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Ribosomal DNA Replication Fork Barrier and *HOT1* Recombination Hot Spot: Shared Sequences but Independent Activities

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In the ribosomal DNA of *Saccharomyces cerevisiae*, sequences in the nontranscribed spacer 3′ of the 35S ribosomal RNA gene are important to the polar arrest of replication forks at a site called the replication fork barrier (RFB) and also to the *cis*-acting, mitotic hyperrecombination site called *HOT1*. We have found that the RFB and *HOT1* activity share some but not all of their essential sequences. Many of the mutations that reduce *HOT1* recombination also decrease or eliminate fork arrest at one of two closely spaced RFB sites, RFB1 and RFB2. A simple model for the juxtaposition of RFB and *HOT1* sequences is that the breakage of strands in replication forks arrested at RFB stimulates recombination. Contrary to this model, we show here that *HOT1*-stimulated recombination does not require the arrest of forks at the RFB. Therefore, while *HOT1* activity is independent of replication fork arrest, *HOT1* and RFB require some common sequences, suggesting the existence of a common *trans*-acting factor(s).

The ribosomal DNA (rDNA) locus in the yeast *Saccharomyces cerevisiae* consists of 9.1-kb tandem repeats with the 35S rRNA gene, the much smaller 5S rRNA gene, and two nontranscribed spacer (NTS) regions (see Fig. 1) (see references 29 and 22 for reviews of sequence elements in the NTS). NTS2, located between the 5′ ends of the two genes, contains the promoter for the 35S rRNA gene, a weak origin of replication named the rDNA *ARS*, and sequences essential for the *cis*-acting mitotic recombination hot spot *HOT1*. The 35S RNA polymerase I transcriptional enhancer lies in NTS1 near the 3′ end of the 35S gene. NTS1 also contains sequences important for the polar arrest of replication forks (replication fork barrier [RFB]) and *HOT1*. The extent of sequence overlap and the interdependence of these two events in DNA metabolism are unknown.

The rDNA RFB was first identified in *S. cerevisiae*, when high-resolution two-dimensional (2D) gel electrophoresis revealed two closely spaced sites where forks arrest (2), herein called RFB1 and RFB2. RFBs appear to be a highly conserved feature of rDNAs, with barriers being found at the 3′ end of the rRNA genes in a number of other organisms (9, 21, 23, 32, 36, 38). The yeast RFBs efficiently block replication forks traveling in the direction opposite to 35S transcription, together impeding ~90% of encountered forks (2). Fork arrest is not a consequence of transcription per se, since replication forks still arrest at the RFB in cells lacking functional RNA polymerase I (2). The RFB sequences are also not inherently difficult to replicate (2), and thus fork arrest is thought to result from the binding of proteins at the RFB sequences. A protein-mediated mechanism of fork arrest in the rDNA RFB has also been implicated in peas and *Tetrahymena thermophila* (24, 37) and reported to involve the transcription-terminating factor TTF-I in mice and humans (8, 23).

HOT1 sequences from the rDNA, when assayed at ectopic sites in the genome, stimulate mitotic homologous recombination between intra- and interchromosomal repeats (14). Subcloning analysis showed that the sequences necessary for *HOT1* recombination are localized to two noncontiguous regions of the rDNA NTS (35); the E fragment contains the enhancer for 35S transcription, and the I fragment contains the 35S promoter and initiation site (see Fig. 1). When the *HOT1* sequences E and I are inserted next to a construct consisting of direct repeats of *his4* sequences on chromosome III (see Fig. 2A), recombination can be elevated more than 350-fold (12). Through studies of recombination at this ectopic site, *HOT1* activity has been shown to require RNA polymerase I transcription of the repeat elements involved in recombination (12, 35). Mutations in four genes, *HRM1* through *HRM4*, reduce *HOT1*-stimulated recombination (19). *HRM1* was later found to be identical to *FOB1* (3), a gene that was identified in a search for mutants defective for both *HOT1* and RFB activities (17). Studies on *FOB1* indicate that the protein is important for the expansion and contraction of the rDNA array (15) and plays a role in regulating life span (3). The *FOB1* protein is a candidate for creating the physical fork barrier at the RFB, but it is not yet known whether the protein functions by directly binding to DNA.

Evidence from *Escherichia coli* that the arrest of replication forks at sequence-specific sites may be recombinogenic (1, 11, 11a; reviewed in references 18 and 31) has led to the hypothesis that forks blocked at the RFB contribute substantially to *HOT1* recombination (15, 17). However, the apparent difference between the transcription requirements for fork arrest at the RFB and for *HOT1*-stimulated recombination and the requirement of the I fragment for only the latter event might suggest that these activities are independent. We report here that fork arrest is not required for *HOT1* recombination. However, we show that RFB activity and *HOT1* recombination

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share some common *cis*-acting sequences in the rDNA NTS1 region.

MATERIALS AND METHODS

Construction of RFB plasmids. Plasmid pBB3NTS (see Fig. 3A) was provided by Katherine Friedman and was constructed as follows. Vector pBB3 was constructed by ligating the 967-bp *Nde*I-*Sma*I *URA3* fragment to the 2.435-kb *Nde*I-*Sma*I pUC18 vector. Yeast RM14-3a DNA prepared by glass bead lysis (10) was cleaved with *Eco*RI. Fragments were separated by gel electrophoresis, and a visible rDNA band of approximately 2.5 kb was excised and electroeluted. This fragment was cloned into the unique *Eco*RI site of vector pBB3. The resulting plasmid was partially digested with *Eco*RV, and a 425-bp *Nhe*I-*Hin*dIII fragment containing *ARS1* was blunt-ended with Klenow enzyme (Boehringer) and cloned into the *Eco*RV site of the rDNA. *ARS1* was oriented so that its *Hin*dIII site is closest to the RFB. The *Hin*dIII-*Hpa*I fragment in plasmid pBB3NTSΔHH was deleted by digesting plasmid pBB3NTS with *Hin*dIII and *Hpa*I, filling in the *Hin*dIII site with Klenow polymerase, and ligating the resulting ends.

YEp24 plasmids containing the *Hin*dIII-*Hpa*I RFB fragment were constructed in two steps following the procedure used by Kobayashi et al. (16). The *Hin*dIII site of the *Hin*dIII-*Hpa*I fragment from pBB3NTS was blunted with Klenow polymerase, and the fragment was ligated into the *Hin*cII site of the polylinker of pUC18. Plasmids were screened for orientation of the insert by sequencing across the plasmid with the M13 sequencing primer 1211 (New England Biolabs). For each orientation, the *Sph*I-*Bam*HI fragment of the pUC18 derivatives was cloned between the *Sph*I and *Bam*HI sites of YEp24 to create plasmids YEp24HH$^+$ and YEp24HH$^-$. YEp24HH$^+$ (see Fig. 5A, top) contains the *Hin*dIII-*Hpa*I fragment in the orientation expected to block replication forks coming from the 2μm origin of replication.

Two plasmids were constructed for in vitro mutagenesis. The first construct, used to make mutations M2 through M11, in which blocks of DNA in the region required for RFB activity were replaced, was made by inserting the 837-bp NTS1 *Eco*RI-*Pvu*II fragment from pBB3NTS into the *Eco*RI and *Pvu*II sites of the polylinker of pUC118 to create pUC118RFB. Mutated *Hin*dIII-*Hpa*I fragments were excised from pUC118RFB and ligated between the *Hin*dIII and *Hpa*I sites of pBB3NTS in place of the wild-type fragment. All mutations were confirmed by sequencing and then tested for RFB function. The second mutagenesis construct, used to make mutations M1, M12, and M13, consisted of an insertion of the *Nsi*I-*Pvu*II fragment of pBB3NTS in place of the small *Nsi*I-*Pvu*II fragment of a modified YIp5 vector (pMUTBIAS, provided by Katherine Kolor, has a mutation in the ampicillin resistance gene created by filling in a *Pst*I site). The resulting plasmid, pMUTBIASRFB, was ampicillin sensitive. For sequencing and testing of RFB function, the *Nsi*I-*Sph*I fragment of a mutated pMUTBIASRFB was ligated between the *Sph*I and *Nsi*I sites of pBB3NTS in place of the wild-type fragment.

