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Dna2 Helicase/Nuclease Causes Replicative Fork Stalling and Double-strand Breaks in the Ribosomal DNA of *Saccharomyces cerevisiae**

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We have proposed that faulty processing of arrested replication forks leads to increases in recombination and chromosome instability in *Saccharomyces cerevisiae* and contributes to the shortened lifespan of *dna2* mutants. Now we use the ribosomal DNA locus, which is a good model for all stages of DNA replication, to test this hypothesis. We show directly that DNA replication pausing at the ribosomal DNA replication fork barrier (RFB) is accompanied by the occurrence of double-strand breaks near the RFB. Both pausing and breakage are elevated in the early aging, hypomorphic *dna2-2* helicase mutant. Deletion of *FOB1*, encoding the fork barrier protein, suppresses the elevated pausing and DSB formation, and represses initiation at rDNA ARSs. The *dna2-2* mutation is synthetically lethal with Δ*rrm3*, encoding another DNA helicase involved in rDNA replication. It does not appear to be the case that the rDNA is the only determinant of genome stability during the yeast lifespan however since strains carrying deletion of all chromosomal rDNA but with all rDNA supplied on a plasmid, have decreased rather than increased lifespan. We conclude that the replication-associated defects that we can measure in the rDNA are symbolic of similar events occurring either stochastically throughout the genome or at other regions where replication forks move slowly or stall, such as telomeres, centromeres, or replication slow zones.

Replication fork stress has been implicated as a major cause of genome instability in bacteria and yeast. In *Escherichia coli*, replication forks initiated at the origins frequently stall because of mutations in replication proteins, template blocks, or pauses at natural replication terminator sites. A common intermediate in restoring replication forks after stalling is a double-strand break (DSB),[1] which is thought to lead to recombination, producing genomic instability.

Evidence that replication forks pause in *Saccharomyces cerevisiae* is also convincing (1, 2). In the presence of the replication inhibitor HU, forks stall and give rise to single-stranded regions at the forks. In the absence of checkpoint function, the stalled forks are converted to regressed forks, a Holliday-like structure arising by branch migration and reannealing of nascent DNA strands (3). Primase mutants also show high levels of stalled and regressed forks (1). There is also a naturally occurring replication fork barrier (RFB) within the rDNA (ribosomal DNA) repeats, and the structure of forks paused at the RFB has been characterized (4–6). Finally, recent evidence suggests that the yeast ATR homolog, Mec1, protects replication forks from collapsing and giving rise to DSBs in replication slow zones throughout the chromosome (7). Evidence is also accumulating that replication fork failure leads to recombinogenic structures that result in gross chromosomal rearrangements (7–10). Such events may lead to the well documented genomic instability observed in DNA replication and checkpoint mutants (8, 9).

We have proposed that replication fork stalling and recombinogenic lesions in hypomorphic mutants of the essential replication helicase/nuclease Dna2 lead to genomic instability that shortens the life span of yeast (11). Dna2 is thought to collaborate with *FEN1* in maturation of Okazaki fragments on the lagging strand and in DNA repair in the gap-filling step (12–25). Given the lagging strand role, *dna2* mutants might be expected to lead to recombinogenic intermediates resembling those that are found in primase mutants (1). We previously provided evidence of elevated rDNA recombination in *dna2* mutants, as measured by loss of an *ADE2* marker from the rDNA array in the mutant, and we showed that Dna2p associates with rDNA maximally during S phase (11, 26). This evidence for replication-related recombination did not show directly, however, that *dna2* increased replication fork pausing, nor did it demonstrate the occurrence of lesions in the rDNA.

Now, we show that replication pausing and Holliday junction formation are accompanied by DSB formation at near the naturally occurring replication fork block in the rDNA and that all of these events are increased in a *dna2-2* mutant. We present the rDNA only as a model for other regions of the chromosome that we propose are undergoing similar replication fork failure, since we show that altering the physical structure of the rDNA by encoding rRNA only on plasmids, under either an RNA pol I or an RNA pol II promoter, shortens, rather than extends, the lifespan of yeast.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Cell Growth—Strains used in this study are W303, *MATa trp1 his3 ade2 ura3; dna2-2*, *MATa trp1 his3 ade2 ura3 dna2-2 (::LEU2); fob1Δ*, *MATa trp1 his3 ade2 ura3 fob1(::HIS3); dna2-2 fob1Δ*, *MATa trp1 his3 ade2 ura3 dna2-2 (::LEU2) fob1(::HIS3)*. The

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[1] The abbreviations used are: DSB, double-strand break; RFB, replication fork barrier; pol, polymerase; NTS, nontranscribed spacer; RI, replication intermediates; HJI, Holliday junction intermediate; nt, nucleotide; TBE, Tris borate/EDTA.

dna2-2 strain was generated by backcross of the original *dna2-2* mutant (21) to W303 five times. Isogenic *fob1*Δ strains were constructed as described previously using plasmid YEp*FOB1*::*HIS3* kindly provided by T. Kobayashi (National Institute of Basic Biology, Okazaki, Japan) (27). The strains were verified for disruption of the *FOB1* gene by Southern blot. Plasmid YEplac195*FOB1* was used for complementation of bleomycin resistance, also provided by T. Kobayashi (27). Plasmid transformation and general genetic techniques were performed by standard methodology (28, 29). For synthetic lethality (Table I), strain 4741-994, *MATa rrm3*Δ::*Kan leu2*Δ *met15A ura3*Δ *his3*Δ was crossed with the isogenic 4742-2-2-11B, *Matα dna2-2*:*LEU2 leu2*Δ *met15A ura3*Δ *his3*Δ and sporulated and analyzed by standard genetic techniques. For lifespan analysis strain NOY505 *MATa ade2-1 ura3-1 his3-11 leu2-3 112 trp1-1 can1-100* was the parent of NOY891 and NOY908 described in the text. The rDNA plasmids are described in Refs. 30 and 31. These strains were provided by H. Wai and M. Nomura, UC, Irvine. Lifespans were determined beginning with logarithmic phase cultures as previously described (11).

Two-dimensional Gel Analysis—All strains were grown in 300 ml of YPD medium at 30 °C to an OD_{600} of 0.6–1 for a two-dimensional gel and DSB analysis. Genomic DNA was prepared as described by Huberman *et al.* (32) except that DNA was extracted with phenol/choloroform/isopropyl alcohol instead of being purified with CsCl gradients. The purified DNA was digested with *Bgl*II, and BND-cellulose (benzoylated naphthoylated DEAE cellulose, Sigma) was used to enrich branched DNA molecules (32). The procedure for Neutral/Neutral two-dimensional agarose gel electrophoresis described by Brewer and Fangman (33) was used with minor modifications. The first dimension gel (0.5% agarose in TBE) was run at 1 V/cm at 4 °C for 36 h. The second gel (0.9% in TBE containing 0.3 μg/ml ethidium bromide) was run at 4 V/cm at 4 °C in circulating TBE containing 0.3 μg/ml of ethidium bromide for 9–12 h.

