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Contrasting Roles of Checkpoint Proteins as Recombination Modulators at Fob1-*Ter* Complexes with or without Fork Arrest[▽]

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The replication terminator protein Fob1 of *Saccharomyces cerevisiae* specifically interacts with two tandem *Ter* sites (replication fork barriers) located in the nontranscribed spacer of ribosomal DNA (rDNA) to cause polar fork arrest. The Fob1-*Ter* complex is multifunctional and controls other DNA transactions such as recombination by multiple mechanisms. Here, we report on the regulatory roles of the checkpoint proteins in the initiation and progression of recombination at Fob1-*Ter* complexes. The checkpoint adapter proteins Tof1 and Csm3 either positively or negatively controlled recombination depending on whether it was provoked by polar fork arrest or by transcription, respectively. The absolute requirements for these proteins for inducing recombination at an active replication terminus most likely masked their negative modulatory role at a later step of the process. Other checkpoint proteins of the checkpoint adapter/mediator class such as Mrc1 and Rad9, which channel signals from the sensor to the effector kinase, tended to suppress recombination at Fob1-*Ter* complexes regardless of how it was initiated. We have also discovered that the checkpoint sensor kinase Mec1 and the effector Rad53 were positive modulators of recombination initiated by transcription but had little effect on recombination at *Ter*. The work also showed that the two pathways were Rad52 dependent but Rad51 independent. Since *Ter* sites occur in the intergenic spacer of rDNA from yeast to humans, the mechanism is likely to be of widespread occurrence.

Recombination is an important part of DNA replication because it promotes uninterrupted fork progression and complete duplication of the genome by facilitating restart of prematurely arrested replication forks caused by extrinsic or intrinsic factors (15). Despite this important beneficial effect, recombination, if not stringently controlled, poses the inherent risk of inducing genome instability by causing loss or gain or transposition of sequences. The ribosomal DNA (rDNA) of budding yeast provides an excellent model system for investigation of the control of replication fork arrest-induced recombination at *Ter* sites (see reference 45 for a recent review). Although much information is available on the choice between intrachromatid and interchromatid recombination in rDNA (24), much less is known about the initiation of recombination and its control at *Ter* sites.

Budding yeast rDNA is present as ~200 tandem repetitions of a ~9.1-kb unit sequence in chromosome number XII (34). Each rDNA unit contains, from left to right (Fig. 1A), the sequence encoding the 35S precursor rRNA that is transcribed by RNA polymerase I; an intergenic, nontranscribed spacer region I (IGS I) that contains tandem replication termini *Ter2* and *Ter1* (also called replication fork barriers) (8); and the sequence encoding 5S RNA that is transcribed by RNA polymerase III, followed by a second intergenic spacer (IGS II) that contains an origin of DNA replication (*ARS*) (7). Replication is initiated from the *ARS* and initially progresses bidirectionally until it meets the *Ter1* and *Ter2* sites, at which it is arrested in a polar mode. The *Ter1* and *Ter2* sequences specifically bind to the multifunctional terminator protein called Fob1, which causes polar fork arrest at the protein-DNA complexes (23, 32). The programmed fork arrest prevents the replication forks from invading the region of the 35S transcript progressing from the opposite direction. The convergence of oppositely moving transcription and replication is known to cause fork stalling and physiologically unscheduled recombination (40).

Ter sites are known to be recombinogenic in prokaryotes (16), and in budding and fission yeasts, these act as hot spots for recombination. The process is regulated at multiple levels (45). Interchromatid recombination presumably helps to maintain the homogeneity of the rDNA repeat sequences, whereas maintenance of the rDNA repeat length homeostasis requires controlled illegitimate, intrachromatid exchanges (25, 26). Ribosomal repeat length expansion or contraction, which presumably occurs in response to physiological cues, is caused by Fob1-mediated recombination (25). Recombination at *Ter* is regulated by Fob1-mediated recruitment of the histone deacetylase called Sir2. Sir2 and Net1 are two of the component proteins of the RENT (*re*gulators of *n*ucleolar silencing and *t*elophase exit) complex (38), which inhibits intrachromatid recombination by recruitment of cohesin in two ways. First, it represses transcription initiated at the bidirectional promoter called *E-pro*, located in the *EXP* (expansion) sequence adjacent to *Ter* in the IGS I (Fig. 1A) (24). Transcription directed toward the *Ter* region apparently disrupts cohesin assembly and removes this impediment to intrachromatid recombination (24). Second, Fob1 physically interacts with the RENT complex, which in turn interacts with Tof2 and the monopolin complex. The latter attracts cohesins to the *Ter*