Site-directed mutagenesis. Site-directed mutations were made in the *Hin*dIII-*Hpa*I fragment by oligonucleotide-directed mutagenesis (4). The annealing reaction mixture consisted of 70 ng of vector, 25 pmol of each kinase-treated oligonucleotide, 2 μl of solution TN (0.2 M Tris-HCl [pH 7.5], 0.5 M NaCl), and 2 μl of 0.1 M $MgCl_2$ in a total volume of 20 μl. This reaction mixture was incubated at 100°C for 3 min and then chilled for 5 min on ice. The synthesis reaction mixture consisted of the 20-μl annealing reaction, 3 μl of solution TDD (5 mM deoxynucleoside triphosphates, 0.1 M Tris-HCl [pH 7.5], 20 mM dithiothreitol), 1 μl (3 U) of T4 DNA polymerase (New England Biolabs), and 1 μl (400 U) of T4 DNA ligase (New England Biolabs) in a total volume of 30 μl. This reaction mix was incubated at 37°C for 90 min. The synthesis reaction was stopped by the addition of 3 μl of solution SE (0.25% sodium dodecyl sulfate, 5 mM EDTA) and a 5-min incubation at 65°C.

For mutagenesis of plasmid pUC118RFB, 5 μl of the synthesis reaction mixture was used to transform the mismatch repair-defective *E. coli* strain DSM3 (33). Primary transformants were cultured in 10 ml of Luria-Bertani medium with 10 μg of ampicillin per ml overnight. Plasmids were recovered with a Qiagen Midi Column procedure. Plasmid DNA (1 μg) was digested with *Sca*I in a 20-μl reaction mix to select against the parental plasmid which had not incorporated the selection oligonucleotide (CTGTGACTGGTGACGCGTCAACCAAGTC). Then 5 μl of the digest was transformed into *E. coli* DH5α. Plasmids which had lost the *Sca*I site were then screened for the presence of the new restriction site indicative of a mutation in the *Hin*dIII-*Hpa*I fragment. Approximately 75% of plasmids that had incorporated the selection oligonucleotide had also incorporated the mutagenesis oligonucleotide.

For mutagenesis of plasmid pMUTBIASRFB, 5 μl of the synthesis reaction mix was used to transform the mismatch repair-defective *E. coli* strain DSM3, and after a 2-h incubation at 37°C, transformants were spread on plates containing ampicillin (10 μg/ml). Incorporation of the selection oligonucleotide (CAC CACGATGCCTGCAGCAATTGGCAAC) restores the *Pst*I site in the ampicillin resistance gene so that cells containing the plasmid are ampicillin resistant. Ampicillin-resistant colonies were screened by restriction digest to determine if the plasmid had incorporated the mutagenesis oligonucleotide. The wild-type and mutant sequences for each *Hin*dIII-*Hpa*I mutation (M1 to M13) are shown in Table 1.

Construction of plasmids containing *HOT1* mutations. The C20 single-base-pair mutation (12) was reconstructed in the RFB test plasmid by two in vitro mutagenesis steps. First, a 2-bp mutation which created an *Mlu*I site and included the C20 single-base-pair conversion was produced by using the pMUTBIASRFB construct as explained above. Second, the *Nsi*I-*Pvu*II fragment that included the 2-bp mutation was moved into a fresh pMUTBIAS vector, mutagenized to the C20 single-base-pair mutation, and screened for the loss of the *Mlu*I site. The C20 mutation was cloned into pBB3NTS for sequencing and testing for RFB function as explained above.

TABLE 1. Mutations in the *Hin*dIII-*Hpa*I region

Mutation	Wild-type sequence	Mutant sequence	New restriction site
M1	TTTCCTATAGTT	GGGATCCGCTGG	*Bam*HI
M2	GAAAAGCTCA	AGGCCTAGAG	*Stu*I
M3	AGAGAATTGA	CTCTCCGGAC	*Bsp*EI
M4	GTATAAGTTT	TGCGCCCGGG	*Sma*I
M5	ATGAGTGCTT	CGTCCCATGG	*Nco*I
M6	AGCGGCAAAC	CTATTGGCCA	*Msc*I
M7	GCACCATCAG	TACGTCGACT	*Sal*I
M8	AGTTTTTTCC	CCGGGGGGAA	*Sma*I
M9	TTCATGGAGC	GCACGTTCTA	*Fsp*I
M10	GACAGTTTGC	TCACTGGGAA	*Eco*RI
M11	GATTTGCCCG	ACGCGTAAAT	*Mlu*I
M12	AGCGTGAAAG	CTATGTTCTA	*Xba*I
M13	AAGCTTCCCG	CCATGGAAAT	*Nco*I

To house the other *HOT1* mutations for 2D gel analysis, pBB3NTS was modified to create plasmid L3520 by replacing the *Hpa*I site with an *Xba*I linker and deleting the *Eco*RI site distal to the enhancer. The 320-bp *Eco*RI-*Xba*I enhancer-containing fragment of L3520 was deleted and replaced with an *Eco*RI-*Hin*dIII-*Xba*I polylinker to create L3520ΔE. The *HOT1* mutants G182, G188, G190, and N35 (mutant sequences are described in reference 12) were recovered as a 320-bp *Eco*RI-*Xba*I fragment and ligated into the polylinker site of L3520ΔE for assay by 2D gel.

2D agarose gel conditions. DNA for 2D gels was isolated from asynchronous, log-phase cultures as described previously (7). The yeast strain RM14-3a (*MAT***a** *cdc7-1 bar1 ura3-53 trp1-289 leu2-3,112 his6*) was used to construct the strains for all 2D gel experiments. From 1 to 4 μg of DNA was used for each 2D gel. The conditions for the first dimension of the gels to test the mutant plasmids and integrated constructs were 0.5% agarose and 1 V/cm for approximately 22 h at 23°C. Conditions for the second dimension were 1.5% agarose, 0.3 μg of ethidium bromide per ml, and 4 to 5 V/cm for 5 to 6 h at 4°C. Probes were labeled by the hexanucleotide priming method (5).

***HOT1* quantitative intrachromosomal recombination assay.** Mutations M4, M5, M6, M10, and M11 were isolated from the pBB3NTS clones as a 320-bp *Eco*RI-*Hpa*I fragment. The *Eco*RI and *Hpa*I ends of the fragment were converted to *Bam*HI and *Xba*I, respectively, by the addition of linkers. The resulting fragment replaced the corresponding region of the plasmid G141 (12). All plasmids were digested at the unique *Cla*I site and targeted to the *his4* locus of chromosome III in RLK 88-3C (35). Mutant chromosomal constructs were confirmed by Southern blot hybridization, and the *HOT1* activity of these mutants was assayed as previously described (12).

RESULTS

Does replication fork arrest contribute to *HOT1* recombination? An appealing hypothesis to explain *HOT1* mitotic hyperrecombination is that replication forks stalled at the RFB are fragile (17, 31). DNA strand breakage at the RFB may generate a lesion that is repaired by homologous recombination. This possibility led us to test whether the two phenotypes are related mechanistically. That is, is the level of *HOT1*-stimulated recombination detected at the chromosome III site dependent on the efficiency of the RFB at this site? The ultimate test of this hypothesis is to determine whether forks are actually being blocked at the chromosome III *HOT1* assay site and whether the presence or absence of barriers correlates with the functional status of *HOT1*.