DSB Analysis—Isolation of intact yeast DNA was basically performed as described previously with modifications (28). Briefly, cells (5×10^7) were suspended in 50 μl of cold L buffer (0.1 M EDTA, pH 8, 0.01 M Tris, pH 7.6, and 0.02 M NaCl) in an Eppendorf tube containing 5 μl of cold zymolyase (Zymolyase-20T, *Arthrobacter luteus,* 20,000U/gm; ICN) at a stock concentration of 20 mg/ml. The cell suspension was warmed by incubating the tube in a 42 °C water bath for a few seconds and mixed briefly with 50 μl of 1% low melting agarose prepared in L buffer and cooled down to 42 °C. The mixture was placed in an ice water bath. The gel plug was transferred into a 20-ml round-bottomed glass tube containing 5 ml of 0.5 M EDTA, pH 8 and 0.01 M Tris, pH 7.6 plus 1% β-mercaptoethanol (the gel plug could be easily flushed out into the glass tube with the buffer). The gel plug was incubated at 37 °C for 24 h. The buffer was removed and replaced with 0.5 ml of the same buffer containing 0.5 mg/ml zymolyase. The gel plug was incubated at 37 °C for 24 h. The buffer was removed and replaced with 1 ml of L buffer containing 1 mg/ml proteinase K and 2.5% Sarkosyl. The gel plug was incubated at 50 °C for 24 h. After the gel plug was cooled on ice, the buffer was removed and replaced with 0.5 ml of the same buffer containing proteinase K and Sarkosyl. The incubation was repeated at 50 °C for 24 h. The gel plug was incubated in 5 ml of Tris-EDTA (TE) (pH 7.6) containing 40 μg/ml phenylmethysulfonyl fluoride at 50 °C. After 1 h of incubation, the gel plug was incubated in 5 ml of TE at 50 °C for another hour. The plug was further incubated with 100 μg/ml RNase A at 50 °C for 3 h in restriction enzyme buffer (1 ml). Buffer was removed, and the plug incubated in 0.5 ml of restriction enzyme buffer containing *Bgl*II (100 units) at 37 °C overnight. The gel plug was soaked in 5 ml of TE at 4 °C for 0.5 h. After the buffer was drained, 20 μl of 6× loading buffer (28) was added to the gel plug. After incubation on ice for 0.5 h, the gel plug was loaded into the well of a 1% agarose gel, which was run in TBE at 1.5–2 V/cm at 4 °C for 24 h. The gel was stained in 1 μg/ml ethidium bromide for 2–4 h and inspected under UV and used for Southern blot.

Probes and Labeling—Probe DNA was prepared by PCR from a rDNA plasmid with the following pairs of primers: probe A, ER5A-up, 5′-GCC ATT TAC AAA AAC ATA ACG-3′ and ER5A-lower, 5′-GGG CCT AGT TTA GAG AGA AGT-3′; probe B, X35S-up, 5′-ATA TCA ACC CTG ACG GTA GAG-3′ and X35S-lower, 5′-CAT GGT ATA ACT GTG GTA ATT CTA GAG-3′; probe C, *Bgl*II end-up, 5′-ACA GAT GTG CCG CCC CAG CCA AAC TCC-3′ and *Bgl*II end-lower, 5′-CCT GGA TAT GGA TTC TTC ACG GTA ACG-3′. The PCR products were gel-purified. Random-primed labeling kit (Roche Applied Science GmbH) was used to label the probe DNA in the presence of four [α-^{32}P]dNTPs.

Southern Hybridization—DNA in the gel was vacuum-transferred onto a gene screen plus hybridization transfer membrane (positive charged, PerkinElmer Life Sciences) in 0.1 N NaOH. After transfer, the membrane was washed in 0.5× SSC and 0.5% SDS twice and vacuum-dried at 80 °C for 20 min. The membrane was prehybridized in 6× SSC, 5× Denhardt, 0.5% SDS, 100 μg/ml boil-denatured calf thymus DNA, and 200 μg/ml bovine serum albumin at 65 °C. It was hybridized in 6× SSC, 5× Denhardt, and 0.5% SDS containing heat-denatured labeled probe at 65 °C in a water bath for 1 h or overnight. When the temperature of the water bath went down to 30 °C, the membrane was washed briefly with 0.5× SSC and 0.5% SDS twice, further with 1–2 liters of prewarmed (30 °C) 0.5× SSC 0.5% SDS at 30 °C for 2–4 h. After washing briefly with 0.5× SSC at room temperature to remove SDS, the membrane was air semi-dried on tissue (complete drying was avoided) and then put in a phosphorimaging screen cassette plus an x-ray film. Exposure was at 4 °C for 1–3 weeks.

Gel Image Analysis—Phosphorimager files of two-dimensional gels were translated into Photoshop files (Adobe Photoshop Elements) for image processing and quantification. Image quantification was performed using NIH Image 1.62 and plotted with CA-Cricket Graph III 1.52. Briefly, images of a Photoshop file was pasted to a blank window of NIH Image Analysis. The same tool was used to select the image object for measurement of area, integrated density, and background. The numerical data were pasted onto a blank work sheet of Cricket Graph. Calculations are conducted as follows: Unit signal of individual object, USIO = (integrated density − background)/area. Individual objects include 1 N spot (US 1N), RFB (US RFB), fork convergence or double-Y (US Y), and Holliday junction (US HJ, see Fig. 1*D*). The ratios for comparison include US RFB/US 1N, US Y/US 1N, and US HJ/US 1N. All three ratios were set to 1 for the wild type with which the mutants were compared.

RESULTS

Two-dimensional Gel Analysis of rDNA Replication and Recombination Intermediates—We have chosen to study rDNA replication in yeast, both because instability of the rDNA locus is correlated with short lifespan in yeast and because the rDNA repeats contain a naturally occurring RFB, similar in some respects to the bacterial termination system (see Ref. 34 for review). In yeast, the rDNA occurs as about 150 tandem repeats of a 9.1-kb sequence on chromosome XII. The rDNA is organized into a nucleolar structure where rRNA is synthesized, processed, and ribosome assembly is begun. Each rDNA repeat contains two transcribed regions (35 S and 5 S rRNA) and two nontranscribed spacers (NTS1 and NTS2). NTS1 houses the polar RFB (130 bp), an enhancer for RNA polymerase I (pol I), and a *cis*-acting mitotic recombination hot spot (HOT1); while NTS2 contains a promoter for 35 S rRNA recognized by pol I and an origin of replication, ARS (4, 5, 35, 36).

When replication initiates from the ARS in NTS2, the movement of replication forks in the same direction as 5 S rRNA transcription is blocked at the RFB in NTS1, whereas the forks progressing in the direction of 35 S rRNA transcription are allowed to pass through the RFB (4, 5). Thus, the RFB imposes polar replication arrest in rDNA units containing an active ARS (Fig. 1) (37). Characterization of forks arrested at the RFB shows that after the replication fork arrives, the Okazaki fragments on the stalled lagging strand are completely processed (6, 38). The nascent lagging strand arrests three bases ahead of the leading strand (6).

To study replication pausing and recombination at the rDNA RFB, we used N/N (neutral-neutral) two-dimensional agarose gel electrophoresis (two-dimensional gel) (33, 37) with modifications (see "Experimental Procedures" for details). Genomic DNA was digested with *Bgl*II, which generates two fragments in the rDNA, a 4.6-kb fragment containing the RFB in the center and the ARS near the right end (Fragment *Bgl*II-A in Fig. 1*A*), and a second fragment containing most of the 35 S RNA coding region (*Bgl*II-B in Fig. 1*A*). The digest was fractionated by two-dimensional gel electrophoresis, in which the first dimension is run under conditions that separate DNA by mass, and the second dimension under conditions that separate according to the shape of replication intermediates (RIs). The gel was then blotted and the RIs visualized by hybridization to

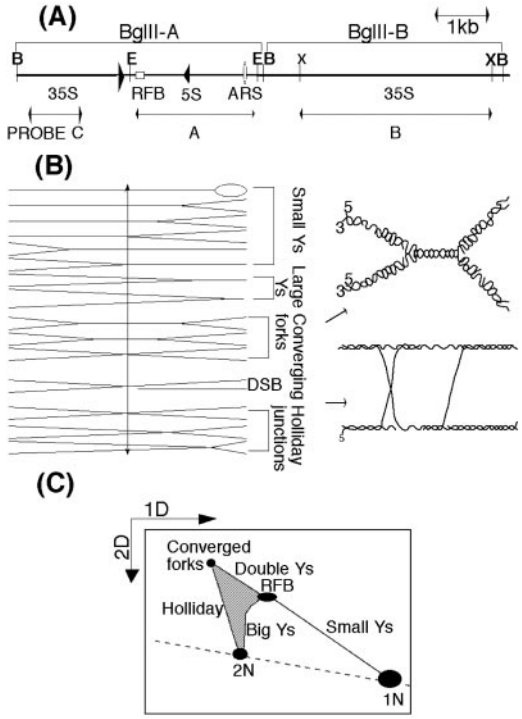

FIG. 1. **Schematic rDNA replication and recombination.** *A*, map of the rDNA and probes used. Illustrated is a 9.2-kb rDNA unit from chromosome XII drawn to scale. *B*, *Bgl*II; *E*, *Eco*RI; *X*, *Xba*I; *35S* and *5S*, transcription units for 35 S and 5 S RNA, respectively. In the *Bgl*II-A fragment, the replication fork barrier, RFB, is located in the *center* and the ARS near the *right end*. *B*, schematic of expected replication and recombination intermediates. On the *right*, a possible difference in structure between Holliday junctions and converging forks (77, 78) is illustrated. *C*, schematic of two-dimensional gel pattern of rDNA replication and recombination intermediates expected for the *Bgl*II-A fragment. *Arrow* shows directions of first dimension (*1D*) and second dimension (*2D*) electrophoresis. See text for details.

