Binding of the Replication Terminator Protein Fob1p to the Ter Sites of Yeast Causes Polar Fork Arrest*

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Fob1p protein has been implicated in the termination of replication forks at the two tandem termini present in the non-transcribed spacer region located between the sequences encoding the 35 S and the 5 S RNAs of Saccharomyces cerevisiae. However, the biochemistry and mode of action of this protein were previously unknown. We have purified the Fob1p protein to near-homogeneity, and we developed a novel technique to show that it binds specifically to the Ter1 and Ter2 sequences. Interestingly, the two sequences share no detectable homology. We present two lines of evidence showing that the interaction of the Fob1p with the Ter sites causes replication termination. First, a mutant of FOB1, L104S that significantly reduced the binding of the mutant form of the protein to the tandem Ter sites, also failed to promote replication termination in vitro. The mutant did not diminish nuclear transport, and interaction of the mutant form of Fob1p with itself and with another protein encoded in the locus YDR026C suggested that the mutation did not cause global misfolding of the protein. Second, DNA site mutations in the Ter sequences that separately and specifically abolished replication fork arrest at Ter1 or Ter2 also eliminated sequence-specific binding of the Fob1p to the two sites. The work presented here definitively established Ter DNA-Fob1p interaction as an important step in fork arrest.

DNA replication in many prokaryotes is often terminated at sequence-specific replication termini (Ter sites). Replication terminator proteins specifically bind to the Ter sites and arrest forks in an orientation-specific mode with respect to the replication origin (1). In many eukaryotes, although every replicon in the multiorigin chromosomes does not have specific Ter sites, such sites are present in the nontranscribed spacer sequences in the rDNA from yeast to man (1–15).

In Escherichia coli and Bacillus subtilis, the Ter sites bind to replication termination proteins, called Tus and Rtp, respectively, that are contrahelicases (3, 4) and impede replication fork movement not only by binding to the Ter sites but also by protein-protein interaction between the helicase and the terminator protein (5). The rDNAs of Saccharomyces cerevisiae (6, 7), Schizosaccharomyces pombe (8), Xenopus (9), mouse (10), pea (11), and humans (12) have similar replication fork arrest systems located in their nontranscribed spacer elements. In S. pombe, replication fork arrest has been shown to occur at and near the mating type switch locus (13–15).

The rDNA locus of S. cerevisiae (Fig. 1) consists of 100–200 tandem copies of 9.1-kb DNA units present in chromosome number XII. Each unit of the rDNA consists of a 35 S rRNA and a 5 S rRNA gene that transcribe in opposite directions. A non-transcribed spacer called NTS2 containing an origin of replication called ARS (autonomously replicating sequence) is located between the genes encoding the 35 S and the 5 S RNAs (6, 16).

The two replication forks initiated at the ARS face unequal fate. The rightward moving fork moves through the 35 S rDNA in the same direction as that of transcription until it meets the fork coming from the opposite direction. But the leftward moving fork, after passing through the 5 S RNA gene, is arrested at two Ter sites located in the nontranscribed spacer called NTS1 and is thus prevented from entering the 35 S RNA gene. The two Ter sites (known as replication fork barrier or RFB sites) located in NTS1 arrest replication forks in an orientation-dependent manner (6, 7). The Ter1 and Ter2 sites are separated from each other by a few nucleotides and can be further separated by inserting foreign DNA between them without affecting their activities (7).

The Ter sites in prokaryotes and eukaryotes are recombinogenic (2). The yeast rDNA also contains a recombination hot spot HOT1 that consists of two elements called enhancer and initiator that include the RNA polymerase I enhancer and promoters, respectively (see Fig. 1). The enhancer region includes the tandem Ter sites. The initiator and enhancer elements together stimulate high levels of recombination between tandemly repeated sequences even when placed at ectopic sites in a chromosome (17–20).

Deletions of Ter sites (present in the enhancer element) and/or that of the promoter (present in the initiator element), as expected, reduce not only the HOT1 activity (18, 21) but also fork arrest (22). A colony sectoring assay has been developed to identify mutations in genes that reduce HOT1 activity, and this technique has resulted in the identification of several genes that are involved in the recombination process (19, 21). The FOB1 gene was discovered using such a colony sectoring assay and was shown by two-dimensional gel analysis to be involved in fork arrest at the Ter sites of rDNA (22). FOB1 also plays a role in rDNA circle formation and aging (23) and in expansion and contraction of rDNA repeats (24–26).