The E and I fragments of the rDNA NTS (Fig. 1) that are required for *HOT1* activity were assayed for their ability to stimulate recombination at an ectopic site on chromosome III (Fig. 2A) (12, 35). Previous work revealed that both orientations of the E element conferred similar levels of *HOT1* recombination (35). If fork arrest at the polar RFB does play a role in *HOT1* activity, then replication forks must proceed

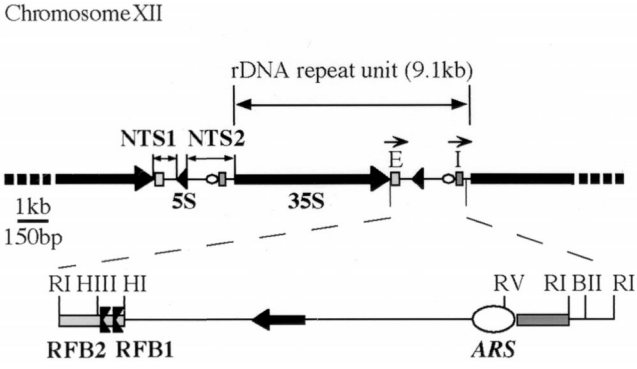


FIG. 1. Features of the NTS region of *S. cerevisiae*. (Top) Repetitive nature of the rDNA array, where 100 to 200 rDNA repeat units (9.1 kb) are found in tandem on chromosome XII. The NTS lies between 35S genes and is separated into NTS1 and NTS2 by the intervening 5S gene. Fragments containing the 35S rDNA transcriptional enhancer and initiator (called E and I, respectively) are essential to *HOT1*-stimulated recombination (35) and are labeled with the arrows oriented in the direction of RNA polymerase I transcription of the 35S gene. (Bottom) A 2.68-kb *Eco*RI (RI) fragment that spans most of the NTS region. Only relevant restriction sites are shown. The positions of RFB1 and RFB2 within the 129-bp *Hin*dIII-*Hpa*I (HIII-HI) fragment of the E fragment are delineated in this report. RFB1 and RFB2 block replication forks polarly, inhibiting forks traveling in the direction opposite of 35S gene transcription (2). The rDNA *ARS*, near the *Eco*RV (RV) site, is also noted. BII, *Bgl*II.

through the E fragment equally in both directions within different cells in a population to establish a similar number of blocked forks. The E fragment is located between *ARS305* and *ARS306*, which fire at similar times early in S phase (30) and initiate very efficiently (28). However, the asymmetrical location of E, closer to *ARS306* than to *ARS305*, predicts that E would be replicated by forks from *ARS306* (Fig. 2A). The normal test orientation of the E fragment in the *HOT1* chromosome III assay is such that replication forks reaching this locus from *ARS306* travel in the direction where they do not confront the barrier activity in E. Consistent with this expectation, a test for barriers in the *Xho*I fragment containing *HOT1* sequences gave no evidence for the RFB (Fig. 2C). The 2D gel pattern was comparable to that of the construct that was missing the 320-bp *Eco*RI-*Hpa*I E fragment (Fig. 2B). These data suggest that this region is replicated by forks traveling from *ARS306*. To confirm this proposal, we performed 2D gel analysis of the direction of replication fork movement (7) in these constructs. We observed that >95% of the replication forks arrive at the *HOT1*-RFB locus from *ARS306* (data not shown). Therefore, >95% of the forks replicate through the E region in the permissive direction.

The possibility that the RFB is not active at this chromosome III location was tested by inverting the E element so that it would now be expected to arrest forks arriving from *ARS306*. High-resolution 2D gel analysis shows that both arrest sites in the *HOT1 Xho*I fragment, RFB1 and RFB2, are functional (Fig. 2D). This observation was confirmed by examining two other restriction fragments in which the RFB was located at different positions (data not shown). From these results, we conclude that a replication fork reaches the E region from *ARS306* and that, if oriented properly, the RFB is capable of efficient fork arrest. Therefore, the two constructs that differ in the orientation of E have significant differences in fork blocking at the RFB. If arresting forks did contribute to *HOT1* activity, we would expect a significant increase in levels of *HOT1*-stimulated recombination from the construct that impeded forks. However, as observed earlier (35) and reconfirmed for the present study, the two constructs were indistinguishable in the level of excisive recombination (bottom of Fig. 2C and D and data not shown). These findings clearly demonstrate that the stimulation of recombination associated with the *HOT1* sequences is not dependent upon stalled replication forks.

Role of the 129-bp *Hin*dIII-*Hpa*I fragment in RFB1 and RFB2. Although *HOT1* recombination does not require replication fork arrest, it is possible that common *cis*-acting sequences play a role in these two activities. To test this possibility, it was first necessary to better define the sequences involved in the rDNA RFB activity.

Previous mapping of the barrier in the yeast rDNA NTS by 2D gel analysis demonstrated that forks initiating at the rDNA *ARS* in NTS2 arrest between the *Hin*dIII and *Hpa*I sites (Fig. 1) (2, 16). High-resolution 2D gel analysis of the chromosomal RFB revealed two discrete arrest sites of unequal strength in this region, RFB1 and RFB2 (2). Because the barriers continue to function when transplanted to plasmids (2, 16), it was possible to perform deletion analysis to identify the sequences important for RFB activity. Kobayashi et al. (16) determined that a 69-bp region within the 129-bp *Hin*dIII-*Hpa*I fragment was sufficient to generate reduced RFB activity on a plasmid. However, their 2D gel conditions failed to resolve the two arrest sites, and it is not known whether the 69-bp region or the 129-bp *Hin*dIII-*Hpa*I fragment containing it is sufficient for arrest at both RFB1 and RFB2.

To determine if sequences for both RFB1 and RFB2 activity are present in the 129-bp *Hin*dIII-*Hpa*I fragment, we analyzed replication of an NTS region from which this fragment was deleted. First, a plasmid was constructed to include the 2.46-kb *Eco*RI fragment that spans most of the NTS region of the rDNA (Fig. 1). The rDNA *ARS* that lies within this *Eco*RI fragment proved to be inefficient in episome maintenance, with the plasmid often integrating into chromosomal rDNA. Therefore, the efficient *ARS1* origin was inserted at the *Eco*RV site near the rDNA *ARS* (Fig. 1), creating plasmid pBB3NTS (Fig. 3A). The location and orientation of the RFB with respect to the origin in pBB3NTS ensured that the replication fork proceeding counterclockwise (CCW) from *ARS1* will encounter the RFB before the fork moving clockwise (CW) (Fig. 3A). Therefore, replication intermediates in which the CCW fork arrests at the RFB until it is met by the CW fork will accumulate. To detect the accumulation of these branched molecules, we examined the 2.2-kb *Ssp*I fragment from pBB3NTS under 2D gel conditions in which the two arrest sites, RFB1 and RFB2, were revealed (Fig. 3C). Although the plasmid RFB generated less intense spots relative to chromosomal barriers (2), the plasmid RFB blocks replication forks long enough for site-specific termination events to occur. Accumulation of the two expected replication termination structures, TER1 and TER2, that correspond with the arrest at RFB1 and RFB2, respectively, are observed along the hybridization line of X-shaped molecules (Fig. 3B and C).

Next, the role of the *Hin*dIII-*Hpa*I fragment in RFB1 and RFB2 was assessed by deleting it from pBB3NTS to create pBB3NTSΔHH. Under high-resolution 2D gel analysis, the replication intermediates from pBB3NTSΔHH lacked any trace of either arrest site and either specific termination site (Fig. 3D). These results indicate that the *Hin*dIII-*Hpa*I fragment is necessary for the function of both RFB1 and RFB2 on a plasmid. They also demonstrate that no other regions within the *Eco*RI fragment, which spans most of the NTS region, were sufficient to significantly arrest the progress of replication forks.