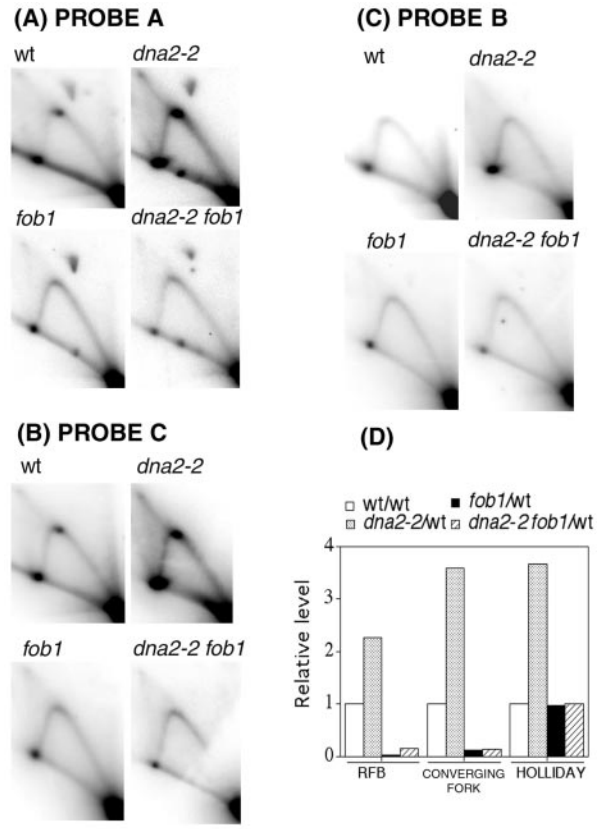

FIG. 2. **rDNA replication and recombination in the *dna2-2* and *fob1*Δ mutants.** *A* and *B*, genomic DNAs from indicated strains were digested with *Bgl*II, fractioned by neutral-neutral two-dimensional gels, and analyzed by Southern hybridization using probes *A* or *C* described in the legend to Fig. 1*A*. The gels show rDNA replication and recombination intermediates in the *Bgl*II-A fragment (containing the ARS and the RFB as indicated in the legend to Fig. 1). *A*, probe A; *B*, same blot, stripped and reprobed with probe C. *C*, lack of replication fork pausing in the *Bgl*II-B fragment that lacks both the ARS and the RFB. The same blots used in *A* and *B* were reprobed with probe B, which hybridizes only to the *Bgl*II-B fragment (Fig. 1). This experiment controls for the specificity of pausing at the RFB. *D*, quantification of rDNA replication and recombination intermediates shown in *A* averaged from three gels as described under "Experimental Procedures." Similar values (not shown) were obtained using data from probe C (*B*).

various probes (Fig. 1*A*). Because the ARS from which bidirectional replication initiates is proximal to the right end of *Bgl*II-Fragment A, the initiation bubble rapidly reaches the right end and is converted to a fork upon restriction enzyme digestion. RIs arising from Fragment A therefore generate a so-called Y-arc on the gel (big and small Ys in Fig. 1, *B* and *C*). In a repeat unit where initiation has occurred at an active ARS, forks moving leftward from the ARS arrest at the RFB greater than 90% of the time, and therefore produce primarily molecules with arms no longer than half of the length of the *Bgl*II-A fragment (small Ys in Fig. 1, *B* and *C*). When the leftward forks are arrested at the RFB, RIs with a branch point near the RFB accumulate, generating a spot at the apex of the Y-arc as shown in Fig. 1, *B* and *C*. The spot intensity reflects the extent of stable replication pausing. Forks entering from the left and moving rightward, by contrast, pass the RFB in repeats lacking an active ARS and generate a composite of small and large Y-forks, forming a complete Y-arc on a two-dimensional gel (Fig. 1, *B* and *C*).

When a rightward fork is approaching a leftward fork stalled at the RFB, a population of RIs having a double-Y structure is produced, and distributes along a line emanating from the RFB (Fig. 1, *B* and *C*). (Double-Y molecules, X-shaped molecules, and broken forms of these species also migrate in the shaded cone-shaped area; Refs. 2 and 39). When the two forks meet at the RFB, the convergence produces a spot at the end of the line, with a mass of about 2N (9.2 kb), labeled converged forks (Fig. 1*C*). The intensity of these species is correlated with the extent of pausing at the RFB. Usually, only a small fraction of forks converge, however, because only about 10% of rDNA ARSs fire and are able to generate leftward forks. One can also observe recombination intermediates, X-shaped, or Holliday structures, in the gels. Migration of the Holliday junction generates a composite of X-shaped HJIs, which are distributed along a spike growing up from 2N spot and ending at the same spot as converged forks (Fig. 1, *B* and *C*). DNAs in this spike have been shown to be cleaved by RuvC and are therefore considered to represent HJIs rather than forks converging randomly along the rDNA, which would have the same migration pattern (40). The intensity of the spike of HJIs is therefore thought to reflect primarily the frequency of the recombination events.

Two-dimensional gel analyses of the *Bgl*II-A fragment (*probes A* and *C* in Fig. 1*A*) using various mutants are shown in Fig. 2, *A* and *B*. The RFB is clearly seen in the wild-type strain, both with a probe to the right end of the fragment (*probe A* in Figs. 1*A* and 2*A*) and after re-probing with a fragment hybridizing only to the left end (*probe C* in Figs. 1*A* and 2*B*). The spike to the far left of the cone area in wild type and more prominently in *dna2-2* is attributable to multibranched structures (39). Probe B hybridizes to the *Bgl*II-B fragment, which contains neither an ARS nor an RFB, and therefore serves as a control. Clearly, there is no specific pausing in this fragment (Fig. 2*C*). This is consistent with previous findings that the

major specific replication fork pause in the rDNA is at the RFB.

Quantification of Hybridization Signals—In order to quantitatively compare the effects of various mutations on rDNA replication and recombination, we quantified the probe A-hybridization signals due to the RFB, converging forks, and HJIs. Then we normalized individual signals in relation to the intensity of the linear 1N spot (4.6 kb) for each strain (see "Experimental Procedures" for details) (41) and averaged each value from three experiments using probe A (see Fig. 2*D*). For ease of comparison, the averaged ratio of each of the signals to 1N was set to one for the wild type. Although we found some variations in the intensity of the 1N spot between experiments due to the loading variations, the ratio of the RFB to 1N signal remained unchanged in all replicate experiments.

*fob1*Δ *Reduces Fork Pausing at RFB*—Fob1p (fork blocking less, Ref. 42) a nucleolar protein (43), recently proposed to belong to the viral integrase family (44), is required for accumulation of paused forks at the rDNA RFB, although its mechanism of action is unknown (42). As expected, *fob1*Δ cells had 0.03 times as many RIs accumulated at the RFB and 0.12 times as many converging forks as wild type. The level of HJIs was low in both wild-type and in *fobl*Δ cells (Fig. 2, *A*, *B*, and *D*). These results support previous findings that inactivation of *FOB1* reduces accumulation of paused replication forks at the RFB (42).

