increase in replication initiation, and forks arrested or converged at the RFB are more likely to break. In addition, rightward-moving forks pause near the beginning and end of the 35S gene, at the 5S rRNA gene, and at inactive ARSs. These replication defects and DNA breakage are associated with elevated rDNA recombination, including a large increase in rDNA circles (Keil and McWilliams 1993; Ivessa et al. 2000). Rrm3p, which is rDNA-associated in vivo, acts catalytically and probably directly to promote rDNA replication (Ivessa et al. 2000).

Sites whose replication depends on Rrm3p are assembled into stable, nonnucleosomal protein–DNA complexes. One model to explain the effects of Rrm3p on DNA replication is that the Rrm3p DNA helicase promotes movement of replication forks past protein–DNA complexes. This model predicts that disruption of the protein complex at a given site will render replication of that site Rrm3p-independent. In this paper, we test this hypothesis using rDNA. We report that Fob1p is required to cause rrm3-induced replication defects at the RFB. However, pausing at other sites in the rDNA, was unaffected by loss of Fob1p. Additionally, deletion of FOB1 did not eliminate the increased recombination, cell cycle progression defects, or synthetic lethal interactions seen in rrm3 cells. These data suggest that local protein–DNA complexes make rDNA replication-dependent on Rrm3p and show that Rrm3p-dependent pauses
at sites other than the RFB contribute to the rDNA fragility and genome instability of rrm3 cells.

Results and Discussion

The histone deacetylase Sir2p represses transcription in three regions—rDNA, telomeres, and the silent mating type loci—where replication is Rrm3p-dependent (Rusche et al. 2003). To determine if Sir2p-mediated chromatin modifications render rDNA replication dependent on Rrm3p, we used two-dimensional (2D) gel electrophoresis to examine rDNA replication in rrm3 sir2 cells. If silent chromatin is responsible for rrm3-dependent pausing, then replication fork progression should be unimpeded in rrm3 sir2 cells. However, rDNA replication was indistinguishable in rrm3 and rrm3 sir2 cells (Fig. 1C–E). Therefore, the deacetylase Sir2p and its associated regional chromatin changes did not make the replication of rDNA Rrm3p-dependent.

Next we asked if site-specific protein–DNA complexes confer Rrm3p-dependent replication. FOB1 encodes a 65-kD protein that binds to the RFB and is required for RFB activity and most rDNA recombination (Kobayashi and Horiiuch 1996; Kobayashi et al. 1998; Huang and Moazed 2003). Fob1p is also required for the RFB binding of Sir2p and Net1p, two proteins that affect transcriptional repression in the rDNA (Huang and Moazed 2003). Thus, Fob1p is needed for the formation of a multiprotein complex at the RFB.

In wild-type cells, the RFB is a polar block to fork progression as only leftward-moving forks stop at the RFB (Brewer and Fangman 1988; Linskens and Huberman 1988). In rrm3 cells, the fraction of replication intermediates at the RFB in BglII-digested DNA is twice that of wild-type cells (Ivessa et al. 2000). As ~90% of leftward-moving replication forks in wild-type cells arrest at the RFB (Brewer et al. 1992), the twofold increase in rrm3 cells cannot be explained by increased numbers of leftward-moving forks arresting at the RFB. Because leftward- and rightward-moving forks stopped at the RFB migrate to the same position in BglII-digested DNA (Fig. 1C), this increase is likely due to rrm3-dependent pausing of rightward-moving forks at the RFB.

To establish that rightward-moving forks pause at the RFB in rrm3 cells, we examined DNA digested with both HaeII and SmaI (Fig. 2A–C). In this double digest, the positions of rightward- and leftward-moving forks stopped at the RFB are easily distinguished. In wild-type cells, forks arrested and converged at the RFB (Fig. 2B,C, panel 1), and these structures were absent in fob1 cells (Fig. 2B,C, panel 3). DNA from rrm3 cells showed the expected increase in converged forks (labeled X) and pauses at the ARS (pause d) and near the 35S terminator (pause a; Fig. 2B,C, panel 2). In addition, a pause between the 35S terminator and the RFB was evident (Fig. 2C, panel 2, pause b indicated by arrow). This pause was ~40% of the way through the fragment, the expected position for rightward-moving forks paused at the RFB (Fig. 2A, left panel). Leftward-moving forks arrested at the RFB, expected at ~60% of the way through the fragment, generated the strong signal at the structure labeled RFB (Fig. 2C, panel 2, marked with an asterisk). Rightward-moving forks paused at the 5S gene were not visible in this digest because they congregate with leftward-moving forks arrested at the RFB.