Sequences within the *Hin*dIII-*Hpa*I fragment responsible for RFB1 and RFB2. Since it appeared that the *Hin*dIII-*Hpa*I

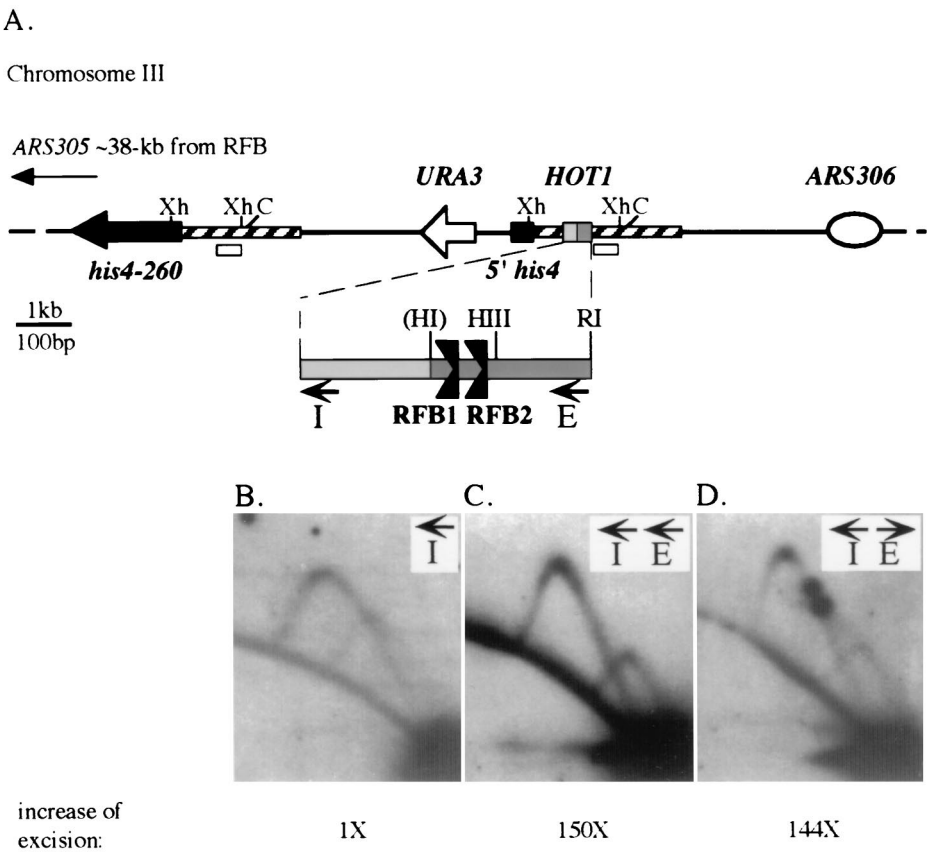


FIG. 2. *HOT1*-stimulated recombination independent of RFB fork arrest. (A) Construct for *HOT1* quantitative intrachromosomal recombination assay. The sequences that lie between the *Cla*I (C) sites were integrated into the *his4* locus on chromosome III of RLK 88-3C as previously described (12). The E-I *HOT1* sequences are inserted to the 5′ side of the repeated *his4* sequences to stimulate homologous recombination between these sequences. Intramolecular recombination between repeated sequences of the 5′ end of *his4* or the flanking chromosomal DNA, indicated by the striped area, can result in excision of the intervening *URA3* marker. The two flanking origins are indicated: *ARS305* and *ARS306* are located ~38 and ~6 kb, respectively, from the E-I region. The bottom map displays the orientation of the 255-bp I and 320-bp E fragments and the RFBs. The arrows for E and I and restriction sites in E are indicated as in Fig. 1. The *Hpa*I (HI) site is not present in this construct. Xh, *Xho*I. (B, C, and D) Autoradiograms of high-resolution 2D gel of three *HOT1* constructs at the *his4* locus on chromosome III. For each gel, the *Xho*I fragment was probed with chromosomal sequences (open rectangles in panel A). The *Xho*I fragment of interest is a 1.7-kb fragment for B and a 2-kb fragment for C and D. Due to the duplicated chromosomal and *his4* sequences in the *HOT1* cassette, the probe hybridized to a second *Xho*I fragment near the *his4-260* gene. Hybridization to this smaller fragment, 1.4 kb, is observed as a second simple Y arc in the lower right corner of the 2D gels. The number beneath each gel is the fold stimulation of excision by *HOT1*, taken from Voelkel-Meiman et al. (35). These data were reconfirmed in the present study (data not shown). The strains used were M51 (B), M39 (C), and M78 (D) (35).

fragment is necessary to generate RFB1 and RFB2 at an ectopic site on a plasmid, we subjected the 129-bp *Hin*dIII-*Hpa*I region to systematic mutagenesis of consecutive 10-bp regions (12 bp in the case of M1). Mutations in the *Hin*dIII-*Hpa*I region were generated in a mutagenesis plasmid (see Materials and Methods) and then transferred to pBB3NTS, in which RFB function could be tested in the context of almost the entire NTS region. Each base pair within the sequence targeted for mutagenesis was replaced with another base pair. An effort was made to create the least conservative changes possible: adenines were replaced with cytosines, guanines were replaced by thymines, and vice versa. However, for ease of screening, each mutation created a new restriction site that sometimes made it impossible to make the least conservative change in the region. In no case did any base within the mutated sequence remain the same as it was in the wild-type sequence (Table 1). All mutated *Hin*dIII-*Hpa*I regions were sequenced to confirm that no unintended base pair changes were created.

High-resolution 2D gel analyses of *Ssp*I fragments from each of the 13 block mutations were performed. Figure 4A summarizes all of the 2D results obtained, and the autoradiograms of wild-type plasmid and some mutant plasmids are shown in Fig. 4B. Eight of the 13 mutations do not differ significantly from wild-type RFB behavior (Fig. 4A; M3, M6, and M13 in Fig. 4B). All exhibit two intense spots along the simple Y arc, corresponding in location to RFB1 and RFB2 and the two prominent termination signals. Mutations M4 and M5 displayed only one barrier, located at the position of RFB2 and a single termination species at the position of TER2 (Fig. 4B). This result indicates that the sequences within the 20 bp covered by mutations M4 and M5 are required for RFB1 but not for RFB2 when the NTS region is on a plasmid.

Similar analysis of mutations M10, M11, and M12 uncovered the importance of a nearby 30-bp stretch in causing the arrest of forks at RFB2. M10 and M11 completely abolished arrest at RFB2 (Fig. 4B). These changes in an RFB activity are again reflected in the shift of terminating structures, in this case to TER1. M12 produced a somewhat reduced accumulation of arrested forks at RFB2 and termination structures at TER2. Overlapping autoradiograms of M5 and M10, offset horizon-