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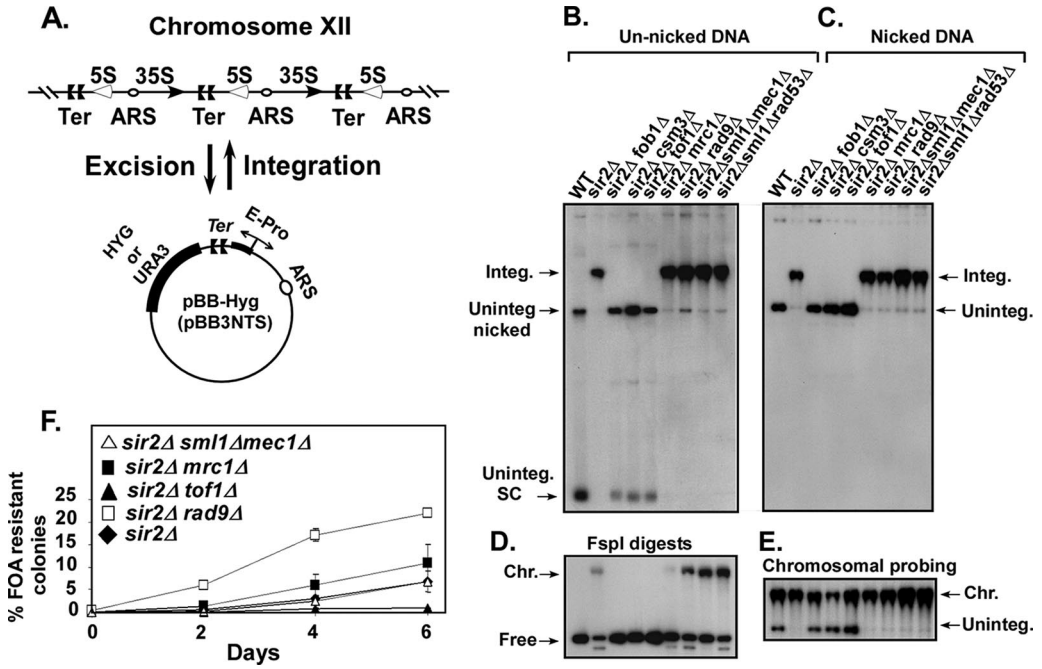

FIG. 1. Effect of checkpoint genes on plasmid integration into and excision from the *Ter* sites of rDNA. (A) Schematic representation of the plasmid integration and excision assays; the plasmid pBB3NTS (pBB-Hyg) contained *EXP* sequence (which includes the *Ter* sites and the *E-pro* promoter). (B) Autoradiogram of a Southern blot showing the intracellular distribution of the reporter plasmid pBB-Hyg DNA resolved without nicking the DNA in an 0.5% agarose gel; a labeled plasmid-specific probe was used for detection of the plasmid DNA. integ., integrated; uninteg., unintegrated; SC, supercoiled DNA; WT, wild type. The lanes are self-explanatory. (C) Autoradiogram of a Southern blot showing the DNA samples shown in panel B but after nicking with NB.BsrD1. (D) Same as in panel B except that the DNA samples were digested with FspI; the lanes from left to right are as labeled in panels B and C. (E) Same as in panel C but probed with labeled chromosomal rDNA to identify the location of the integrated plasmid bands. (F) Kinetics of excision of pBB3NTS (*URA3*) plasmid rDNA from strains containing the integrated form of the plasmid reporter. Effects of individual deletions of the checkpoint genes *tof1*, *mrc1*, and *rad9* (all present in a *sir2*Δ background) on loss of *URA3* plasmid as a function of time of growth in nonselective medium. The nine lanes in panels D and E correspond exactly to the nine lanes in panels B and C.

region by protein-protein interaction (17, 18). Sir2 also suppresses recombination at rDNA by preventing its accessibility to recombination proteins through alteration of the chromatin structure (14).