Although several functions of FOB1 have been described by in vivo experiments, very little is known about its biochemical properties and its precise mechanistic role in promoting repli-

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1 The abbreviations used are: ARS, ARS (autonomously replicating sequence); GST, glutathione-S-transferase; DAPI, 4,6-diamidino-2-phenylindole; wt, wild type; GFP, green fluorescent protein; IR, inverted repeat; IRAS, inverted repeat associated sequence; TAP, tandem affinity purification.
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**EXPERIMENTAL PROCEDURES**

**Strains**—The yeast strains used for sectoring assay are K2307 and its HRM1-1 derivative (gift from Dr. Ralph Keil) (21). The mutant form of *fob1* in HRM1-1 was deleted as described below. This strain (called Δ*fob1*) was used in sectoring assay and two-dimensional gel analysis. *Fob1p* gene was deleted from a protease-deficient strain SUB62 (Mata lys2-801 leu2-3, 112 ura3-52 his3-200 trp1-100) by setting the blender speed to 7 and running the blender 6 times for 30 s each with 4-min intervals between each run. KC1 was added to 150 mM final concentration. The lysate was first centrifuged in a Sorvall RC5B HS4 rotor at 5000 rpm for 10 min and then centrifuged in a Beckman ultracentrifuge at 30,000 rpm for 20 min in a Ti70 rotor, and the supernatant (fraction I) was collected. Ammonium sulfate was added to the fraction I to 70% final concentration, and it was centrifuged at 20,000 rpm for 1 h. The ammonium sulfate pellet was suspended in (150 ml) buffer B (10 mM Tris-HCl, pH 8.0, 5% glycerol, 10 mM β-mercaptoethanol, 1 mM MgCl₂, 1 mM imidazole, 2 mM CaCl₂, 2 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, a mixture of leupeptin, pepstatin, and aprotinin), and conductivity was adjusted to that of buffer C (buffer B + 150 mM NaCl). 5 ml of calmodulin beads (Stratagene) were washed with buffer C, and the lysate was bound to the calmodulin beads at 4 °C for 2 h. The slurry was poured into a column, and the beads were washed with 100 ml of buffer C followed by 50 ml of buffer D (buffer B + 0.65 M NaCl). The protein was eluted with 3 fractions of 1 ml each of buffer E (buffer C with 2 mM EDTA instead of 2 mM CaCl₂). The last four fractions containing the Fob1p fusion protein were pooled and loaded on Mono Q column in a fast pressure liquid chromatograph. The column was washed with 3 ml of buffer E and eluted with buffer C containing a gradient of 150 mM to 1.5 M NaCl. The fractions containing Fob1p protein were pooled and bound to 2.5 ml of glutathione-Sepharose beads (Amersham Biosciences). The beads were washed with 50 ml of buffer C and 50 ml of buffer F (buffer E) and eluting with 10.5 mM fractions of buffer E. Fractions 2 and 3 were used for all experiments.

**Purification of GST-TAP-Fob1p Protein for DNA Binding Assay**—The SUB62 cells expressing TAP-Fob1p fusion protein were grown in 500 ml of medium and harvested. The cells were lysed in a bead beater and centrifuged as above. The supernatant was added to the fraction I to 70% final concentration, and it was centrifuged. The ammonium sulfate pellet was suspended in (150 ml) buffer B (10 mM Tris-HCl, pH 8.0, 5% glycerol, 10 mM β-mercaptoethanol, 1 mM MgCl₂, 1 mM imidazole, 2 mM CaCl₂, 2 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, a mixture of leupeptin, pepstatin, and aprotinin), and conductivity was adjusted to that of buffer C (buffer B + 150 mM NaCl). 8 ml of calmodulin beads (Stratagene) were washed with buffer C, and the lysate was bound to the calmodulin beads at 4 °C for 2 h. The slurry was poured into a column, and the beads were washed with 100 ml of buffer C followed by 50 ml of buffer D (buffer B + 0.65 M NaCl). The protein was eluted with 5 fractions of 1 ml each of buffer E (buffer C with 2 mM EDTA instead of 2 mM CaCl₂). The last four fractions containing the Fob1p fusion protein were pooled and loaded on Mono Q column in a fast pressure liquid chromatograph. The column was washed with 3 ml of buffer E and eluted with buffer C containing a gradient of 150 mM to 1.5 M NaCl. The fractions containing Fob1p protein were pooled and bound to 2.5 ml of glutathione-Sepharose beads (Amersham Biosciences). The beads were washed with 50 ml of buffer C and 50 ml of buffer F (buffer E) and eluting with 10.5 mM fractions of buffer E. Fractions 2 and 3 were used for all experiments.

**DNA Preparation and Two-dimensional Gel Analysis**—Replication intermediates were purified from yeast cells growing in appropriate medium to an OD₆₀₀ of 0.8–0.9. Cell lysis, DNA preparation, and two-dimensional gel analysis were carried out as described previously (32). The DNA was transferred to Nytran membrane (Schleicher & Schüll) and hybridization was carried out as described (33).