dna2-2 Stimulates Fork Pausing and Recombination at the RFB—We next examined the *dna2-2* mutant. We chose this allele for the following reasons. The helicase motifs are clustered in the C-terminal region and nuclease motifs in the N-terminal region of the *DNA2* gene. The helicase activity appears to act in DNA damage repair and lagging strand synthesis (21). The R1253Q mutation in *dna2-2* directly alters helicase motif IV and causes sensitivity to MMS and synthetic lethality with a mutation in *CTF4,* encoding a protein interacting with DNA polymerase α (21). A mutation of the same arginine residue in motif IV of two other helicases also abolishes the helicase activity of the mutant proteins (45, 46). We expected that the *dna2-2* mutation would cause replication to stall at the RFB. In the *dna2-2* cells, the intensity of RIs at the RFB detected with probe A was 2.3-fold greater than wild type and that of converged forks, 3.6-fold greater (Fig. 2, *A* and *D*). Quantitation of the data using probe C in Fig. 1*A* also showed a 2-fold greater pause in the *dna2-2* mutant compared with wild type (not included in plots in Fig. 2*D*). The level of HJIs increased up to 3.7-fold in the *dna2-2* mutant (*probe A*, Fig. 2, *A* and *D*). To further verify that the increased pausing was due to the helicase defect, we investigated the effect of a K1080A mutation in helicase motif I, which eliminates the helicase activity of Dna2 (15). The K1080A mutation increased replication fork stalling at the RFB more than 2-fold (data not shown). The level of increased pausing in the *dna2-2* strain is the same as that observed with the yeast *rrm3*Δ mutant (41, 47). As in the *rrm3*Δ mutant (41), pausing was independent of *RAD52* in the *dna2-2* strain (data not shown). Increases in HJIs within the rDNA have been demonstrated previously for other DNA replication mutants, where the accumulation of HJIs is independent of Rad51, the yeast RecA homolog (40).

*fob1*Δ *Suppresses dna2-2-induced Elevated Fork Pausing and Recombination*—We also examined replication in the *dna2-2 fob1*Δ cells. The RFB signal in the *dna2-2 fob1*Δ double mutant was reduced 14-fold, while the converging forks and recombination were reduced 25-fold compared with *dna2-2*, confirming that events at the RFB give rise to the RIs observed in the *dna2-2* mutant (Fig. 2, *A* and *D*). The intensity of the small and large Y arc signal also decreased (Fig. 2*A*). No pausing was seen in the *Bgl*II-B fragment for any of the strains,

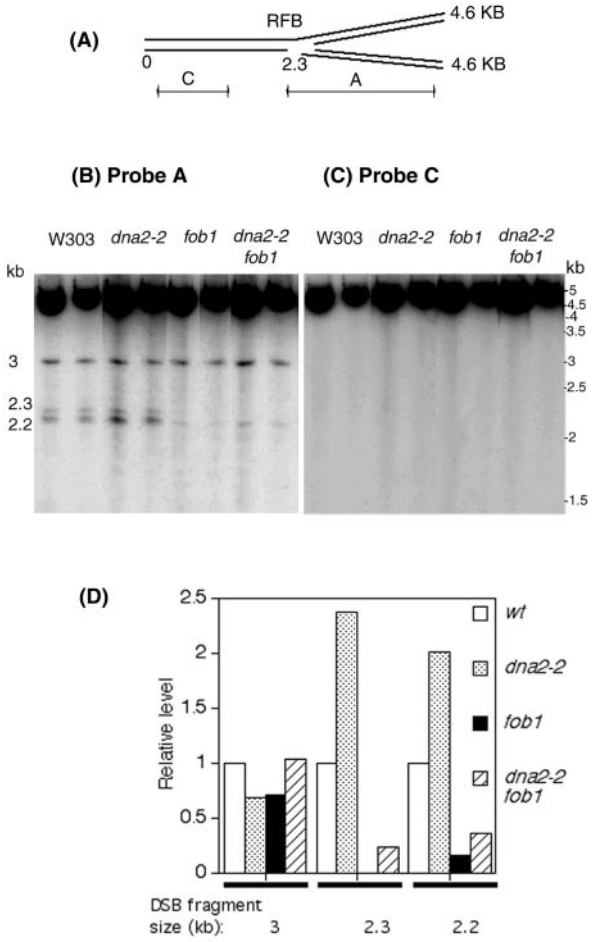

FIG. 3. **Formation of DSBs near the RFB in the *dna2-2* and *fob1*Δ mutants.** *A*, schematic of species found in *panels B* and *C*; *B*, agarose gel electrophoresis showing DSBs. Genomic DNAs from indicated strains were extracted in an agarose plug, digested *in situ* with *Bgl*II, fractioned by neutral one-dimensional agarose gel electrophoresis, and analyzed by Southern hybridization using probe A (see Fig. 1*A* and Fig. 3*A*). For each strain, two gel blocks were prepared from 5×10^7 and 2.5×10^7 cells, respectively, and loaded in two adjacent wells. *C*, reprobing blot with probe C (see Fig. 1*A*) after stripping probe A. *D*, quantification of DSB fragments. The intensities of the 2.3- and 2.2-kDa bands in each lane were determined and normalized to the intensity of the 4.6-kb band. The wild-type value was set at 1, as in Fig. 1*D* and "Experimental Procedures," and relative values, that is the value in the mutant compared with wild type, was determined. The values shown represent the average of results from the two equivalent lanes for each strain (*B*).

including the *dna2-2* mutant (Fig. 2*C*, *probe B*). This demonstrates that replication forks pause and/or accumulate primarily at the RFB for the rDNA regions studied. The *dna2-2* mutant differs in this respect from *rrm3*Δ mutants, which show pausing not only at the RFB but also at the promoter region for the 35 S and 5 S rRNAs (41).

DSBs Are Generated Near the RFB—Neutral agarose gel electrophoresis was used to determine the level of DSB lesions near the RFB in the 4.6-kb *Bgl*II-A fragment shown in Fig. 1*A* (Fig. 3*A*) (28). In order to prevent shearing, DNA is prepared in an agarose plug and digested with restriction enzyme directly within the agarose (see legend of Fig. 3 and "Experimental Procedures"). If DSB formation is random, the broken linear DNA will not form distinct DNA bands when the DNA is run out of the plug into an agarose gel electrophoresis system, whereas site-specific DSBs will give detectable DNA bands. Occurrence of DSBs at the RFB should give rise to fragments shorter than 4.6 kb that hybridize to a *Bgl*II-A (Fig. 1*A*, *probe*

A and Fig. 3*A*). Three such fragments, with sizes of 3, 2.3, and 2.2 kb, were detected in DNA from wild-type cells. The relative intensity of each fragment, defined by the ratio of individual fragment intensity to the intensity of the 4.6-kb fragment, was determined and averaged (Fig. 3*D*). Because mechanical shearing of chromosomes is unlikely during preparation in agarose plugs and the sizes are shorter than 4.6 kb, the fragments must result from DSBs at specific sites near the center of the *Bgl*II fragment, at or near the RFB, which lies 2.2–2.3 kb from the end of the restriction fragment (Fig. 3*A*). This suggests that DSBs are formed at three nearby sites in a proportion of the cells.

Since the RFB is at the center of the 4.6-kb *Bgl*II-A fragment and the length of the DSB fragments are 2.2 and 2.3 kb, and since probe A hybridizes only to the 2.3-kb region between the ARS and the RFB (Fig. 3*A*), the DSBs arising *in vivo* may originate near or at RFB. After the same membrane had been stripped to remove probe A, it was hybridized with probe C located on the left half of the RFB in the 4.6-kb *Bgl*II-A fragment shown in Figs. 1*A* and 3*A*. No DNA fragments shorter than 4.6 kb were detected (Fig. 3*C*). The same probe does hybridize to replication intermediates that represent the entire *Bgl*II-A fragment, however, as shown in Fig. 2*B*. Apparently, truncated fragments were only generated on the right side of the RFB, between the ARS and the RFB, but not the left side (Fig. 3*A*). Both the 2.2- and 2.3-kb fragments could arise from breakage at the RFB, which is 129 bp in length. Both contain the 5 S RNA promoter region, but pausing at the 5 S RNA could only give rise to a 2.2-kb fragment by extensive branch migration before cleavage. This suggests that one arm of the leftward-moving fork was released, as might occur due to breaks in a single-stranded gap or cutting by a resolvase at a regressed fork (see Fig. 8). Gruber *et al.* (6) have shown that fork regression at the RFB is extremely limited, which would be consistent with the lengths of fragments we observe and our interpretation of the break site.