Many extrinsic (e.g., hydroxyurea) and intrinsic factors can cause genome-wide unscheduled fork arrest either at DNA lesions or due to depletion of the deoxynucleoside triphosphate pool. The stalled forks are prone to replisomal disassembly and fork collapse that can cause genome instability. Cells respond to stalled forks by invoking a signal transduction pathway called S-phase checkpoint control that delays cell cycle progression, turns on DNA repair genes, and stabilizes the stalled forks against replisomal disassembly and collapse (13, 41). The stalled fork is stabilized by a fork protection complex consisting of Tof1, Csm3, and Mrc1 proteins (21). The S-phase checkpoint pathway of budding yeast consists of a sensor protein kinase called Mec1 and its associated protein Ddc2; the latter binds to a single-stranded DNA binding protein complex, RPA (replication protein A), which accumulates on unraveled single-stranded DNA present near stalled forks. The sensor is followed in the cascade by mediators (Mrc1, Tof1, Csm3, and Rad9) that in turn activate an effector kinase called Rad53 that phosphorylates the target genes to cause cell cycle arrest and turns on genes needed for repair of DNA lesions at which the forks have stalled (13). Checkpoint proteins are not just activated by stress but are also important modulators of DNA transactions under normal conditions (11, 36).

Although unscheduled fork arrest caused by genotoxic stress can induce a checkpoint response, physiologically programmed fork arrest at *Ter* sites of rDNA does not seem to elicit such a response. The intra-S checkpoint proteins Tof1 and Csm3, but not Mrc1, promote stable programmed fork arrest at *Ter* (9, 31, 44) by counteracting Rrm3 helicase/"sweepase" (31). Rrm3 is known to facilitate fork passage through many nonhistone protein-DNA complexes that are tightly bound to DNA throughout the genome (43).

Given that fork arrest at *Ter* triggers recombination and in the light of the information presented above, one would hypothesize that Tof1/Csm3 should promote recombination at replication termini of rDNA by counteracting the negative effect of Rrm3 on fork arrest. One would further predict that Mrc1 and Rad9, the mediators of replication and DNA damage response checkpoint pathways, respectively (48), which do not play a role in promoting fork arrest (9, 31, 44), should not promote recombination at *Ter*. Although some information on the roles of checkpoint proteins in recombination caused by nonphysiological fork arrest is available (29), the possible regulatory role of checkpoint adapter proteins on recombination at natural *Ter* sites has not been completely elucidated in the nucleolar milieu.

Investigations of recombination at ectopically placed *Ter* sites outside the rDNA array have yielded different results in different systems. For example, placement of inverted *Ter* sites at an ectopic location in budding yeast showed no evidence of enhanced recombination at this site and fork arrest at this location was not dependent on Mec1 and Rad53, the sensor and the effector kinases of the checkpoint pathway, respectively (9). On the other hand, an ectopically placed sequence consisting of the promoter (I element) and enhancer (E element) that includes the *Ter* sites, when located in chromosome III, promoted illegitimate recombination (HOT1 activity). HOT1-activated recombination requires both transcription by RNA polymerase I and Fob1 binding to *Ter* located in the E element (22, 35, 46). Fob1 protein induces HOT1 recombination not by arresting replication forks (47) but by acting as a transcription factor that promotes RNA polymerase I transcription (34). In *Schizosaccharomyces pombe*, placement of *Ter* sites (*RTS*) at ectopic locations caused enhanced recombination and genome instability when the cognate terminator protein Rtf1p and the intra-S-phase checkpoint protein SWI1 (and presumably SWI3) were provided (2, 27).

The proteins encoded by the *RAD52* epistasis group of genes that promote homologous recombination (HR) have been reviewed previously (33). Rad52 and Rad59 are single-stranded DNA-annealing proteins that act together but somewhat differently from each other; Rad54 is a motor protein; Rad55 and Rad57 are facilitators of the homology search protein Rad51, which is the equivalent of prokaryotic RecA protein. Rad50 and yKu80 are proteins involved in the nonhomologous end-joining (NHEJ) pathway.