**Purification of GST-TAP-Fob1p Protein**—An overnight culture of Δ*fob1* SUB62 cells containing the GST-TAP-FOB1 plasmid was inoculated into 10 liters ofYPD with 2% raffinose and 200 µg/ml hygromycin. Galactose was added to 2% final concentration at midlog phase, and the cells were harvested after 5 h and stored at −70 °C. Cells were quickly chilled in ice and suspended in 10 ml of buffer A (10 mM Tris-HCl, pH 8.0, 0.65 M NaCl, 0.5 mM MgCl₂, 0.5 mM dithiothreitol, 2 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, a mixture of leupeptin, pepstatin, and aprotinin). Acid-washed glass beads (half the total volume of buffer A) were added to the cells, and the cells were lysed in a Waring Blender by setting the blender speed to 7 and running the blender 6 times for 30 s each with 4-min intervals between each run. KC1 was added to 150 mM final concentration. The lysate was first centrifuged in a Sorvall RC5B HS4 rotor at 5000 rpm for 10 min and then centrifuged in a Beckman ultracentrifuge at 30,000 rpm for 20 min in a Ti70 rotor, and the supernatant (fraction I) was collected. Ammonium sulfate was added to the fraction I to 70% final concentration, and it was centrifuged at 20,000 rpm for 1 h. The ammonium sulfate pellet was suspended in (150 ml) buffer B (10 mM Tris-HCl, pH 8.0, 5% glycerol, 10 mM β-mercaptoethanol, 1 mM MgCl₂, 1 mM imidazole, 2 mM CaCl₂, 2 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, a mixture of leupeptin, pepstatin, and aprotinin), and conductivity was adjusted to that of buffer C (buffer B + 150 mM NaCl). 5 ml of calmodulin beads (Stratagene) were washed with buffer C, and the lysate was bound to the calmodulin beads at 4 °C for 2 h. The slurry was poured into a column, and the beads were washed with 100 ml of buffer C followed by 50 ml of buffer D (buffer B + 0.65 M NaCl). The protein was eluted with 5 fractions of 1 ml each of buffer E (buffer C with 2 mM EDTA instead of 2 mM CaCl₂). The last four fractions containing the Fob1p fusion protein were pooled and loaded on Mono Q column in a fast pressure liquid chromatograph. The column was washed with 3 ml of buffer E and eluted with buffer C containing a gradient of 150 mM to 1.5 M NaCl. The fractions containing Fob1p protein were pooled and bound to 2.5 ml of glutathione-Sepharose beads (Amersham Biosciences). The beads were washed with 50 ml of buffer C and 50 ml of buffer F (buffer E) and eluting with 10.5 mM fractions of buffer E. Fractions 2 and 3 were used for all experiments.

**Two-hybrid Assay**—For two-hybrid assay, the pGBT9 and pGAD424 plasmids and their derivatives containing FOB1 gene, its mutants, or deletions were transformed sequentially into the yeast strain PJ69-4A (31) and patched on SD/Leu Trp-, SD/Leu Trp- Ade-, or SD/Leu Trp- His- (with 2 mM aminotriazole) plates, and the interactions were subsequently confirmed by liquid β-galactosidase assay (described in the Chien manual)
affects HOT1 activity and replication termination at Ter1.

C20 factor. In an orientation-dependent manner. The NTS2 has an origin of replication called ARS. The NTS1 has two Ter sites that arrest replication fork coming from the NTS2 region in an orientation-dependent manner. The middle panel shows an expanded view of the NTS1 area containing Ter1 and Ter2 sites. The major restriction sites in the area shown are as follows: RI, EcoRI; H, HindIII; Hp, HpaI; and PvuII. Bottom panel shows a magnified view of the features between the EcoRI and HpaI sites. REBI, the transcription terminator site that binds to Reb1p; ABF1, binding site for a transcription factor. IR and poly-T, inverted repeat and poly(dT) region that are involved in replication termination and HOT1 activity. N35, mutation in IR affects HOT1 activity and replication termination at Ter2. C20 and C26 affect HOT1 activity and replication termination at Ter1.

**DNA Binding Assay**—To the TAP-Fob1p protein bound to calmodulin beads, 20–50 fmol of labeled DNA was added in the presence of 5 μg of poly(dI-dC) in a volume of 200 μl of buffer C (calmodulin binding buffer), and the binding was carried out at room temperature for 15 min. The reaction mixture was loaded on a Bio-Rad polyprep column (catalogue 731-1550), and the beads were washed with 4 × 100 ml of buffer E (EGTA buffer). 200 μl of the eluate was mixed with DNA loading dye without or with 0.25% SDS and fractionated on a polyacrylamide gel and autoradiographed.

**Fluorescence Microscopy**—Cells grown in raffinose and galactose were spotted on slides, treated with DAPI for several minutes, and directly visualized by a Nikon microscope with visible, UV, and fluorescence filters. A Spot RT color camera (Diagnostic Instruments, Inc.) was used to take the photomicrographs.

**RESULTS**

**Replication Fork Arrest at Ter Sites**—The locations and the landmarks around the replication termini derived from published works is shown in Fig. 1. The EcoRI-HpaI fragment should contain both of the Ter sites, and we confirmed this and calibrated our two-dimensional gel technique by cloning the hygromycin resistance marker into the pBB3NTS plasmid (7) (resulting in the plasmid pBB3-H; Fig. 2A). Both the fob1 and wild type yeast cells were transformed with the plasmid, and cultures were grown in rich medium containing hygromycin, and the plasmid DNA was extracted as described above. A typical two-dimensional gel of the SspI cut plasmid that was probed with 32P-labeled NTS1 DNA is shown in Fig. 2C. The interpretation of the pattern is shown in diagrammatic form in Fig. 2B. Consistent with published observations, two tandem termination spots, Ter1 and Ter2, were observed on the Y arc. Most of the forks seemed to be getting arrested at Ter1 and any that leaked through were apparently arrested at Ter2. In addition to the twin Ter spots on the Y arc, two corresponding spots were observed in the X arc. The latter spots are generated by progression of forks initiated from the ARS, moving counterclockwise which merged with the forks that were initiated from the ARS and moving clockwise and arrested at Ter1 and Ter2, thus generating X-shaped intermediates. Previous work had shown that although all of Ter1 was located entirely within the HindIII and HpaI fragment (Fig. 1, center, H to Hp), Ter2 consisted of sequences located not only in this fragment but also of an inverted repeat sequence located just upstream of the HindIII site (see Fig. 1, lower, marked IR) (7). The poly(dT) region located immediately upstream of the IR sequence is needed for enhanced activity of Ter2 (7). The mutations C20 and C26 specifically knocked out Ter1, and the N35 mutation located in the inverted repeat knocks out Ter2 but not Ter1. Experiments utilizing some of these mutations are described below.