Is Formation of DSBs Associated with Replication Pausing at the RFB?—In order to further connect DSB formation with pausing at the RFB, we carried out the same analysis in a *fob1*Δ mutant. As shown in Fig. 3, the 2.2- and 2.3-kb bands were dramatically reduced in intensity (Fig. 3, *B* and *D*). The relative intensity of the 2.3- and 2.2-kb fragments in the *fob1*Δ mutant was close to zero and 0.16-fold that of the wild type (Fig. 3, *B* and *D*). Thus, the decrease in replication pausing at the RFB in the *fob1*Δ strain (Fig. 1) is accompanied by a decline in frequency of DSB formation near the RFB. However, this is not correlated to total recombination events, in that the level of HJIs in the *fob1*Δ strain is similar to that in the *dna2-2 fob1*Δ strain and in the wild-type strain (Fig. 1*D*). Our explanation is that replication fork stalling due to endogenous DNA damage at nonspecific (*i.e.* RFB-independent) sequences throughout the rDNA gives rise to a background level of HJIs, which is dependent on *RAD52,* as shown previously (40). In our work, these HJIs are barely seen in a *rad52*Δ mutant (not shown).

The 3-kb fragment also hybridizes only to the right half of the *Bgl*II-A fragment (probe A but not probe C, Figs. 1*A* and 3*A*), and the amount of the 3-kb fragment does not decrease in the *fob1*Δ strain (Fig. 3*B*). We propose that leftward replication forks in a portion of cells that fail to stall at the RFB may pause and break near the termination site of the 35 S transcript. Pausing is observed at the 35 S region in *rrm3*Δ mutants (41).

*The Increase in DSB Formation in Strain dna2-2 Is Suppressed by fob1*Δ—We also carried out DSB analysis in *dna2-2* and *dna2-2 fob1*Δ mutants, to judge if the increased pausing in *dna2-2* mutant was accompanied by increased breakage. As shown in Fig. 3, the intensity of the 2.2- and 2.3-kb fragments

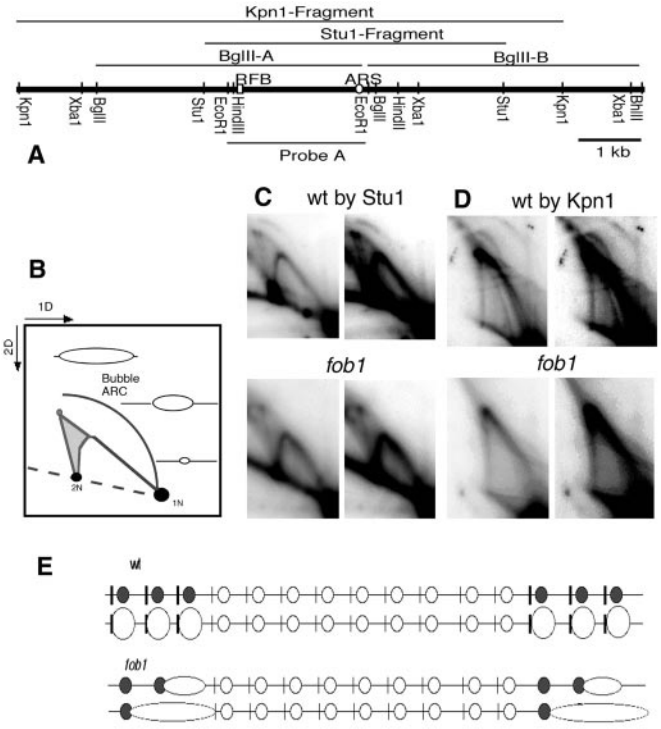

FIG. 4. **Frequency of initation at the rDNA ARS in the *fob1* and wild type strains.** See text and "Experimental Procedures." for details. *A*, restriction map of relevant sites in the rDNA and probes used in the two-dimensional gel analysis. *B*, schematic of replication intermediates expected in these fragments. *C*, two-dimensional gel analysis of RIs from the *Stu*I fragments with the probe A indicated in *A*. The duplicated are normal and overexposed images from the same gel. *D*, two-dimensional gel analysis of the *Kpn*I fragment with probe A, shown in (*A*). *E*, schematic interpretation of the mechanism of suppression of rDNA initiation in the absense of Fob1 (see text for explanation). *Open circles*, inactive ARS; *closed circles*, normally active ARSs; *vertical bars*, RFB.

derived from the *Bgl*II-A fragment in the *dna2-2* mutant was more than 2-fold greater than in the wild-type strain (Fig. 3, *B* and *D*). In the *dna2-2 fob1*Δ strain, the 2.2- and 2.3-kb fragments were dramatically decreased, suggesting that they arise due to breakage after fork arrest at the RFB. The relative intensity of the 2.2- and 2.3-kb fragments from the 4.6-kb *Bgl*II-A fragment (Figs. 1*A* and 3*A*) in the *dna2-2 fob1*Δ double mutant was 0.36- and 0.23-fold that of the wild-type strain (Fig. 3, *B* and *D*). Compared with the *dna2-2* single mutant, the relative level of the two fragments (2.2 and 2.3 kb) was reduced 6- and 10-fold in *dna2-2 fob1*Δ. This indicates that the elevated levels of DSBs arising from events at or near the RFB in the *dna2-2* cells are suppressed by the *fob1*Δ mutation (Fig. 3, *B* and *D*). Therefore, the elevated levels of Fob1-dependent replication fork stalling at the RFB correlates with increased accumulation of HJIs and DSB formation in the *dna2-2* mutant.

The intensity of the 3-kb linear fragment did not increase in the *dna2-2* mutant. Thus, this generation of the fragment is independent of both the *dna2-2* and *fob1*Δ mutations. The mechanisms behind generation of this fragment remain for further study.

*Replication Initiation at rDNA ARSs in fob1*Δ *and in the dna2-2 fob1*Δ—During analysis of replication initiation in the rDNA in various mutants studied here, we noticed an interesting effect of the *fob1*Δ mutation. The enzyme *Stu*I produces a relatively short fragment carrying the rDNA ARS in the center (Fig. 4*A*), allowing one to visualize replication bubbles, as shown in Fig. 4, *B* and *C*. The RFB spot is now seen proximal

TABLE I
Combining dna2-2 and rrm3Δ mutations

Strain *dna2-2* was crossed with the *rrm3Δ* strain described under "Experimental Procedures." Tetrads (26 tetrads on glucose, 19 tetrads on sorbitol) were analyzed after sporulation of the diploids. Spore viability was high.

Genotypes	No. of spores YPD plates
DNA2 RRM3	20
dna2–2 RRM3	28
DNA2 rrm3Δ	31
dna2–2 rrm3Δ	0
	YPD plates plus sorbitol
DNA2 RRM3	6
dna2-2 RRM3	12
DNA2 rrm3Δ	12
dna2-2 rrm3Δ	0

to the 2N spot. The enzyme *Kpn*I gives rise to a larger fragment carrying both the ARS and the RFB in a more central position (Fig. 4, *A* and *D*), which allows visualization of both the RFB among the small Y′s near the apex of the Y arc and also replication bubbles (bubble arc). Since most rDNA ARSs are dormant, visualization of the bubble arcs occurs on a high background of Y arcs from the repeats with inactive ARSs. Thus, as shown in Fig. 4, *C* and *D* for each strain, the bubble arcs of necessity appear weak. Longer exposure times result in such dark Y arcs that the bubble arcs are obscured. Thus, these gels are not of low quality; their quality is inherent in the nature of the rDNA. When genomic DNA was digested with *Stu*I (Fig. 4*C*), we found that the bubble arc is not affected significantly in intensity in the *dna2-2* mutant (data not shown), compared with that in the wild type, indicating that initiation at the rDNA ARSs is normal in the *dna2-2* mutant. However, in both *fob1Δ* (Fig. 4*C*) and *dna2-2 fob1Δ* strains (not shown) the bubble arc was significantly reduced compared with wild type. To ensure that the observation with the *Stu*I fragment is not due to an artifact, we studied the *Kpn*I-fragment and obtained the same results (Fig. 4*D*). The effect of eliminating Fob1 could imply a direct effect of Fob1 on origin firing. More likely, however, the effect is indirect. That is, it is now known that the active rDNA ARSs are clustered adjacent to each other rather than at random locations in the rDNA (48). Firing of closely spaced origins is usually prevented by origin interference (49). Presumably the RFB prevents origin interference. We propose that lack of pausing at the RFB in the *fob1Δ* strains causes adjacent replication origins to suppress each other resulting in replication of the entire rDNA locus from fewer replication origins. This is the first demonstration of an effect of Fob1 on the frequency of rDNA ARS utilization.