If the presence of Fob1p at the RFB makes replication through this site Rrm3p-dependent, then rightward- and leftward-moving forks should not stop at the RFB in rrm3 fob1 cells. Consistent with this possibility, in rrm3 fob1 cells, the strong signal caused by leftward-moving forks arrested at the RFB was not detectable in HaeII–SmaI-digested rDNA (Fig. 2C, panel 4, position marked by asterisk). The minor pause visible at this site can be attributed to rightward-moving forks pausing at the 5S
rRNA gene. Likewise, rightward-moving forks no longer paused at the RFB [Fig. 2C, panel 4; arrow indicates site of paused rightward-moving fork in rrn3 cells]. However, forks still paused at the inactive ARS (pause d) and near the end of the 35S rRNA gene (pause a) as well as at the 5S gene (pause c) in rrn3 fob1 cells. The same conclusions are reached by examining XbaI-digested DNA [Fig. 2D–F, panel 4; asterisk marks site of arrest of leftward-moving forks at the RFB; arrow marks site of pause of rightward-moving forks at the RFB]. Although leftward-moving forks did not arrest and rightward-moving forks did not pause at the RFB in rrn3 fob1 DNA, the pauses at the end of the 35S rRNA gene [pause a], at the 5S gene [pause c], and at the inactive ARS [pause d] were not Fob1p-dependent [Fig. 2E,F, panel 4]. Additionally, two new pauses were detected [d-l and e-l]. These pauses can be attributed to leftward-moving forks paused at the start of the 35S transcription unit [pause e-l] and at the inactive ARS [pause d-l]. These pauses are not seen in FOB1 rrn3 cells, because leftward-moving forks arrest at the RFB when Fob1p is present and thus do not reach these sites. Therefore, when Fob1p is absent such that replication of the rDNA is no longer unidirectional, the RFB, the promoter region for the 35S transcript and inactive ARSs impeded movement of both rightward- and leftward-moving forks in an Rrm3p-dependent way. We conclude that loss of Fob1p binding at the RFB in rrn3 cells eliminates pausing and arrest at the RFB but does not reduce pausing at other sites.

Because the number of rDNA circles is reduced in fob1 cells, circles are thought to arise from breakage of forks arrested at the RFB [DeFossez et al. 1999; Versini et al. 2003]. Paused and broken replication intermediates, as well as rDNA circles, are much more abundant in rrn3 than in wild-type cells [Vessa et al. 2000]. If rDNA circles arise solely from forks stalled at the RFB, then circles will be equally abundant in fob1 and rrn3 fob1 cells. Alternatively, if breakage also occurs at non-RFB sites of rrn3-induced pausing, then the circle content of rrn3 fob1 cells will be higher than that of an fob1 strain but lower than in an rrn3 strain. We used 2D gels to determine the fraction of rDNA in circles [Fig. 3A,B]. In these gels, linear chromosomal rDNA is indicated by an asterisk, and supercoiled monomer circles are indicated by an arrow. Other circular forms, such as relaxed circles and multimers, run in an arc above linear DNA. The fraction of rDNA in circles was determined by dividing the amount of hybridization in all circular forms by the total rDNA hybridization in the gel. As expected, wild-type cells had more rDNA circles (7.5%, Fig. 3, panel 1) than fob1 cells (3%, Fig. 3, panel 3) and less than rrn3 cells (38%, Fig. 3, panel 2). The rDNA circle content of the rrn3 fob1 strain (19%, Fig. 3, panel 4) was intermediate between that of the fob1 and the rrn3 strains [Fig. 3]. Although the absolute fraction of circles varied from experiment to experiment, in four of four experiments the fraction of rDNA in circles in the fob1 rrn3 strain was lower than in the rrn3 strain and higher than in either the fob1 or wild-type strain. Thus, forks paused at sites other than the RFB must also break and recombine to generate rDNA circles.
Replication and genome instability in rrm3 cells