**HOT1 Induced Recombination and Isolation of fob1 Mutants**—We wished to investigate the possible role of Fob1p in replication termination by endeavoring to isolate a series of mutants using the HOT1 recombination assay described (18, 21). The principle of the HOT1 assay is diagrammatically shown in Fig. 3A. A yeast strain constructed by Dr. R. Keil (20, 21) contained chromosomally integrated tandem alleles of leu2 and a HOT1 element inserted into the left leu2 sequence. In between the tandem leu2 sequences is a reporter sequence ADE5 whose presence in the ade2, ade5 background of the host generated a red pigment. Loss of the ADE5 marker generated white sectors. We deleted the FOB1 gene from this host by one-step gene disruption as described above and introduced a plasmid expressing FOB1 or a blank plasmid into the host. In the presence of functional FOB1, the HOT1 sequence promoted recombination between the tandem leu2 sequences resulting in a recombinant product excised as a circle and containing the ADE5 marker but without a replication origin. The circle was therefore eliminated from the cell generating white sectors (in red colonies) due to the loss of the ADE5 marker (Fig. 3B, panel

![DNA-Protein Interaction at Yeast Replication Termini](image-url)
When the blank plasmid (without FOB1) was present in the cell, there was very infrequent loss of ADE5 resulting in mostly solid red, non-sectoring colonies (Fig. 3B, panel I). We mutagenized the FOB1 sequence in vitro by PCR and introduced the mutagenized pool of plasmid DNA into the indicator host strain, and we looked for solid red colonies that potentially contained nonfunctional fob1 mutants. By specific site-directed mutagenesis, we also isolated mutant forms that did or did not knock out its recombinogenic activity. We therefore proceeded systematically to analyze the various properties of the mutant forms of Fob1p and the wild type control as described below.

**Interaction of Fob1p with Itself and with Another Protein YDR026C—**Fob1p is known to interact with itself (36) and with a protein encoded by a yeast gene called YDR026C that has no known function (37). We performed systematic two-hybrid analyses of the wild type and the mutant forms described above to investigate both homologous and heterologous protein-protein interactions. First, we proceeded to examine FOB1-FOB1 interaction of the wild type and the various mutant forms.

![Diagram showing HOT1 activity of wild type FOB1 gene and its mutants as revealed by the colony color sectoring assay.](image_signature)

**Fig. 3.** HOT1 activity of wild type FOB1 gene and its mutants as revealed by the colony color sectoring assay. A, schematic diagram showing HOT1 activity. A duplication of leu2 mutants flanking an ADE5 gene and pBR322 sequences is present in chromosome III of the yeast strain used in the sectoring experiment. The 570-bp HOT1 element has been inserted in one of the copies of leu2. The strain that is ade2, ade5 and has the ADE5 gene (inserted in chromosome III) is red when it is non-recombinogenic (in the ∆fob1 background). In the presence of a wild type FOB1 gene that acts at the HOT1 site, the ADE5 gene is lost from the cell by recombination and makes the cell white. Thus the strain with wild type FOB1 gives red colonies with white sectors. B, phenotype of wild type and mutant fob1 gene transformed into the ∆fob1IH strain. I, red colony produced by transformation of the vector pGADh. II, sectoring produced by transformation of plasmid containing wild type FOB1 gene. III, red colony produced by transformation of plasmid containing L104S; and IV, sectoring produced by transformation of plasmid containing K168A.
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FIG. 4. Two-hybrid analyses of protein-protein interactions between wild type FOB1 or YDR026C and FOB1 or its mutant forms and lack of replication fork arrest activity of YDR026C. A and B show FOB1-FOB1 interaction, and C and D show FOB1-YDR026C interaction. A and C show growth on SD/Trp-Leu' plates, and B and D show growth on SD/Trp-Leu' Ade' plates. All cultures patched on the plates had pGBT9 plasmid containing 1–430 amino acids of wild type FOB1 or YDR026C gene as bait. The plasmids tested were pGAD424, pGAD.FOB, pGAD.L104S, pGAD.K168A, and pGAD.C246R. Whereas the wild type and L104S showed Fob1p-Fob1p interaction and Fob1p-YDR026C interactions, the K168A showed interaction with YDR026C but no Fob1p-Fob1p interaction. The C246R mutant neither showed Fob1p-Fob1p interaction nor Fob1p-YDR026C interaction. E and F, two-dimensional gel analysis of wild type and ΔYDR026C strains. Both wild type and ΔYDR026C strains show similar termination spots.