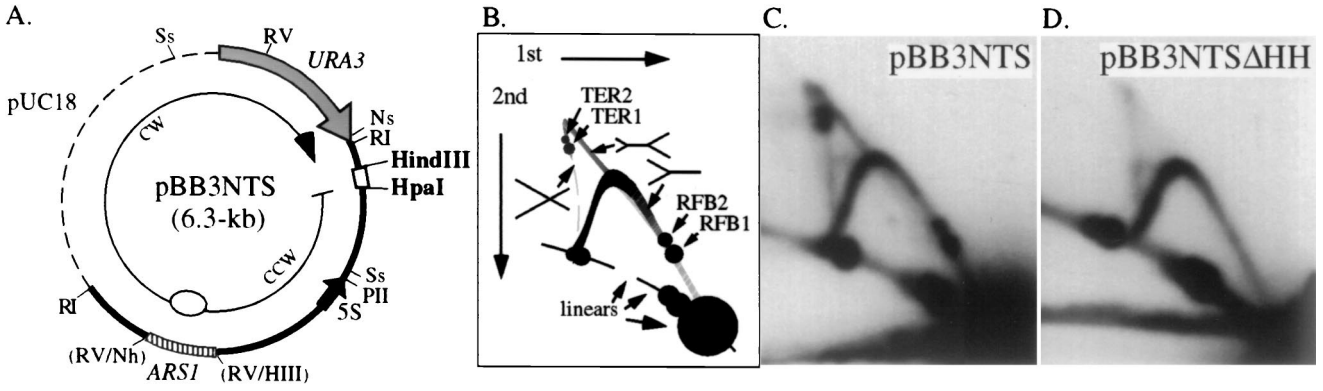


FIG. 3. Deletion of the *Hin*dIII-*Hpa*I region eliminates RFB1 and RFB2. (A) Map of the 6.3-kb plasmid pBB3NTS. The dashed line is vector sequence from pUC18. Only relevant restriction sites are noted. The thicker line between the *Eco*RI (RI) sites corresponds to the 2.46-kb *Eco*RI NTS region (a subfragment from the restriction map in Fig. 1) from the rDNA of RM14-3a. The locations of *ARS1* and *URA3* are indicated. A 425-bp *Nhe*I-*Hin*dIII (Nh-H3) fragment containing *ARS1* was blunt-ended and inserted into the *Eco*RV (RV) site near the rDNA *ARS* (Fig. 1) to improve the efficiency of extrachromosomal maintenance of the plasmid. Bidirectional replication initiating from *ARS1* creates a CCW fork that is blocked by the RFBs before meeting the CW fork. Ss, *Ssp*I; Ns, *Nsi*I; Sp, *Sph*I; PII, *Pvu*II. (B) Schematic diagram of the migration of different replication intermediates in 2D gels shown in C and D. Accumulation of arrested forks results in the two intense spots of hybridization (RFB1 and RFB2) along the arc of Y intermediates. The nearly vertical dashed line represents the pattern of hybridization seen for X-shaped, or terminating, molecules. Termination at RFB1 and RFB2 results in the accumulation of X-shaped molecules TER1 and TER2, respectively. The thicker diagonal gray line corresponds to the hybridization pattern for double-Y replication intermediates. (C and D) High-resolution 2D gels of the 2.2-kb *Ssp*I (Ss in panel A) fragment from pBB3NTS and pBB3NTSΔHH, respectively, probed with *URA3* sequences.

tally by 4 mm, allow the unambiguous assignment of RFB1 and RFB2 in the two mutations (last panel of Fig. 4B).

To test whether the plasmid results are valid in the context of the chromosomal rDNA locus, plasmids containing the mutation at either M5 or M11 were integrated into the rDNA on chromosome XII. The plasmid used for chromosomal integration was similar to pBB3NTS but lacked the *ARS1* sequence and contained a fragment of λ DNA to serve as a unique hybridization probe. Transplacement of just the NTS region was attempted; however, when 10 transformant cultures of each mutant type were screened for the presence of the unique restriction site created by the mutation, all were found to have lost the restriction site, indicating that the mutations had been repaired through gene conversion to wild-type sequence. A lower rate of gene conversion was seen when the entire plasmid was integrated into the rDNA in the *Bgl*II site at the 5′ end of the 35S gene (Fig. 1); 25 to 50% of the transformants screened retained the restriction site indicative of the mutated constructs. High-resolution 2D gel analysis of the chromosomal M5 mutation showed the complete loss of RFB1 at the rDNA locus (data not shown), a result which is consistent with the plasmid analysis. Similarly, the chromosomal M11 mutation abolished RFB2 activity (data not shown). The results for the rDNA integrants of mutations M5 and M11 support the validity of using the plasmid model to investigate RFB function.

Sequences sufficient for RFB1 and RFB2 activity. Kobayashi et al. (16) found that the 129-bp *Hin*dIII-*Hpa*I fragment alone had RFB activity, although it seemed reduced. We recreated their minimal *Hin*dIII-*Hpa*I plasmid (YEp24HH$^+$; Fig. 5A, top) to test whether their minimal construct was sufficient for both RFBs or only one. Analysis by high-resolution 2D gels showed the presence of only a single RFB species (Fig. 5A, bottom). Further deletion of the 129-bp fragment by Kobayashi et al. reduced the sequences sufficient for RFB activity to a 69-bp fragment (white bar in Fig. 5B and C, top). Since these sequences include M4 and M5, which are required for RFB1, but do not include those sequences necessary for RFB2 (M10 to M12), we can conclude that the 69-bp region is sufficient for RFB1.

The 69-bp sequence sufficient for RFB1 activity cannot be further trimmed by deletion without complete loss of RFB activity (16), yet our mutagenesis data suggest that the sequences within these deletions, about 35 of these 69 bp (M6 to mid-M9), may not be necessary for RFB1 (white bar inside map in Fig. 5B and C, top). One possibility is that the M6 to M9 region contains functionally redundant sequence elements that contribute, together with sequences in M4 and M5, to RFB1 activity. If so, moving the M4-M5 and M6-M9 regions apart might affect RFB1 function. As a test of this idea, we inserted a 111-bp fragment of pUC18 DNA into the restriction site of the M6 mutant sequence (Fig. 5B, top). The insertion mutation in the *Hin*dIII-*Hpa*I region was tested for RFB function in plasmid pBB3NTS (Fig. 3A). High-resolution 2D gel analysis shows that RFB1 activity is eliminated (Fig. 5B, bottom). As a control, the same 111-bp fragment was inserted outside of the 69-bp region, in the restriction site of the mutant M9 sequence (Fig. 5C, top). This construct retains both RFB1 and RFB2 activity (Fig. 5C, bottom), with the distance between the two spots increased compared with the barrier spacing of the wild-type *Hin*dIII-*Hpa*I fragment (compare with Fig. 3C). These findings support the functionally redundant sequence hypothesis and indicate that the spacing between these functionally redundant sequences and M4-M5 is crucial for RFB1 fork arrest.

From these data, we conclude that the *Hin*dIII-*Hpa*I NTS fragment contains discrete sequences that uniquely contribute to the specification of RFB1 and RFB2. It should be noted that the identified sequences required for RFB1 and RFB2 function do not necessarily coincide with the sequences at which the nascent strands are arrested. Sequences essential for RFB1 map to the 20-bp region defined by mutations M4 and M5 and unspecified, redundant sequences in the region between M6 and M9. RFB2 depends absolutely on the sequences defined by M10 and M11 and to a lesser extent on sequences in the region defined by M12. However, these 30 bp are not by themselves sufficient for RFB2; on low-resolution 2D gels, Brewer et al. (2) determined that sequences that lie 35S gene-proximal to the *Hin*dIII site contributed to RFB activity. In addition to the *Hin*dIII-*Hpa*I fragment, sequences within the 188-bp