In this work, we endeavored to test some of the hypotheses stated above by measuring the frequencies of integration of a plasmid containing the *EXP* sequence (which includes *Ter* and *E-pro*) into chromosomal rDNA in the absence of the *SIR2* gene (5, 30) and of the excision of the integrated sequences from rDNA. We investigated the roles played by the various checkpoint proteins in recombination at Fob1-*Ter* complexes with or without associated fork arrest. We show that the intra-S-phase checkpoint genes *TOF1* and *CSM3* promoted recombination at *Ter* within the rDNA array but suppressed recombination at the ectopically placed *HOT1* locus that included the enhancer (including *Ter*)-promoter sequences of the precursor 35S rRNA. In contrast, *MRC1* and *RAD9* mediators/adapters suppressed recombination at both *Ter* and *HOT1* to various degrees. The Mec1 sensor and Rad53 effector were positive modulators of *HOT1* recombination triggered by RNA polymerase I transcription. The results revealed that the intra-S-phase checkpoint proteins Tof1 and Csm3 have dual and contrasting roles in regulating recombination at Fob1 binding sites depending on whether the recombination was triggered by fork arrest at *Ter* or by an alternative mechanism involving transcription by RNA polymerase I. Recombination was also modulated either positively or negatively by other members of the replication checkpoint signal transduction pathway.

MATERIALS AND METHODS

Construction of strains. All strains constructed as a part of this work and those received from other sources are listed in Table 1. The strain LPY11 (W303 Δ*sir2*:*HIS3*; courtesy of Loraine Pillus) was used to delete all checkpoint and recombination genes using appropriate markers such as G418 or TRP1 (28).

TABLE 1. Yeast strains

Strain	Genotype	Source
W303	*MAT***a** (*leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*)	R. Rothstein
LPY11	W303a *sir2*Δ:*HIS3*	L. Pillus
Lfob1	LPY11 *fob1*Δ:*G418*	This study
Lcsm3	LPY11 *csm3*Δ:*G418*	This study
Ltof1	LPY11 *tof1*Δ:*G418*	This study
Lmrc1	LPY11 *mrc1*Δ:*G418*	This study
Lrad9	LPY11 *rad9*Δ:*G418*	This study
Lrad50	LPY11 *rad50*Δ:*G418*	This study
Lrad51	LPY11 *rad51*Δ:*G418*	This study
Lrad52	LPY11 *rad52*Δ:*G418*	This study
Lrad54	LPY11 *rad54*Δ:*G418*	This study
Lrad55	LPY11 *rad55*Δ:*G418*	This study
Lrad59	LPY11 *rad59*Δ:*G418*	This study
Lrrm3	LPY11 *rrm3*Δ:*G418*	This study
Ltr13	LPY11 *tof1*Δ:*G418* (*crelox G418 lost*) *rrm3*Δ:*G418*	This study
Lmus81	LPY11 *mus81*Δ:*G418*	This study
Lslx4	LPY11 *slx4*Δ:*G418*	This study
Lms9	LPY11 *mus81*Δ:*G418* (*crelox G418 lost*) *slx*Δ:*G418*	This study
WDHY1638	W303 *RAD5 sml1*Δ *mec1*Δ	W. D. Heyer
Lmec1	WDHY1638 *sir2*Δ:*G418*	This study
W2105-17b	W303 *sml1*Δ:*URA3 rad53*Δ:*HIS3 rad5*	R. Rothstein
Lrad53	W2105-17b *sir2*Δ:*G418*	This study
K5665	RLK1-3C *MAT*α *his4-260 ade2-1 ura3-52 can*^{R}	R. Keil
Kfob1	K5665 *fob1*Δ:*G418*	This study
Kcsm3	K5665 *csm3*Δ:*G418*	This study
Ktof1	K5665 *tof1*Δ:*G418*	This study
Kmrc1	K5665 *mrc1*Δ:*G418*	This study
Krad9	K5665 *rad9*Δ:*G418*	This study
Krad51	K5665 *rad51*Δ:*G418*	This study
Krad52	K5665 *rad52*Δ:*G418*	This study
Krad54	K5665 *rad54*Δ:*G418*	This study
Krad55	K5665 *rad55*Δ:*G418*	This study
Krad59	K5665 *rad59*Δ:*G418*	This study
Krrm3	K5665 *rrm3*Δ:*G418*	This study
Ktr1	K5665 *rrm3*Δ:*G418* (*crelox G418 lost*) *tof1*Δ:*G418*	This study
Ksml1	K5665 *sml1*Δ:*G418*	This study
Kmec1	K5665 *sml1*Δ:*G418* (*crelox G418 lost*) *mec1*Δ	This study
Krad53	K5665 *sml1*Δ:*G418* (*crelox G418 lost*) *rad53*Δ	This study