Combining the dna2-2 and rrm3Δ Mutations but Not the dna2-2 and pif1Δ Mutations Is Lethal—Two additional 5′ to 3′ yeast helicases, Rrm3 and Pif1, have been found to have effects on fork progression at the RFB (41, 47). Pif1, like Fob1, is involved in maintenance of the RFB. Rrm3 seems to promote fork progression throughout the rDNA, since *rrm3Δ* mutants show dramatic RFB pauses and long-lived converged forks in the rDNA. The paused forks may eventually be converted to DSBs that stimulate recombination, since *rrm3Δ* mutants show an increased frequency of extrachromosomal ribosomal circular DNAs. Since the effects of the *dna2-2* mutation are similar to those of the *rrm3Δ* but opposite to the *pif1Δ* mutation, we hypothesized that the *dna2-2* mutant might be lethal in combination with the *rrm3Δ* but not with the *pif1Δ* mutation. Genetic crosses show that the *dna2-2* mutant is synthetically lethal with the *rrm3Δ* mutation (Table I). Sorbitol in the growth medium suppresses some of the phenotypes of *dna2* mutants (11), but the *dna2-2 rrm3Δ* was synthetically lethal on sorbitol as well as on glucose medium. The *dna2-2 pif1Δ* double mutant, in contrast to *dna2-2 rrm3Δ*, was viable. Although lethality may involve multiple events, the fact that both DNA helicases act at replication forks implies a similar effect of the *dna2-2* and *rrm3* mutations on replication fork progression, possibly at the RFB and/or in telomere regions where they both reportedly function (26).

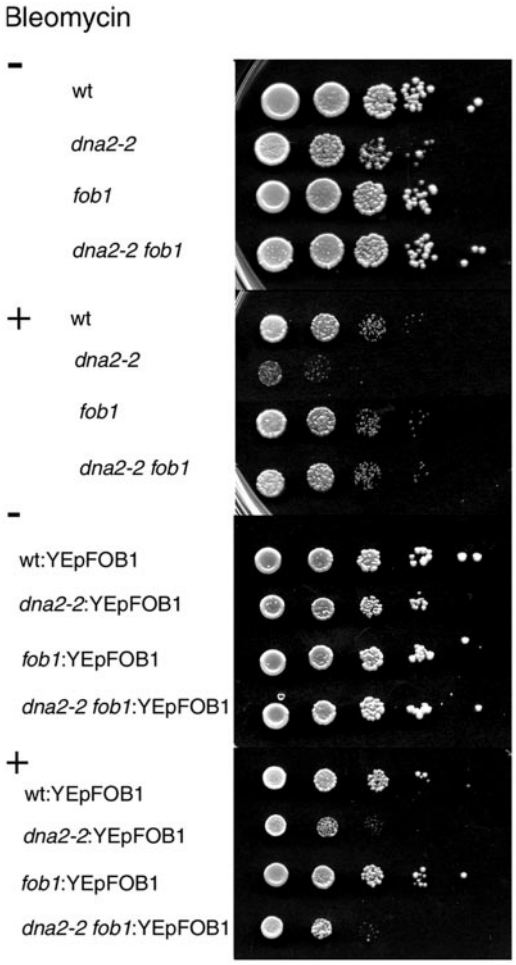

FIG. 5. **Suppression the bleomycin sensitivity of *dna2-2* strains by the *fob1Δ* mutation.** Serial dilutions (10-fold) of the same number of log phase cells from the same culture were inoculated onto each of two YPD plates with and without 15 μg/ml of bleomycin. The plates were incubated at 30 °C for 3 days. Strains are indicated in the figure.

Suppression of Sensitivity of the dna2-2 Mutant to Bleomycin by the fob1Δ Mutation—We have shown that *fob1Δ* suppresses the *dna2-2* rDNA DSB phenotype, and wished to see if *fob1Δ* would also suppress the sensitivity of the *dna2-2* mutant to more global DSB-inducing agents. Since *dna2* mutants are sensitive to bleomycin (26), we used bleomycin, a radiomimetic DNA-damaging agent that causes DSBs with no demonstrated specificity for the rDNA to investigate this. Bleomycin was of particular interest because it causes relocalization of Dna2p away from telomeres *in vivo* (26). We tested the effect of *fob1Δ* on drug sensitivity using a dilution assay for bleomycin sensitivity (Fig. 5). Clearly, the *fob1Δ* mutation suppressed the sensitivity of *dna2-2* to bleomycin. Introduction of a plasmid-encoded *FOB1* gene into the *dna2-2 fob1Δ* strain restored the strain's blemycin sensitivity, verifying that the bleomycin resistance of *dna2-2 fob1Δ* was due to the deletion of *FOB1*. The result suggests that in addition to its role at the RFB in rDNA, *FOB1* may be involved in bleomycin-induced DBS repair pathways that require *DNA2*. Identical results were observed using MMS (not shown).

Lengthening of Telomeres in the dna2-2 fob1Δ Mutant—In

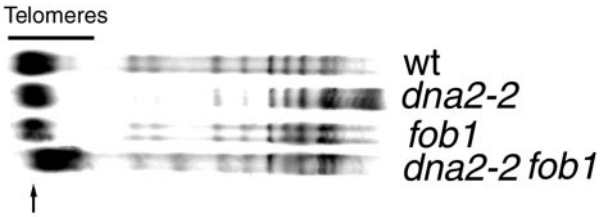

FIG. 6. **Telomere lengthening in *dna2-2 fob1*Δ mutants.** Genomic DNAs from indicated strains grown to log phase were digested with *Xho*I, fractioned by neutral one-dimensional gel electrophoresis, and analyzed by Southern hybridization using a telomere probe as previously described (26). The *arrow* marks the broad band of yeast telomeres that represents the terminal *Xho*I fragment from Y′ telomeres, which are slightly heterogenous, and is used to determine relative telomere length.

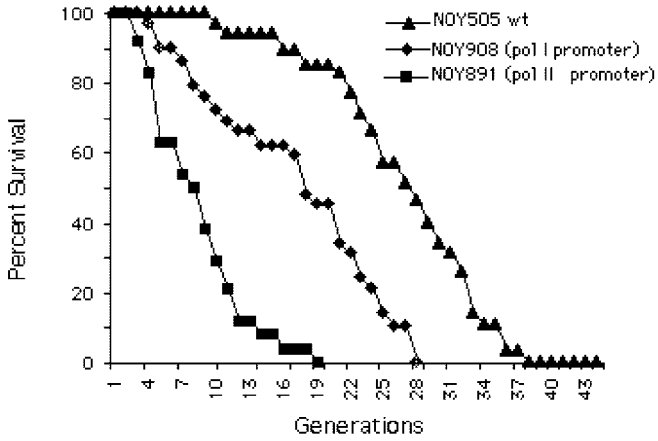

FIG. 7. **Effect of deletion of the rDNA from chromosome XII on yeast lifespan.** Median lifespan data are given in the text where the strains are described. Lifespans were determined on about 50 cells as previously described (11).

yeast, not only rDNA but also telomere integrity affects lifespan; longer telomeres correlate with shorter lifespan (50). A mutation in *FOB1* partially suppresses the phenotype of reduced lifespan of the *dna2-2* mutant (11). Dna2, as well as Rrm3, and Pif1 helicases have all been shown to play roles at telomeres as well as in the rDNA (21, 26, 41, 47, 51). Although Dna2p is associated with rDNA during early S-phase, it is recruited to telomeric regions in late S-phase (26). Since *DNA2* and *FOB1* are important in maintenance of rDNA stability, we wanted to investigate their effects on telomere maintenance. We isolated genomic DNA and digested with *Xho*I, which creates a broad band, due to the heterogeneity of yeast telomeres, of about 1.3 kb that represents the termini of the Y′ telomeres (26). The DNA was resolved by one-dimensional agarose gel electrophoresis and hybridized with a telomere probe. Telomere length is slightly affected, if at all, in the *dna2-2* or *fob1*Δ single mutant. Significant lengthening of the telomere was observed in the *dna2-2 fob1*Δ double mutant, however (Fig. 6). This is the first piece of evidence that Fob1 may act outside of the rDNA, possibly affecting telomere replication and recombination or the levels of Sir2 in telomere chromatin. Longer telomeres in *dna2-2 fob1*Δ might account for the fact that introduction of *fob1*Δ into the *dna2* strain did not restore lifespan to wild-type levels (26), since telomere length has been shown to be inversely proportional to lifespan in yeast (50, 52).