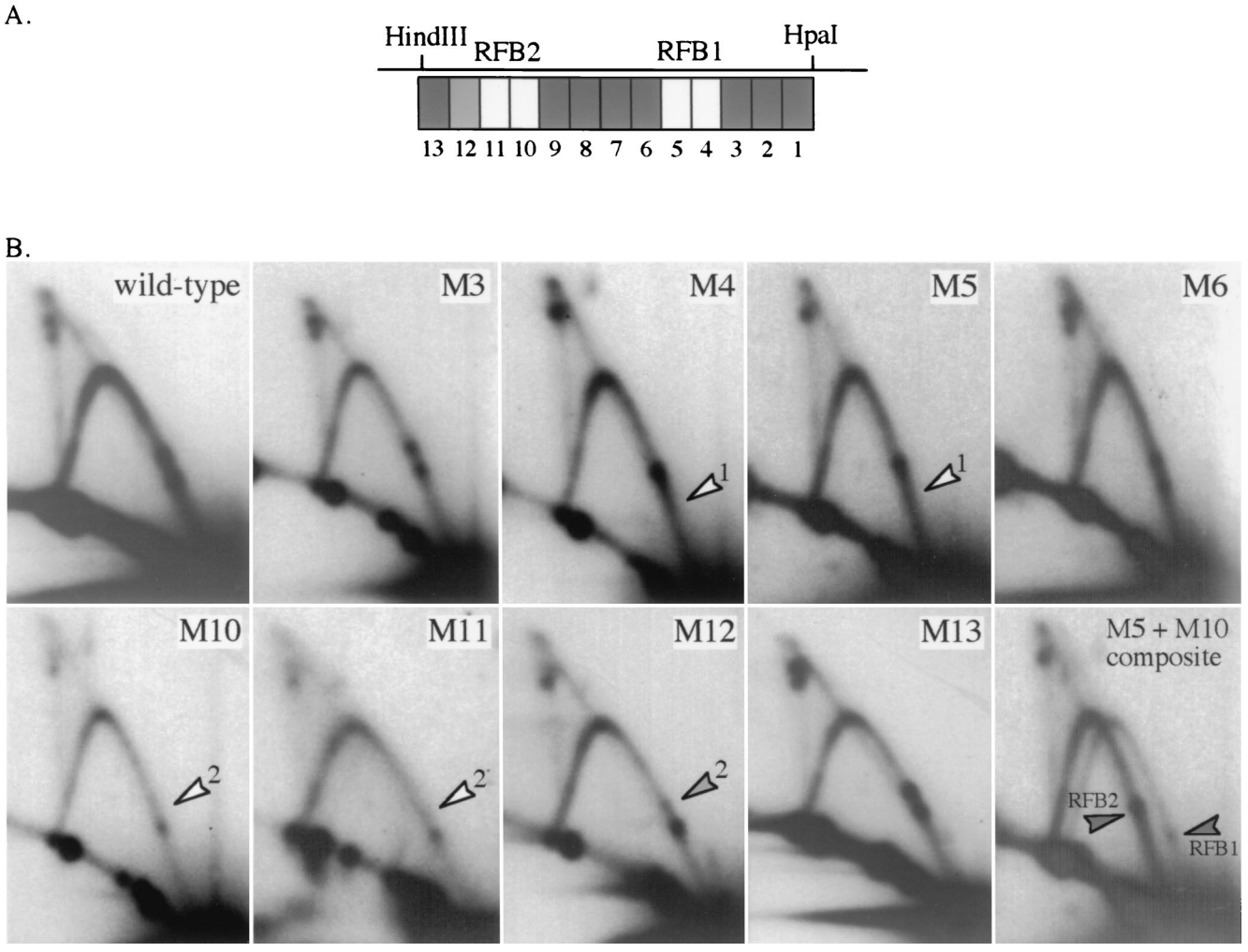


FIG. 4. Scanning mutagenesis of the 129-bp *Hin*dIII-*Hpa*I region. (A) Diagram showing the locations of the 12 10-bp (M13 to M2) and 1 12-bp (M1) block mutations within the *Hin*dIII-*Hpa*I fragment. The mutant and the wild-type sequences are listed in Table 1. The raw data in panel B are summarized by the shading of the boxes in A: black, similar to wild type; gray, reduced fork arrest; white, absence of fork arrest. (B) High-resolution 2D gel analysis of the block mutations in the *Hin*dIII-*Hpa*I region. Mutations were tested for RFB function in plasmid pBB3NTS (see Fig. 3A for map). The 2.2-kb *Ssp*I fragment was probed with *URA3* sequences. A 2D gel of pBB3NTS containing the wild-type *Hin*dIII-*Hpa*I sequence is shown for comparison. Open arrowheads point to loss of an RFB, and gray arrowheads point to a significantly decreased RFB. The M5 + M10 composite is an overlay of the M5 and M10 autoradiograms shifted laterally to display the relative and easily distinguishable positions of RFB1 and RFB2, indicated by solid arrowheads.

*Eco*RI-*Hin*dIII fragment most likely comprise the region sufficient for full RFB2 activity.

Correlation between RFB and *HOT1* sequences. Mutations in the E fragment that abolished *HOT1* activity were identified by Huang and Keil (12) in a screen using the chromosome III *HOT1* recombination assay (Fig. 2A). Those mutations were scattered in the right halves of both the *Hin*dIII-*Hpa*I (E_b) and *Eco*RI-*Hin*dIII (E_a) fragments of E (see Fig. 7A). Five of these mutations (G182, N35, G188, G190, and C20) were moved into the RFB test plasmid pBB3NTS, and the presence of RFB activity was determined by high-resolution 2D gels. Results for the wild-type construct (L3520) and three of the mutations are shown in Fig. 6. C20, a single-base-pair mutation in the M5 region, cleanly abolished RFB1 (Fig. 6B). Within the inverted repeat region of E_a, the point mutation N35 and the scrambled sequence block mutations G188 and G190 eliminated RFB2 (Fig. 6C and data not shown). G182, a block mutation in the poly(T) region and the most distal mutation from the *Hin*dIII-*Hpa*I fragment tested, had a more modest effect on RFB2, showing a reduced efficiency of fork arrest at this site (Fig. 6D). As would be predicted, deleting the entire E fragment results in the elimination of both barriers (data not shown). All of these mutations also result in the drastic reduction of *HOT1* recombination (Fig. 7B) (12). These data show that sequences required for RFB1 and RFB2 overlap sequences essential to *HOT1*-stimulated recombination.

Since the screen for *HOT1* mutations may not have been a saturating screen, the failure to find *HOT1* mutations that mapped to the M10 and M11 region may not be meaningful. To test the role of the M10-M11 region in *HOT1* recombination and to test additional 10-bp linker mutations, both normal and mutant for RFB function, five of the mutations were cloned into the E fragment of the *HOT1* assay cassette on chromosome III (Fig. 2A) and their effects on *HOT1* recombination were assessed (Fig. 7B). M5, as expected from the previous C20 result, completely eliminated *HOT1* activity. However, the adjacent M6 mutation, which had no effect on either RFB, reduced *HOT1* activity substantially (about five-