Plasmid integration assay. The plasmid integration assay was carried out essentially as described in references 5 and 30) with slight modifications. Plasmid pBB3NTS (45) containing a *URA3* marker was transformed into appropriate strains by the lithium acetate method and plated on SD-Ura$^-$ plates. A few colonies of ~1 mm in diameter were mixed by the inoculation loop and then streaked on a new SD-Ura$^-$ plate. After three or six cycles of streaking, a few colonies were pooled and then inoculated into 10 ml of SD-Ura$^-$ medium. DNA was prepared from overnight cultures (32). Strains containing plasmid pBB-Hyg were grown in the same way except that the culture medium was yeast extract-peptone-dextrose (YPD) plus hygromycin (200 μg/ml). DNA samples, unnicked or nicked by NB.BsrD1 (New England Biolabs), were fractionated in an 0.8% agarose gel in the presence of ethidium bromide. Southern transfer and probing by pUC18 were carried out as described in reference 31. Plasmid integration was carried out using both pBB3NTS and pBB-Hyg plasmids, and they are shown in the appropriate figure legends. Excision assays were carried out with strains in which plasmid pBB3NTS was completely integrated into the chromosome.

Excision assay. Strains containing the integrated pBB3NTS (*URA3*) plasmid were first grown overnight in liquid SD-Ura$^-$ medium. Overnight cultures were then inoculated into fresh SC complete medium to allow cells to lose the integrated plasmid. At appropriate times (days), cultures were diluted and plated on YPD and SD medium containing 1 g/liter of 5-fluoroorotic acid (5-FOA). Plates

were incubated at 30°C for 3 days before colonies were counted. Each day, the liquid cultures were diluted in SC medium, grown for 24 h, and plated on the appropriate day.

HOT1 assay. The wild-type strain or its derivatives were grown overnight in SD-Ura$^-$ medium. Cultures were inoculated into fresh YPD medium and grown at 30°C. Cultures were diluted at different time points and spread on YPD and SD-FOA$^+$ plates in triplicate. Plates were incubated at 30°C. The percent HOT1 activity was calculated from the ratio of FOA-resistant colonies and the total number of colonies growing on YPD plates multiplied by 100.

2D gel electrophoretic assay. Two-dimensional (2D) gel analysis of fork arrest was carried out as described in reference 6.

RESULTS

Effect of checkpoint proteins on recombination at *Ter*. First, we wished to investigate the possible impact of replication checkpoint proteins of the adapter class on recombination provoked by fork arrest at *Ter*. Two of the adapter proteins, namely, Tof1 and Csm3, but not Mrc1 and Rad9, are required for promotion of stable fork arrest at *Ter* sites (9, 31, 44). We investigated the impact of the loss of the various individual checkpoint proteins on recombination at *Ter* by utilizing the observation that a plasmid containing the recombinogenic *EXP* region that includes the tandem *Ter* sites (24) is readily integrated into the chromosomal rDNA array in the absence of Sir2 in a Fob1-dependent fashion (5). It was essential to eliminate Sir2 activity because it is known to suppress intrachromatid but not interchromatid recombination in rDNA (26). While intrachromatid recombination was critical for the plasmid integration and excision assay (Fig. 1A), interchromatid recombination was phenotypically "silent." We transformed the reporter plasmid pBB3-Hyg (32) into the *sir2*Δ strain (LPY11) and its isogenic derivatives, which contained deletions of various checkpoint genes of the adapter/mediator class such as *TOF1*, *CSM3*, *MRC1*, and *RAD9.* In addition, we also transformed the plasmid into the strains from which *SIR2* was deleted along with the checkpoint sensor *MEC1* gene and the effector *RAD53* gene (Table 1 shows all strains). We measured the percentage of the total intracellular plasmid DNA that was integrated into the chromosome of each of these strains. The transformants were grown for 40 to 60 generations on YPD-plus-hygromycin plates. We determined plasmid integration by extracting and resolving the intracellular DNA in agarose gels followed by Southern blotting and hybridization of the blots to a labeled plasmid-specific probe. The free and integrated forms of plasmid DNA were quantified with a phosphorimager (Fig. 1B and C). The intracellular pBB-Hyg plasmid DNA in the wild-type cells remained almost exclusively in the unintegrated form in either the supercoiled or the relaxed state (Fig. 1B and C). In the *sir2*Δ strain, almost the entire population of plasmid DNA was integrated and it migrated in a band that corresponded to the chromosomal DNA (Fig. 1B and C). By reprobing the blots with a labeled rDNA probe, we confirmed that this band corresponded to sheared chromosomal DNA (Fig. 1E). We confirmed that the plasmid integration was *FOB1* dependent by examining the intracellular plasmid DNA in the *sir2*Δ *fob1*Δ strain and found that it existed almost completely in the unintegrated state (Fig. 1B and C). It should be noted that flipping the orientation of the *Ter* site with respect to the origin by 180° abolished plasmid integration, thereby showing that the integration was dependent on polar fork arrest at the terminus (data not shown).