1–430 showed more vigorous self-interaction either between two truncated forms or between the truncated form and the intact protein. We also observed that the truncated form was functional in the sectoring assay described above and in replication termination (not shown). We used the truncated version of FOB1 and YDR026C as baits in the vector pGBT9 (with TRP marker) to look for protein-protein interaction. The intact FOB1 gene and the mutants L104S, K168A, and C246R were all cloned into pGAD424 (LEU2 marker). The results of two-hybrid assay on SD/Trp 'Leu' and SD/Trp 'Leu' Ade' plates are shown in Fig. 4. Five colonies from each pair of plasmids were patched on each indicator plates. All cultures showed good growth on control plates (Fig. 4, A and C). In the FOB1-FOB1 oligomerization assay, only FOB1 and L104S showed good growth on SD/Trp 'Leu' Ade' plates, whereas the vector, K168A, and C246R did not show any growth (Fig. 4, A and B). Similar results were obtained in SD/Trp 'Leu' His' plates and in β-galactosidase assay in solution (not shown). Thus, we conclude from our experiments that both the wt and the L104S mutant form show self-interaction (oligomerization), whereas the K168A and the C246R mutant forms were completely defective in this interaction. We proceeded to analyze the interaction of wt and the various mutant forms of FOB1 with YDR026C. Initially, we had identified the YDR026C gene in a monohybrid assay that used tandem copies of yeast Ter1 sites cloned at the upstream activation sequence. A library of yeast DNA fused in-frame with the activation domain of Gal4 was transformed into the indicator strain to elicit activation of a His or a β-galactosidase reporter gene. In this way, YDR026C was identified as a potential DNA-binding protein that appeared to bind to the Ter region of yeast rDNA. We and others (37) had observed that YDR026C interacted with FOB1 in a two-hybrid assays. Here we have analyzed the interaction of wt and the mutant forms of FOB1 with YDR026C as an indicator of possible global misfolding caused by the mutations in Fob1p. In the two-hybrid analyses, wt FOB1, L104S, and K168A all showed interaction with YDR026C, whereas the blank vector and C246R showed no such interaction on SD/Trp 'Leu' Ade' plates (Fig. 4, C and D). Similar results were also obtained on SD/Trp 'Leu' His' indicator plates and confirmed by β-galactosidase assay. The results of two-hybrid analysis showed that L104S and K168A mutant proteins interacted with YDR026C, whereas the C246R mutant had lost that ability. The YDR026C locus encodes a Myb-like protein that in a monohybrid assay showed binding to rDNA. Because YDR026C showed interaction with Fob1p, we wished to determine whether this interaction is needed for fork arrest. We performed a knockout of the YDR026C locus and performed two-dimensional gel assays to monitor possible fork arrest in the YDR026C-deleted strain. The results (Fig. 4, E and F) showed that both the wt and the isogenic YDR026C knockout strains were fully capable of fork arrest at the Ter sites. The results and other data discussed below showed that whereas Fob1p-Ter DNA interaction was definitely needed for fork arrest, none of the confirmed protein-protein interactions with FOB1 mentioned were needed for replication termination.

The wt Fob1p and L104S and K168A Proteins Were Localized in the Nucleus—Fluorescence microscopy of Fob1p fused to GFP and expressed in yeast showed that Fob1p is a nuclear protein (23, 35). We analyzed the intracellular localization of the wt and the mutant forms of FOB1 as another possible indicator of the folded state of the protein. We constructed N-terminal fusion of FOB1, L104S, and K168A with GFP under a Gal promoter (29). Cells with a modified pGADh plasmid, which expressed Fob1p-GFP or mutant fob1p-GFP, were grown in rich medium (yeast extract + peptone) containing raffinose, galactose, and hygromycin. The cells were put on a microscope slide and photographed in phase contrast, in filtered UV light for DAPI staining or in GFP fluorescence. Fig. 5 shows the comparison of fluorescence studies among wt, L104S, and K168A. Merger of the DAPI and GFP fluorescence suggests that all of them are localized in the nucleus, and the GFP staining is further delimited to a part of the nucleus (e.g., see the merged pattern for wt in Fig. 5). The localized pattern of the GFP fluorescence within the nucleus along with published data (23, 35) suggest that Fob1p was localized to the nucleus. The results showed that the L104S and the K164A mutations did not abolish the transport and localization of the mutant forms of the protein into the nucleus. The protein-protein interaction data and the nuclear/nucleolar localization data, taken together, strongly suggested that the L104S mutation did not cause a global misfolding of the mutant form of the protein.