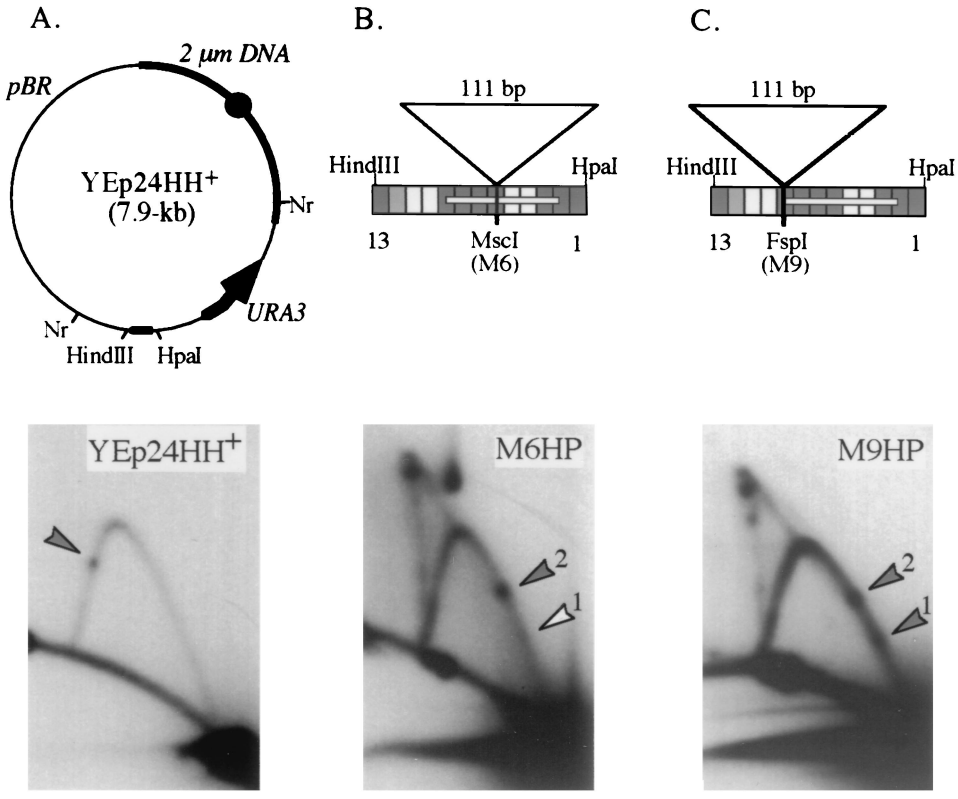


FIG. 5. *Hin*dIII-*Hpa*I sequences sufficient only for RFB1. (A) Map of the 7.9-kb plasmid YEp24HH$^+$ and a high-resolution 2D gel of the 2.4-kb *Nru*I (Nr) fragment. The 2μm origin (solid circle) and the *Hin*dIII-*Hpa*I insert are labeled. Also shown are the locations of the *URA3* gene and pBR322 (pBR) sequences. (B and C) Maps of 111-bp insertion mutations between RFB1 and RFB2. Disruptions in the *Hin*dIII-*Hpa*I region were tested for RFB function in plasmid pBB3NTS (see Fig. 3A for map). The 2.3-kb *Ssp*I fragment was probed with *URA3* sequences. The map above each gel is similar to the diagram in Fig. 4A, and mutations 1 and 13 are noted for orientation. The white bar within the chart shows the location of the 69-bp minimum RFB sequence determined by Kobayashi et al. (16). Mutation M6HP has an 111-bp insertion (from pUC18) in the *Msc*I site created by the M6 mutation. Mutation M9HP contains the same 111-bp fragment inserted just outside of the 69-bp sequence in the *Fsp*I site created by the M9 mutation. The spot located above the double-Y line of replication intermediates in B is background hybridization. See the legend to Fig. 4 for other details.

fold). Of the two mutations that eliminate RFB2, M10 significantly reduced *HOT1* activity (sixfold), whereas M11 decreased *HOT1* by only twofold. The results with M6 and M11 indicate that either *HOT1* or RFB activity can be dramatically decreased or eliminated by mutation without great reduction in the other one. Therefore, we find that the *cis*-acting sequences for RFB and *HOT1* are not entirely coincident.

Mutation M4 behaved anomalously, eliminating RFB1 but producing variable effects on *HOT1* recombination. The recombinational excision rates for 14 independent M4 transformants ranged from less than 1% to more than 100% of the wild-type value (data not shown). Most of the isolates showed a very modest (twofold) decrease in recombination. Sequence analysis showed that the M4 mutation was still present in all 14 transformants examined. The reason for the unique variability of mutation M4 on *HOT1*-stimulated recombination is unknown at this time.

DISCUSSION

***HOT1*-stimulated recombination is independent of blocked replication forks.** Sequences necessary for both RFB activity and *HOT1* recombination reside at the 3′ end of the 35S gene. An attractive model to explain this colocalization is that replication forks arrested at the RFB are prone to strand breaks that can stimulate homologous recombination (17). If this model were correct, then, because the arrest is polar, the direction of replication of the *HOT1* sequences should determine the level of *HOT1*-stimulated recombination. Earlier work showing that the E element functions in an orientation-independent manner in *HOT1* recombination (35) cast doubt on this model. Here we have demonstrated directly, both by fork direction analysis and by 2D gel visualization of fork arrests, that RFB arrested forks are not required for *HOT1*-stimulated recombination. These findings are surprising, considering the current model that proposes that RFB arrested forks stimulate rDNA recombination by initiating a breakage event (3, 15, 30a). Our studies do not support the paradigm that stalled forks are fragile sites at which recombinational repair is induced. Instead, we favor the idea that proteins that stimulate *HOT1* recombination may, as a consequence of DNA binding, have the ability to arrest replication forks.

While there is evidence in *E. coli* that the arrest of replication forks can lead to double-strand breaks (1a, 11, 26), there is no physical evidence that normal fork arrest at the yeast RFB causes breaks in vivo. Indeed, two observations suggest that replication forks arrested at the yeast RFB may have less single-stranded character than moving forks and thus may be more stable. Linskens and Huberman (20) observed that RFB arrested forks behave on BND-cellulose chromatography as if they possessed more double-stranded regions than moving forks. Consistent with this interpretation, Lucchini and Sogo (25) noted that the DNA immediately behind the stalled RFB fork appeared to be mostly double stranded when visualized by

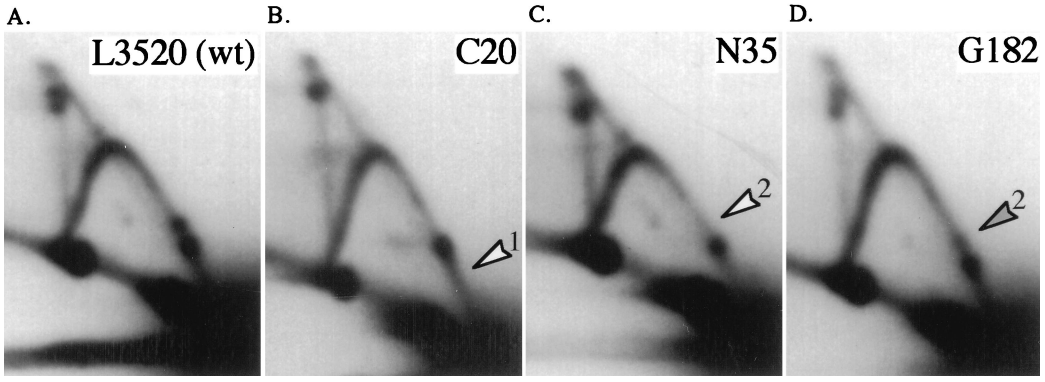


FIG. 6. E fragment mutations that affect *HOT1* and RFB activity. (A) 2D gel of the 2.2-kb *Ssp*I fragment of wild-type (wt) L3520. L3520 differs from pBB3NTS (see Fig. 3A) in two restriction sites (detailed in Materials and Methods). L3520 was used to clone the N35 and G182 mutations, which are shown in the other panels, while C20 was cloned into pBB3NTS. (B, C, and D) 2D gel analysis of the 2.2-kb *Ssp*I fragment of C20, N35, and G182. C20 and N35 are point mutations (see Fig. 7A for wild-type and mutant sequences), and G182 is a sequence-scrambled block mutation (see reference 12 for wild-type and mutant sequences). The locations of these mutations within the E fragment are shown in Fig. 7A. See the legend to Fig. 4 for other details.

electron microscopy after psoralen cross-linking. The lagging strands at forks arrested at the RFB site thus appeared to have completed Okazaki fragment replication and ligation. Therefore, the RFB arrested forks may be less fragile, hence less susceptible to breakage and recombinational repair, than moving forks.

Sequences responsible for RFB1 and RFB2 are distinct and independent. Earlier work revealed that the *S. cerevisiae* rDNA RFB consisted of two discrete arrest sites (2), herein named RFB1 and RFB2. Using high-resolution 2D gel analysis of plasmid replication intermediates, we have now further defined the sequences required for arrest at these two sites. While the 129-bp *Hin*dIII-*Hpa*I region in NTS1 (Fig. 1) is necessary for fork arrest at both RFB1 and RFB2, it is sufficient for a barrier only at RFB1. Sequences located in the adjacent, 35S gene-proximal 188-bp *Eco*RI-*Hin*dIII fragment (the 35S enhancer) most likely contain the additional sequences sufficient for fork arrest at RFB2 (2). No other regions within the *Eco*RI fragment that spans most of the NTS region are sufficient to impede progression of a replication fork.