We then examined the distributions of intracellular plasmid DNA in each of the *sir2*Δ derivatives that contained single deletions of the checkpoint genes. Southern blots of the DNA samples resolved in agarose gels and probed with a labeled pUC18 DNA probe showed that in the *sir2*Δ *csm3*Δ and *sir2*Δ *tof1*Δ strains, the plasmids remained in the free form (Fig. 1B and C). In contrast, ~98% of the intracellular plasmid DNA was integrated into the chromosome in the *mrc1*Δ, *rad9*Δ, *mec1*Δ, and *rad53*Δ derivatives (Fig. 1B and C). In order to simplify the gel electrophoresis patterns and thereby make it easier to quantify the results, we nicked the plasmid DNA samples before electrophoresis with the restriction enzyme NB.BsmI or NB.BsrD1, which nicks but does not cut both strands of the DNA at its recognition site(s). Phosphorimager analysis of the resulting blots was performed, and the data confirmed that, whereas in the *tof1*Δ and *csm3*Δ strains plasmid integration was almost completely blocked, ~98% of the plasmid DNA was integrated into chromosome in the *sir2*Δ *mrc1*Δ, *sir2*Δ *rad9*Δ, *mec1*Δ, and *rad53*Δ strains (Fig. 1C). In order to ascertain that the band migrating above the nicked circular plasmid DNA was chromosomally integrated, we stripped the blot shown in Fig. 1C, reprobed it with a labeled chromosomal rDNA, and found that the upper band in lanes 1 to 7 hybridized to the labeled probe (Fig. 1E). Did the plasmid DNA integrate in a single cluster, or were these integrated at diverse locations in rDNA? We addressed this question by digesting the DNA with FspI. It should be noted that, whereas the plasmid DNA contains a single FspI site, none are present in the rDNA repeats. Digestion of the DNA with FspI showed (assuming complete digestion) that ~90% of the plasmid DNA was integrated in a single cluster (Fig. 1D). In order to confirm that the plasmids were integrated into chromosomal rDNA, we double digested the DNA samples with FspI and NheI (this site is present in the rDNA repeats but not in the plasmid sequence). The DNA blots were also probed with an rDNA probe, and the results were consistent with the conclusion that the bulk of the integrations occurred within the rDNA (not shown).

Although the plasmid integration assay showed that *TOF1* and *CSM3* genes were essential for recombination at *Ter*, it did not have sufficient sensitivity to reveal more subtle effects caused by the deletions of *MRC1*, *RAD9*, *MEC1*, and *RAD53* checkpoint genes. We wished to investigate this possibility by performing the reverse assay, i.e., measurements of the rate of excision of pBB3NTS plasmid present in the integrated state in the rDNA. We allowed plasmid excision to occur in the various genetic backgrounds mentioned above and counted the percentage of 5-FOA-resistant colonies generated as a function of prior duration of growth in nonselective (complete) medium. The generation of *ura3* colonies was not due to silencing of the marker in rDNA because the *SIR2* silencer, which encodes the NAD-dependent histone deacetylase, had been deleted from all the strains. The data not only confirmed the absolute necessity of *TOF1*/*CSM3* for *FOB1*-dependent recombination at *Ter* but also revealed that the rate of excision was increased in the *sir2*Δ *rad9*Δ and the *sir2*Δ *mrc1*Δ strains in comparison with the *sir2*Δ control. While the inhibitory impact of *RAD9* on plasmid excision was unambiguous, the effect of *mrc1*Δ was modest in comparison with the *sir2*Δ control (Fig. 1F). The deletion of *MEC1* had no significant effect on plasmid excision.