The L104S Mutation Abolished Fork Arrest—The colony-
sectoring assay described earlier had shown that the L104S mutation abolished HOT1 activity. Is the loss of HOT1 activity due to failure to arrest replication forks? In order to answer this question definitively, we proceeded to perform two-dimensional Brewer-Fangman gel assays (32) of replication fork passage through the NTS1 region of rDNA. Host cells containing a deletion of the FOB1 locus were transformed with a plasmid pGADh that supplied either the wt or a mutant form of fob1, and the cells were grown to mid-log phase in rich medium containing hygromycin, harvested, and lysed. Replication intermediates were prepared by extracting whole cell DNA. The DNA was digested with BglII and resolved in neutral-neutral, Brewer-Fangman two-dimensional gels. The gels were blotted onto Nytran membranes and probed with a 32P-labeled probe specific to the Ter region. The blank plasmid (without FOB1 insert) present in the Δfob1 host cells, as expected, did not generate a Ter spot. As expected, the wt FOB1 also elicited fork arrest as indicated by the presence of the Ter spot(s) just before the inflection point of the Y arc (Fig. 6). The L104S mutant failed to promote termination of replication as evidenced by the lack of a Ter spot (Fig. 6). The K168A protein on the other hand caused replication fork arrest (Fig. 6). Thus, the loss of fork arrest by the L104S mutation correlated with loss of HOT1-mediated recombination. However, this defect was unlikely to be caused by a global misfolding of the mutant form of the protein because, as described above, other properties like FOB1-FOB1 interaction, FOB1-YDR026C interaction, or nuclear localization were unaffected by the mutation. The K168A mutant form retained replication fork arrest, HOT1 activity, and nuclear localization but had lost FOB1-FOB1 interaction properties and is therefore unlikely to have produced a misfolded protein. It should be noted that although K168A had lost Fob1p-Fob1p interaction, it was still able to arrest replication forks. The C246R mutant was negative on the basis of all of the criteria described above and was probably misfolded in the cell.

Purification of Fob1p Protein—We first constructed a GST-FOB1 fusion and attempted to express and purify this protein from E. coli cells. Although the expression was successful and the protein soluble, it was excessively prone to rapid proteolysis, and the protein failed to bind to Ter DNA with any degree of sequence specificity (not shown). An attempt to test the binding of Fob1p to the Ter sites by monohybrid analysis was marginally positive (not shown). These initial results had raised the possibility that Fob1p might bind to the Ter sites via interaction with a DNA binding adapter protein. However, subsequent expression and purification of the protein from yeast dispelled the notion of a possible involvement of a DNA binding, Fob1-interacting adapter protein. We expressed Fob1p as a TAP-FOB1 fusion in yeast. The TAP tag included an IgG-binding site followed by a tobacco etch virus protease cleavage site that in turn was followed by a calmodulin-binding peptide (28) fused in-frame to the reading frame of FOB1. The fusion protein was expressed under the control of an inducible Gal1 promoter. We have determined that the fusion protein was just as effective as the wt protein in promoting colony sectoring in the HOT1 assay and fork arrest in vivo as shown by two-dimensional gel analysis (data not shown). We have also expressed a fusion protein with both the GST and the TAP affinity tags under the Gal1 promoter. Expression of the fusion proteins was confirmed by Western blotting (data not shown).

The protocol for purification of the GST-TAP-Fob1p protein is described in detail under “Experimental Procedures.” At various steps of purification, including the final step, the protein fractions were assayed by Western blotting to confirm the identity of the protein. DNA binding assays were performed as described later to monitor the biological activity of the protein during purification. A Coomassie Blue-stained SDS-polyacrylamide gel shows the degree of purity of the protein (Fig. 7, lanes 1 and 2). The protein contains traces of a proteolytic fragment of Fob1p in addition to the intact protein.