When G1-arrested cells are allowed to progress through the cell cycle, rrm3 and wild-type strains enter S phase with similar timing, but the rrm3 strain is delayed in moving from late S phase into the next cell cycle [Ivessa et al. 2002]. There are ∼150 rDNA repeats, and forks arrested or converged at the RFB show a high level of breakage in the absence of Rrm3p [Ivessa et al. 2000]. Therefore, the cell cycle delay might reflect a checkpoint response to the increase in forks pausing, converging, and breaking at the RFB. If this model is correct, the cell cycle defect should be mitigated in rrm3 fob1 cells. To test this idea, we used release from α-factor arrest to obtain synchronous rrm3 and rrm3 fob1 cultures and examined their progress through the cell cycle with fluorescent-activated cell sorting (FACS; Fig. 4A). The 23°C- grown wild-type and rrm3 cells were in S phase within 30 min after release. However, whereas many wild-type cells had a G1 DNA content by 135 min, G1-phase cells were not detected in the rrm3 culture until ∼195 min. The fob1 culture was indistinguishable from wild type. Thus, the absence of Fob1p alone had no evident effect on cell cycle progression. However, the rrm3 fob1 strain progressed through the cell cycle more slowly than either the wild-type or fob1 strain although not as slowly as rrm3 cells. G1-phase cells were evident in the rrm3 fob1 culture by 165 min, which was 30 min later than wild-type but 30 min earlier than rrm3 cells. Similar results were obtained for 30°C-grown cells [data not shown]. Thus, the S-phase delay in rrm3 cells is probably caused in part by difficulties in resolving replication forks converged at the RFB. However, replication pausing at other sites must also contribute to the S-phase delay.

The viability of rrm3 cells depends on several genes that act in the intra-S-phase checkpoint or that have roles in restarting replication forks [Tong et al. 2001; Ivessa et al. 2003; Ooi et al. 2003; Schmidt and Kolodner 2004; Torres et al. 2004]. For example, Mrc1p, a transducer for the intra-S-phase checkpoint [Alcasabas et al. 2001], and the Sgs1p DNA helicase, which senses, stabilizes, and restarts stalled replication forks [for review, see Khakhari et al. 2003], are essential in cells lacking Rrm3p. Given the large effects of Rrm3p on fork stalling and breakage at the RFB, the lethality of these doubly mutant strains might be due to damage incurred at the RFB. To test this idea, we constructed rrm3 fob1 mrc1 or rrm3 fob1 sgs1 strains carrying the plasmid pIA20, which has the URA3 and RRM3 genes. Because URA3 cells die in the presence of 5-fluoroorotic acid (FOA), only cells that are viable in the absence of RRM3 will grow on FOA plates. Although wild-type and rrm3 cells grew well on FOA plates, rrm3 mrc1 fob1 and rrm3 sgs1 fob1 cells were dead (Fig. 4B, data not shown). Because deleting FOB1 did not suppress the lethality of rrm3 mrc1 or rrm3 sgs1 cells, replication defects at sites other than the RFB must contribute to making rrm3 viability dependent on the intra-S-phase checkpoint and fork restart activities.

Sites that show Rrm3p-dependent replication are assembled into stable nonnucleosomal protein–DNA complexes. Here we show that deleting the Sir2p histone deacetylase in an rrm3 strain did not alter the rrm3-dependent DNA replication pauses. However, removal of the Fob1p complex eliminated fork stalling at the RFB but did not affect pausing at other rrm3-sensitive sites. Likewise, a mutation that prevents formation of the transcription preinitiation complex at a rDNA gene eliminates the rrm3-dependent exacerbated replication pause at this gene, and mutations that prevent ORC or Rapi1p binding to the HMR-E silent mating type silencer allow replication to proceed unimpeded through this site in rrm3 cells [Ivessa et al. 2003]. Together, these data support a model in which Rrm3p promotes fork movement past nonnucleosomal protein–DNA complexes.

There are multiple examples of DNA helicases that are able to remove proteins from DNA in vitro [Bedinger et al. 1983; Kaplan and O’Donnell 2002; Krejci et al. 2003; Veute et al. 2003]. Thus, Rrm3p might act by removing protein complexes from DNA. Alternatively, Rrm3p might be part of a chromatin-remodeling activity or Rrm3p might assist the replicative helicase. Whatever its mechanism of action, proteins like Rrm3p are likely to be critical for genome stability as sequences whose replication is Rrm3p-dependent become fragile sites in its absence.

Materials and methods

Haploid derivatives of the diploid strain WP1107 [homозygous for ade2 ade3 leu2-3,112 ura3-52 his3-200 leu2-3,112 trp1-101 lys2-801 can1-100] were used for 2D gel and FACS analyses. Null mutations of RRM3 were made as described [Ivessa et al. 2003; Veute et al. 2003]. Thus, Rrm3p might act by removing protein complexes from DNA. Alternatively, Rrm3p might be part of a chromatin-remodeling activity or Rrm3p might assist the replicative helicase. Whatever its mechanism of action, proteins like Rrm3p are likely to be critical for genome stability as sequences whose replication is Rrm3p-dependent become fragile sites in its absence.

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spore clones of the appropriate genotypes were grown to log phase, arrested for 2 h with 2 µg/mL α-factor, washed with YEPE to 30°C, and sampled collected at indicated time points. Cells were analyzed by FACS as described (Taggart et al. 2002).

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