Ribosomal Gene Deletions from the Chromosome Accelerate Rather than Prevent Aging—There is considerable interest in the degree to which the rDNA contributes to lifespan. Since we used the rDNA as a model for studying the role of replication in lifespan, it was important for us to directly address the overall importance of the rDNA. Previous work has shown that hyper-recombination in the rDNA can give rise to extrachromosomal circles, ERCs, that have an origin of replication and can therefore replicate to levels equivalent to the entire genome content of the cell; and this has been shown to be one cause of aging (53–55). If the tandemly repeated rDNA on chromosome XII is the principal determinant of lifespan, then deletion of this rDNA locus from the chromosome might extend lifespan. To further investigate how the rDNA contributes to aging, we took advantage of the strains that Nomura and co-workers (30, 31) have constructed, designated *rdn*ΔΔ, in which all of the copies of the rDNA repeats are deleted from their chromosomal location. The rDNA needs of each of such *rdn*ΔΔ strains are supplied via plasmids carrying a single copy of the rDNA. In our study, these plasmids contain the 2-μm origin of replication and 2-μm partition functions and are expected to segregate symmetrically. NOY908 has a plasmid with the normal promoter for rDNA, recognized by RNA polymerase I (*pol I*, Fig. 7). NOY891 contains a plasmid with a *GAL7* promoter before the rDNA, recognized by RNA polymerase II (*pol II*, Fig. 7). NOY891 grows on galactose but not on glucose medium. The level of rRNA transcription in the strains expressing the rDNA genes under the pol I promoter or the pol II *GAL7* promoter are about one-half and one-third of wild type, respectively (31). Both plasmids are maintained at about the same copy number as the number of *RDN* repeats in a wild-type strain (31, 56). Thus, the level of transcription per unit rDNA repeat is similar for both plasmids and for the wild-type chromosome. The *rdn*ΔΔ strain carrying a plasmid expressing the rDNA from the pol I promoter has a significantly shorter average lifespan (16.2 generations for NOY908) than wild type (25.8 generations for NOY505, Fig. 7). This might be due to the extrachromosomal DNA, since it has been shown that rDNA plasmids from the chromosome shorten lifespan to a similar degree (53). The surprise, however, was that the strain carrying a plasmid expressing the rDNA genes under the *GAL7* inducible promoter has an even more severely shortened average lifespan (7.5 generations, see NOY891 in Fig. 7) compared with wild-type NOY505 (25.8 generations). This suggests that recombination in the rDNA repeats on chromsome XII is not the sole determinant of life span, though it does not rule out some other change in the nucleolus being important, since the *rdn*ΔΔ strains have nucleoli (see "Discussion"). Our previous work showed that deletion of *FOB1* failed to restore full-length lifespan to *dna2* mutants, and this work shows that deletion of *FOB1* lengthens telomeres, supporting the notion that the rDNA might not be the only locus affecting aging in *dna2* mutants.

DISCUSSION

We have used physical analysis of replication intermediates to characterize the replication of the rDNA in the yeast *dna2-2* mutant, defective in a replicative helicase-nuclease. We find that replication pausing at the RFB is increased in the mutant. In conjunction with this pausing, the numbers of converging replication forks, as well as Holliday junction intermediates, are increased. These findings support our previous suggestion that replication fork stalling in the rDNA could account for the apparent instability of the rDNA we observed indirectly as increased frequency of loss of an *ADE2* marker from the rDNA (11). Taken together with other data supporting increased rDNA instability in cells near the end of their lifespan compared with young cells (57), the new data further suggest that the shortened lifespan of replication mutants results, at least in part, from increased replication fork stalling and/or defects in repair of stalled forks leading to hyper-recombination. To further support this hypothesis, we investigated whether the

increased replication fork pausing might be accompanied by the formation of DSBs in the rDNA. We found that, even in wild type, DSBs arise at a low frequency at forks stalled at the RFB in the rDNA. Their frequency is increased in the *dna2-2* mutant by the same amount as pausing at the RFB is increased. We suggest that these DSBs are recombinogenic and lead to instability of the rDNA repeats in both wild type and mutant.

Novel Evidence from Yeast for the Classical Bacterial Models—The appearance of DSBs and correlation with increase in replication fork pausing fit well with the model established in prokaryotes, where replication stalling is correlated with DSBs and recombination, leading to increased chromosome instability if incorrectly repaired. In *E. coli* cells deficient in the DnaB or rep helicase, replication forks stall and are converted to DSBs (58). The RuvABC proteins, catalyzing migration and cleavage of Holliday junctions, are thought to be involved in the DSB formation (59). A model has been proposed that a Holliday junction is generated at arrested replication forks by branch migration and annealing of the newly synthesized strands, and evidence for the structure, a regressed fork known as a chicken foot, has been obtained (3). RuvAB binds and stabilizes the structure whereas RuvC (in conjunction with RecBCD and RecA) cleaves a chromosome arm, leading to resolution of the junctions and restoration of the replication fork (59). Slightly different intermediates arise in forks arrested by mutations in the pol III holoenzyme or at sites of UV damage, where RecG is required for formation of the Holliday junction (60–63). In addition to stalling at random sites due to mutations in replication proteins or due to DNA damage, normal replication forks are blocked at natural, sequence-specific stalling sites, such as RFBs. These sites are recombinogenic (34, 64). In *E. coli*, the replication termination system consists of two components: the *ter* site, a sequence-specific replication terminus, and a *trans*-acting replication terminator protein, Tus, that forms protein-DNA complexes at the *ter* site and antagonizes the action of the replicative helicase. Replication forks are arrested at the *ter* site in a polar fashion (5, 65–68). Recently, an intermediate with double-strand ends that involves reinitiation at the origin of replication rather than a regressed fork has been shown to arise at two *ter* sites inserted at ectopic positions in the *E. coli* genome (69). Thus, a common intermediate in restoring bacterial replication forks after stalling is a DSB, which is thought to lead to recombination, *i.e.* genomic instability. The yeast *dna2-2* mutant might either generate increased numbers of paused forks or be defective in the repair and restoration of DNA replication at stalled forks.

Mechanism of Events at the RFB—A schematic summary of possible interpretations of the replication intermediates is shown in Fig. 8. An initiation bubble is formed as replication initiates from an active ARS inside the rDNA and proceeds bi-directionally (Fig. 8, *A* and *B*). A leftward moving fork is halted at the RFB while a rightward fork bypasses the RFB in a repeat lacking an active ARS (Fig. *8C*). The RFB intermediate must persist until a rightward fork converges at the RFB and normal resolution, as at other replication sites in each replicon, takes place (Fig. 8, *E* and *F*). During the pause, Sogo and co-workers have shown that there is a 3-nt gap on the leading strand but the lagging strand is completely replicated up to the junction (6). This 3-nt gap could serve to nucleate reassociating of the nascent strands and Holliday junction (*chicken foot*, Fig. 8*C*) generation. In the *dna2-2* mutant, slow processing of the lagging strand might enhance both pausing and fork regression or another possible role for *dna2* would be to remove the regressed fork via its helicase activity. Once the Holliday structure forms, it is susceptible to cutting by Holliday junction resolving enzymes analogous to RuvC or presumably Fob1, which shows some homology to RuvC (44) (Fig. 8, *C*, *D*, and *B*). If branch migration is limited, such cleavage could give rise to the 2.2- and 2.3-kb linear fragments that hybridize only to the region between the ARS (the right end of the fragment) and the RFB. The other breakage product would be 4.6 kb and migrate with non-replicating molecules (Fig. 8, *D–F*). If replication proceeds through the RFB but pauses near the 35 S RNA termination region, the 3-kb fragment shown in Fig. 3 might arise from breakage there (Fig. 8*G*). The DSBs could use recombination to restore an active replication fork and since the rDNA is repetitive this could lead to expansion or contraction of the number of repeats as proposed by others (27). The DSBs we observed occur on a fairly small population of RIs, but the broken chromosomal fragment would be recombinogenic and could stimulate rDNA recombination rate.