Fob1p Specifically Bound to the Two Ter Sites—To test if Fob1p protein binds to Ter DNA by itself or in complex with other protein(s), we first tested the partially purified protein for DNA binding. We developed a new method for analyzing protein-DNA interaction as described below. The method is schematically shown in Fig. 8A. The cells expressing the TAP-tagged Fob1p were lysed as described above, and the lysate, after removal of insoluble material and ribosomes by ultracentrifugation, was bound to calmodulin affinity beads in the presence of 2 mM CaCl2. The beads were washed, and the TAP-Fob1p protein-DNA complex was eluted after removal of insoluble material and ribosomes by ultracentrifugation, was bound to calmodulin affinity beads in the presence of 2 mM CaCl2. The beads were washed, and the TAP-Fob1p protein-DNA complex was eluted with 2 mM EGTA as described above. The eluted samples were resolved in a non-denaturing polyacrylamide gel (Fig. 8B). The HaeIII fragments of pUC 18 showed no detectable binding under the experimental conditions to the beads containing Fob1p (Fig. 8B, lanes pUC-I and pUC-B). In contrast, when Ter
DNA alone (Fig. 8B, lane Ter1,2 I) or Ter DNA mixed with the pUC18 fragments (Fig. 8B, lane pUC+Ter1,2 I) was loaded on the column, only the Ter fragment bound specifically to the protein on the column (arrow, Fig. 8B, lanes Ter1,2 B and pUC+Ter1,2 B). In this experiment the eluted DNA was mixed with only DNA loading dye without SDS. In addition to a majority of free Ter DNA, another band characteristic of the Fob1p-DNA complex (asterisk, Fig. 8B) was also resolved. In order to make sure that the sequence-specific binding was caused by Fob1p and not by a Fob1p-accessory protein complex, we repeated the binding experiments with highly purified Fob1p and obtained identical results (not shown). Fob1p bound separately to both the Ter1 and Ter2 sequences, and the binding was physiologically significant. In order to further narrow down the binding sequences and to evaluate the physiological significance of the binding, we made use of several mutants at the Ter1 and Ter2 sites that selectively knock out Ter1 or Ter2 activity (7). The C26 mutant (and C20) that has a two-base substitution at the Ter1 site is known to inactivate Ter1 but not Ter2 and conversely the N35 mutation at the inverted repeats knocks out Ter2 but not Ter1 (7). We wished to determine whether a 40-bp (or 42-bp) DNA (oligonucleotide) that contains a functional Ter1 site specifically bound to Fob1p and if the C26 mutations (which includes C20 mutation) abolished the DNA binding activity. Sequences of 40-base oligonucleotides are shown in Fig. 9B. As shown in Fig. 9C, when the 42-bp oligonucleotide pair containing the wild type Ter1 was loaded (Fig. 9, Ter1 lane I) on the beads it bound to Fob1p protein (Fig. 9, Ter1, lane B). In contrast, when the double-stranded labeled oligonucleotide containing the C26 mutation (DNA containing the same Ter1 sequence with only 2-bp change; Fig. 9B) was loaded (Fig. 9, C26 lane I; the minor band below the major band is un-annealed labeled DNA), there was no binding of the DNA to the Fob1p protein (Fig. 9, C26, lane B). Similarly, when a 40-bp DNA containing the M4M5 substitution (Fig. 9B) was loaded (Fig. 9, M4M5, lane I) on the column, there was no binding to the Fob1p protein (Fig. 9C, M4,5 B). We have carried out the oligonucleotide binding experiments with highly purified protein (Fig. 7) with identical results (Fig. 9D). The Ter2 site includes three sequence segments: a poly(dT-dA) region, an inverted repeat (IR), and an inverted repeat-associated sequence (IRAS) as shown (Fig. 9A). Substitution mutations in the IRAS are known to inactivate Ter2 activity (7). The mutation N35 (Fig. 1 and Fig. 10A) is known to knock out completely replication termination at Ter2 (7). Huang and Keil (20) have shown that the mutations C26 and N35 also reduce the HOT1 activity. We also wished to investigate possible binding of Fob1p to the inverted repeat site and the adjacent (IRAS). Oligonucleotides containing either the wild type IR or the N35 mutation were end-labeled and used to monitor Fob1p binding. When the 42-bp-long, inverted repeat oligonucleotide pair (IR) was loaded (Fig. 10B, IR, lane I) on the calmodulin beads containing Fob1p, it bound to the beads as shown in Fig. 10B, IR, lane B. However, when the (42 bp) DNA containing the N35 mutation (Fig. 10A) was similarly loaded (Fig. 10B, N35, lane I), the binding was drastically reduced (Fig. 10B, N35, lane B). We also designed 32-bp oligonucleotide pairs with mutations in the inverted repeat (IRm; Fig 10A), and when this DNA was

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**Fig. 7.** Coomassie Blue-stained 8% SDS-polyacrylamide gel showing the purified Fob1p protein. Lanes 1 and 2 purified Fob1p (two different fractions from calmodulin column); M, molecular weight markers.

**Fig. 8.** Technique that revealed sequence-specific interaction of Fob1p with the Ter1,2 sequences. A, diagrammatic representation of the experimental scheme. B, TAP-tagged Fob1p protein was bound to calmodulin beads in the presence of Ca²⁺, washed to remove adventitiously bound proteins, and incubated with ³²P-labeled pUC18 HaeIII fragments (used as a negative control), a labeled ~370-bp-long fragment containing both Ter1 and Ter2 DNA fragment or the mixture of pUC18 HaeIII and Ter fragments (shown as I in case of pUC, Ter1,2, and pUC+Ter1,2). The beads were washed to remove unbound DNA, and the affinity-bound protein-DNA complex was eluted with EGTA containing buffer. The input (I) and bound (B) DNA was fractionated in a non-denaturing 4% polyacrylamide gel and autoradiographed. The Ter fragment (shown by arrow) bound specifically to the Fob1p immobilized on the calmodulin beads and eluted with EGTA. The extra band shown by the asterisk is probably the protein-DNA complex between Fob1p and Ter DNA (no SDS was used to disrupt DNA-protein complex).
I input and wash were loaded in the lanes showing lanes (cause loss of binding to the protein (all the lanes showing sequence binds to the Fob1p, the C26 mutation and M4M5 substitutions the partially purified Fob1p protein. Whereas the wild type Ter1 sequence binds to the IRAS and tested its binding to Fob1p. The 40-bp DNA samples of wild type TerI and C26 were loaded on calmodulin beads (shown as black rectangle). The C26 mutation has a C-T and T-C change. The IRm mutant and the IRAS mutants used were N35 and IRm. B, autoradiogram showing the binding activity of wild type and mutant Ter1 sequences to the partially purified Fob1p protein. Whereas the wild type Ter1 sequence binds to the Fob1p, the C26 mutation and M4M5 substitutions cause loss of binding to the protein (all the lanes showing B). The input lanes (I) are qualitative as approximately a few microliters from 4-ml input and wash were loaded in the lanes showing I, D. DNA binding activity of purified Fob1p protein. 40-bp DNA samples of wild type Ter1 or C26 were loaded on calmodulin beads (Input Ter1 and C26) containing purified Fob1p protein, and the EGTA eluted protein-DNA complexes were fractionated in 8% polyacrylamide gel. Whereas Ter1 (shown as Bound wt) bound to the beads, C26 DNA did not bind (Bound C26).