Essential sequences for RFB1 and RFB2 were localized to two distinct regions, 20 and 30 bp in length and separated by 40 bp. The 20-bp region covered by mutations M4 and M5, which abolish RFB1 activity, shows no sequence similarity to the 30-bp region covered by mutations M10, M11, and M12, which affect fork arrest at RFB2. Interestingly, the M4 and M5 region includes 10 matches to a 12-bp stretch of the pea RFB (CTTG TATAAGTT) that Hernandez et al. uncovered in a search for RFB homology between pea and *S. cerevisiae* (9). A shorter 7-bp match to this 12-bp pea sequence was also found within the yeast 188-bp *Eco*RI-*Hin*dIII fragment (9), neighboring the right end of the *REB1* binding site (Fig. 7A). While this additional restriction fragment appears to be important to RFB2, it is not yet known if this specific 7-bp sequence is needed for RFB2 arrest.

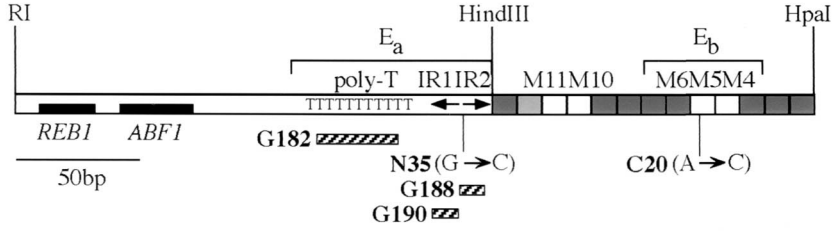
Although the molecular mechanism that results in replication fork arrest at the *S. cerevisiae* rDNA RFB has not yet been determined, the binding of proteins is likely to be involved (2). Our results from mutating different sequences show that fork arrests at RFB1 and RFB2 are eliminated independently from one another. We also demonstrate that the distance between the barriers can be increased by 111 bp without reduction in fork arrest activity at these sites (Fig. 5C, bottom). These data make it unlikely that one protein molecule binds simultaneously at RFB1 and RFB2. Thus, the RFB1 and RFB2 essential sequences most likely correspond to binding sites for either two different proteins or one protein with two independent binding sites.

Sequences essential for *HOT1* recombination and the rDNA RFB overlap. The fact that sequences necessary for *HOT1* and RFB activity colocalize within NTS1 posed the possibility that the two activities may require the same *cis*-acting sequences. While there is some sequence sharing, it is not complete: mutations C20 and N35 (see Fig. 7A for location) reduce *HOT1* activity to less than 6% of the wild-type level while simultaneously eliminating one of the barriers (RFB1 and RFB2, respectively); however, mutation M6 displays normal barrier activities while reducing *HOT1* activity to 22% of the wild-type level, and mutation M11 abolishes the barrier at RFB2, where it has only a modest effect on *HOT1* (Fig. 7B). These results should not be surprising, since *HOT1* and RFB also respond differently to mutations in *trans*-acting factors. For example, *HOT1* recombination is dependent upon 35S rRNA gene transcription by RNA polymerase I (12), while the RFBs function independently of this process (2).

The abundant nuclear transcription factors *REB1* and *ABF1* (*REB2*) have long been known to bind within the E fragment (13, 27). While their sites of binding are in close proximity to the *HOT1* and RFB essential sequences (see Fig. 7A for binding sites), they clearly do not overlap. Deletion of both sites resulted in only a modest decrease in *HOT1* activity (12). Neither protein is required for RFB1 function, considering that their sites are not included in sequences sufficient for RFB1. The *REB1* protein is most likely not involved in RFB2, since both barriers are normal in strains with a temperature-sensitive allele of *REB1* grown at the restrictive temperature for several hours (6). However, the effect of *ABF1* on RFB2 function is not yet known because scanning mutagenesis of the *Eco*RI-*Hin*dIII region has not been done.

Using chromatin immunoprecipitation, two yeast helicases, Pif1p and Rrm3p, were recently found to preferentially associate with rDNA chromatin, including the RFB region (12a). By measuring the generation of extrachromosomal rDNA circles in null mutants, Ivessa et al. (12a) showed that Rrm3p suppressed and Pif1p promoted rDNA recombination. The authors also performed 2D gel analyses on the direction of replication fork movement downstream of the RFB block and showed that a greater number of forks bypassed the RFB in a *pif1* mutant than in the wild type. This finding suggests that Pif1p plays a role in maintaining an efficient fork arrest at the

A.



B.

E fragment DNA sequence present	*HOT1 chromosome III assay*	*RFB 2D gel plasmid assay*	
	% *HOT1* activity (Ura⁻ excision)	**RFB1**	**RFB2**
wt E	**100**	●	●
ΔE	**0**	○	○
M5	**2**	○	●
M6	**22**	●	●
M10	**15**	●	○
M11	**52**	●	○
C20	**7***	○	●
G182	**4***	●	**x**
N35	**3***	●	○
G188	**2***	●	○
G190	**1***	●	○

●RFB present, ○RFB absent, **x** decreased RFB

FIG. 7. Summary of the effect of *HOT1* and RFB activity on E fragment mutations. (A) Map of the 320-bp *Eco*RI-*Hpa*I E fragment located from the rDNA NTS1 (Fig. 1). The *Hin*dIII-*Hpa*I fragment is represented as in Fig. 4A. Only the mutants that were tested for *HOT1* activity are noted. E_a and E_b are the two regions within the E fragment that were identified by linker insertion mutagenesis to be required for *HOT1* activity (34). The striped bars underneath the map represent the *HOT1* block mutations tested in the RFB plasmid assay: G182 in the poly(T) region and G190 and G188 in inverted repeats IR1 and IR2, respectively. Mutant and wild-type sequences for the *HOT1* block mutations are given in Huang and Keil (12). The base pair changes and locations of the point mutations N35 and C20 are indicated. The locations of two identified 35S enhancer-binding proteins, *REB1* and *ABF1*, are noted (13, 27). (B) Summary chart of all the mutant sequences tested. wt E indicates the presence of wild-type E sequences; ΔE signifies that the E fragment is absent. *HOT1* recombination was measured as the relative excision rate of the *URA3* marker in the chromosome III construct (see Fig. 2A) as previously described (12). *, data from reference 12.

RFB. Further evidence also suggests that Rrm3p is an important factor in resolving forks terminating at the RFB. Neither Pif1p nor Rrm3p is essential to RFB activity, since forks are still arrested at the RFB site in the null mutants. However, it seems that Pif1p and Rrm3p are two newly identified *trans*-acting factors that appear to be involved in both RFB activity and rDNA recombination.

The *FOB1* protein is localized to the nucleolus and is essential both for arresting replication forks at the RFB and for *HOT1*-stimulated recombination (3, 17). It plays a role in the maintenance of rDNA repeat number (15) and in the accumulation of rDNA extrachromosomal circles, which appear to influence life span (3). Fob1p does not affect rDNA transcription (R. Prusty, unpublished data) and does not appear to confer any significant growth defects (15). Based on the properties of a *fob1* mutant (17) and on the results reported here, Fob1p must influence protein-DNA interactions at several sites, those determining replication fork arrest at RFB1 and at RFB2 and those mediating *HOT1*-stimulated recombination. It seems likely that the *FOB1* protein facilitates the binding of different proteins to these different sites. Further studies will be needed to clarify the protein-DNA interactions and their functional consequences at this complex locus in the rDNA.

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