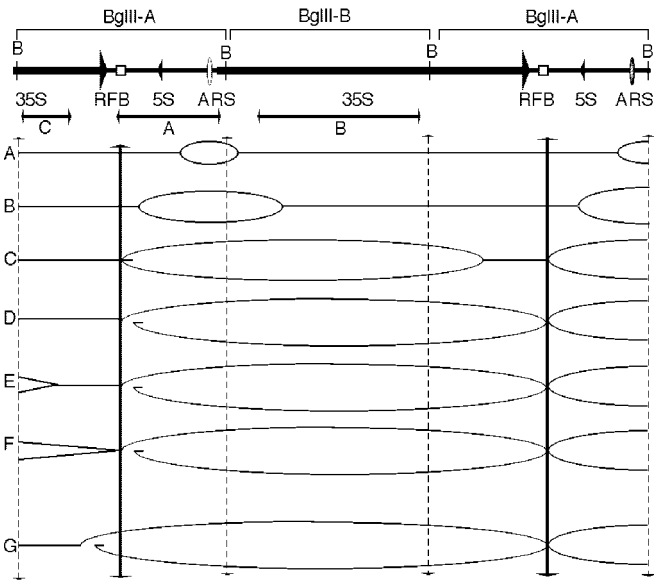

FIG. 8. **Schematic interpretation of results.** Illustrated on the top are 1.5 rDNA units from chromosome XII drawn to scale. *ARS*, replication origin; *RFB*, replication fork barrier; *35S* and *5S*, transcription units for 35 S and 5 S RNA, respectively. *Arrows*, direction of transcription direction. *B*, *Bgl*II. Three probes, called *A*, *B*, and *C* used in our Southern hybridizations are shown below the rDNA. See text for details. *Vertical solid line*, RFB; *vertical dashed line*, *Bgl*II site.

Relationship Between Fork Pausing at the RFB and Lifespan—Pausing and DSB formation in the rDNA are drastically reduced in the absence of the replication fork block protein, Fob1. This may explain why we observed that a *dna2fob1*Δ strain showed slightly increased lifespan (11). The fact that lifespan did not return to wild-type levels in the *dna2fob1*Δ strain might be explained by the fact that repair of the residual DSBs in *fob1*Δ mutants requires *DNA2*. An alternative explanation for the short lifespan of *dna2fob1*Δ is that replication fork pausing in the *dna2-2* mutant is not limited to the rDNA, but occurs throughout the genome due to either stochastic failures of the mutant Dna2 helicase/nuclease during Okazaki fragment maturation or due to other sequence-specific pauses that are not affected by Fob1. Replication fork pausing has been documented at centromeric DNA, in the promoters of highly transcribed genes, at telomeres, and in silenced chromatin (47, 70–72). These pauses are due to the binding of specific proteins, but probably not to binding of Fob1. In an *rrm3*Δ mutant, pausing is increased at all of these chromosomal locations, as well as in the rDNA (47). Our demonstration here that *rrm3*Δ is synthetically lethal with *dna2-2* (Table I), suggests that *DNA2* and *RRM3* may have overlapping or synergistic functions, and thus it is plausible that there is increased paus-

ing at these additional regions in the *dna2-2* mutant accounting for lifespan truncation even when *FOB1* is deleted. DSBs can also occur in replication slow zones if forks are not stabilized by Mec1 (7).

It is interesting that lifespan is extended in wild type and in a *dna2* strain not only in a *fob1*Δ strain but also in strains carrying an extra copy of *SIR2* (11, 73). A recent finding suggests that *FOB1* inactivation and *SIR2* overexpression may have similar effects on rDNA replication and pausing. In a *sir2*Δ strain, replication initiation within the rDNA is increased by 60%, increasing by definition the frequency of pausing by a similar degree (48). Recombination of replication forks stalled at the RFB also increases (74). Extra copies of Sir2 would therefore be expected to decrease initiation and hence replication pausing, supporting our model that faulty processing of replication forks paused at the RFB contributes to genomic instability and decreased lifespan (11). We also show that *fob1*Δ decreases replication initiation in the rDNA.

Role of the rDNA in Aging—The reduced lifespan of the rDNA delete strains suggests that recombination of the rDNA repeats on chromosome XII is not the sole explanation for aging (Fig. 7). In addition, the difference in lifespan between the two *rdn*ΔΔ strains surviving via pol I or pol II is intriguing. The two strains have the same plasmid copy number and about same level of rRNA expression, so it cannot be extrachromosomal DNA *per se* contributing to the shortening of lifespan; nor can it be the level of transcription per rDNA copy. Strains surviving via pol I (pol I nucleoli), and those surviving via pol II (pol II nucleoli) differ in three ways potentially relevant to aging, however. First, they differ both from wild type and from each other in nucleolar morphology/localization. (These strains all have nucleoli.) Second, in poI I nucleoli, pol I is concentrated in the nucleolus while in pol II stains, pol I is more evenly distributed throughout the nucleus. Third, and most relevant to the replicative stress model of aging, in pol I nucleoli, rDNA chromatin is silenced by a Sir2-dependent mechanism (75), whereas in the pol II nucleoli, by definition, the *GAL7* promoter is not silenced, and the chromatin is likely to differ from that in pol I nuclei (30, 31). Since *SIR2* deletion strains show decreased lifespan and an extra copy of *SIR2* extends lifespan, one explanation for the extremely shortened lifespan of the pol II plasmid-containing strain might be failure to recruit Sir2p efficiently to the pol II nucleoli, which might establish a checkpoint signal that affects lifespan (55). We do not know how this might be related to the function Dna2, but Dna2 has also been implicated in checkpoint function.

Repair of Bleomycin-induced DNA Damage—While the suppression of lifespan truncation by the *fob1*Δ was not complete, the suppression of *dna2-2* bleomycin sensitivity by *fob1*Δ was efficient. This suppression could imply that there is a previously unappreciated Fob1-dependent repair pathway that also requires Dna2 helicase. Thus, when Fob1 is absent, Dna2 is not essential for repair because another pathway, such as recombination, is used. Fob1 might create an intermediate during repair that must be processed by Dna2. An alternative explanation for suppression could be antagonism of Dna2 function by Fob1, by analogy to the antagonism between the Tus protein of *E. coli* and the replicative helicase (65, 66). The target of Fob1 at the replication fork barrier is not yet identified. New roles for Fob1 both inside the rDNA and at other sequences are made more likely by recent sequence profile searches that revealed that Fob1p belongs to a super family of polynucleotide transferases, which also includes RuvC (44), the Holliday junction resolvase implicated in cleavage of regressed forks in prokaryotes.

Relationship Between the Effects on rDNA, Telomere, and DNA Damage Repair—Telomeres and rDNA appear to be hot spots on chromosomes that are subject to regulation, affecting chromosome stability and lifespan (8, 76). Like the Sir complexes, Dna2 helicase/nuclease displays dynamic relocalization from rDNA to telomeres, though in the case of Dna2 this is a cell cycle-regulated event. Most of the cellular Dna2 associates with telomeres in G_1 phase. In S phase, there is a dramatic redistribution of Dna2p from telomeres to rDNA and other sites throughout the replicating chromosomes. Dna2p is again localized to telomeres in late S, where it remains through G_2 and until the next S phase. The G_2 localization of Dna2 to telomeres requires the Sir3 silencing protein. Dna2p is functionally required for telomerase-dependent *de novo* telomere synthesis and also participates in telomere lengthening in mutants lacking telomerase. As we have shown here, it is also involved in replication fork processing at the Fob1-dependent RFB in rDNA. The surprising finding that *fob1*Δ suppresses the sensitivity of *dna2* mutants to bleomycin, causes lengthening of telomeres in *dna2* mutants, and affects other phenotypes of the *dna2* mutants as well (not shown) is further evidence that the rDNA is a hot spot for DNA damage sensing and regulation.

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