Fig. 9. Mutations at the Ter1 site that abolish replication termination also eliminate or significantly reduce sequence-specific interaction of the Ter sites with Fob1p. A, features of DNA at the replication fork blockage sites. The Ter1 sequence is shown as a black rectangle. Ter2 sequence may encompass the poly(dT-dA) region, the inverted repeat, and the sequence known as RF2 which we call here inverted repeat associated sequence (IRAS). B, the 40-bp sequence that encompasses the Ter1 region. The two blocks of 10-bp substitution (M4 and M5) are shown below the wild type sequence that is underlined. The C26 mutation has a C-T and T-C change. C, autoradiogram showing the binding activity of wild type and mutant Ter1 sequences to the partially purified Fob1p protein. Whereas the wild type Ter1 sequence binds to the Fob1p, the C26 mutation and M4M5 substitutions cause loss of binding to the protein (all the lanes showing B). The input lanes (I) are qualitative as approximately a few microliters from 4-ml input and wash were loaded in the lanes showing I. D. DNA binding activity of purified Fob1p protein. 40-bp DNA samples of wild type Ter1 or C26 were loaded on calmodulin beads (Input Ter1 and C26) containing purified Fob1p protein, and the EGTA eluted protein-DNA complexes were fractionated in 8% polyacrylamide gel. Whereas Ter1 (shown as Bound wt) bound to the beads, C26 DNA did not bind (Bound C26).

Mutation in Ter2 DNA affects binding to Fob1p protein. A, 27-bp sequence of Ter2 showing the inverted repeat region. The mutants used were N35 and IRm. B, autoradiogram of 8% polyacrylamide gel showing results of Fob1p binding to IR, IRm, N35, IRm, and IRAS sequences. Whereas the wild type IR sequence binds to the protein, N35 has severe defect in Fob1p binding. The IRm mutant and the IRAS sequence do not show binding.

FIG. 10. The L104S mutation that abolished in vivo replication termination also reduces Fob1p-Ter interaction. A, Western blots of the equal amounts of proteins from the wt and the L104S cells bound to calmodulin beads; B, autoradiogram showing results of 32P-labeled Ter1 DNA binding with the wt and L104S proteins immobilized on calmodulin-agarose beads. The input and eluted fractions were resolved in a non-denaturing 8% polyacrylamide gel and autoradiographed and also quantified in a PhosphorImager. Note that the Leu-104 protein shows very low levels of binding to the DNA probe in comparison with the wt protein.

Fig. 11. The L104S mutation that abolished in vivo replication termination also reduces Fob1p-Ter interaction. A, Western blots of the equal amounts of proteins from the wt and the L104S cells bound to calmodulin beads; B, autoradiogram showing results of 32P-labeled Ter1 DNA binding with the wt and L104S proteins immobilized on calmodulin-agarose beads. The input and eluted fractions were resolved in a non-denaturing 8% polyacrylamide gel and autoradiographed and also quantified in a PhosphorImager. Note that the Leu-104 protein shows very low levels of binding to the DNA probe in comparison with the wt protein.

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DISCUSSION

The mechanism of action of Fob1p protein of S. cerevisiae is of considerable interest not only because of its role in replica-
DNA-Protein Interaction at Yeast Replication Termini

Two different domains each one recognizing one of the two sequences have no detectable homology, thus raising the question of the DNA binding domain(s) of Fob1p. Two confirmed protein-protein interactions involving Fob1p have been discovered to date, none of which seem to be involved in fork arrest. First, Fob1p interacts with itself and thus is an oligomeric protein. However, oligomerization is apparently not required to terminate replication as exemplified by the K168A mutant form that is capable of effecting fork arrest but has lost the ability to oligomerize as determined in a two-hybrid assay. Second, Fob1p interacts, as shown in this work and by others (37), with YDR026C, which encodes a Myb-like protein. But the data presented here showed that the interaction is not involved in fork arrest.

A model building and homology search have suggested that Fob1p has similarity to the integrase sequence of retroviruses (44). In this context it is interesting to note that a plasmid (pBB3NTS; Ref. 7) with Ter sites can integrate into the chromosomal rDNA in the presence of a functional Fob1p and in the absence of a functional SIR2 gene (45), which encodes a histone deacetylase (46–49). In the presence of wt SIR2, such integration was not observed (45). We have accumulated a large number of point mutations of fob1 including some at the region having similarity to the integrase sequence. We plan to investigate whether these mutants block the integration event and to determine whether the integration event is dependent on Fob1p-mediated fork arrest.

It should be pointed out that our work presents a novel technique for the detection of sequence-specific DNA-protein interaction using a calmodulin-binding peptide affinity tag and a calmodulin affinity column. This technique is highly sensitive and has very high signal to noise ratio and should be generally useful.

In conclusion, purification Fob1p and the demonstration that it specifically interacts with the Ter1 and Ter2 sequences provide critical evidence with regard to the mechanism of replication termination.

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