The data are consistent with the conclusion that *RAD9* and probably *MRC1* were negative modulators of plasmid excision (Fig. 1F). While performing the excision experiments, we have considered the possibilities that the growth rates of the 5-FOA-resistant colonies of different genotypes, the location of integration, etc., might affect excision rates. However, experiments were performed with independent isolates, and the results were generally consistent with those conducted on plasmid integration whenever it was possible to do this comparison (as in Fig. 1).

Effect of the *tof1*Δ *rrm3*Δ double deletion on recombination. We have previously reported that Tof1 and Csm3 promote stable fork arrest by counteracting the effect of the helicase Rrm3, which allows fork passage through the Fob1-*Ter* complex (30). Rrm3 is a 5′-3′ helicase that is known to promote genome-wide fork passage through nonhistone protein-DNA barriers (19, 42). Tof1 and Csm3 partially restored fork arrest at *Ter* by counteracting the tendency of Rrm3 helicase to facilitate fork passage past Fob1-*Ter* complexes. We wished to determine if the negative modulation of Rrm3 by the Tof1-Csm3 complex was also manifested in a similar manner in plasmid integration and excision in a *sir2*Δ background. We constructed a *sir2*Δ *rrm3*Δ *tof1*Δ strain and compared integration of pBB-Hyg in this strain with its integration in *sir2*Δ, *sir2*Δ *tof1*Δ, and *sir2*Δ *rrm3*Δ strains (Fig. 2). The autoradiograms of Southern blots of agarose gels showed that, after three cycles of serial streaking and growth on selective medium, >98% of the input plasmid DNA had integrated into chromosomal rDNA in the *sir2*Δ and *sir2*Δ *rrm3*Δ cells. In contrast, as expected, no plasmid integration was detectable in the *sir2*Δ *tof1*Δ strain. Furthermore, in the *sir2*Δ *tof1*Δ *rrm3*Δ cells, less than 30% of the input plasmid DNA was found in the integrated state after three rounds of streaking and growth on selective medium. Complete plasmid integration could be achieved only after a total of six cycles of streaking and serial growth of the latter strain (Fig. 2A). The data are consistent with the interpretation that Tof1 activity, although essential for plasmid integration in the presence of the Rrm3 sweepase, was not strictly necessary when there was no Rrm3 present in the cell milieu. The lower rate of plasmid integration appeared to be commensurate with the partial restoration of fork arrest in the *sir2*Δ *fob1*Δ *rrm3*Δ strain in comparison with the *sir2*Δ control. In order to confirm the data by another approach, we measured plasmid excision kinetics in the same genotypes used for plasmid integration. We transformed the plasmid pBB3NTS into *sir2*Δ, *sir2*Δ *rrm3*Δ, and *sir2*Δ *fob1*Δ *rrm3*Δ strains. We deleted the *TOF1* gene from the *sir2*Δ strain in which integration had occurred. As expected, there was a 1.5-fold increase in the rate of excision in the *sir2*Δ *rrm3*Δ strain in comparison with the *sir2*Δ strain, but plasmid excision was completely abolished in the *sir2*Δ *tof1*Δ strain (data from two independent isolates are shown). The rate of plasmid excision, in contrast, was restored to nearly the level of that of the *sir2*Δ control in the *sir2*Δ *tof1*Δ *rrm3*Δ genotype (Fig. 2B).

We wished to make sure that, in the genetic background used here, *sir2*Δ did not alter the requirements for regulation of fork arrest that have been investigated before in *SIR2* genotypes by performing 2D gel electrophoresis (31). The autoradiograms of 2D gels of replication intermediates confirmed that fork arrest was abolished in the *sir2*Δ *tof1*Δ strain in comparison with the *sir2*Δ control. It was slightly elevated over that of the control in the *sir2*Δ *rrm3*Δ strain. Also consistent with our previous work, in the *sir2*Δ *tof1*Δ *rrm3*Δ strain the fork arrest at *Ter* was partially restored in comparison with the *sir2*Δ control (Fig. 2C). The data support the conclusion that in the *sir2*Δ genotype, Tof1 promoted stable fork arrest by counteracting the Rrm3 sweepase, thereby restoring fork arrest-mediated recombination at *Ter*. Deletions of the *MRC1* and *RAD9* mediators did not abolish fork arrest (Fig. 2C). The absence of these genes did not abolish plasmid integration or excision triggered by replication termination (Fig. 2A and B). Rather, these checkpoint mediator proteins seemed to suppress recom-

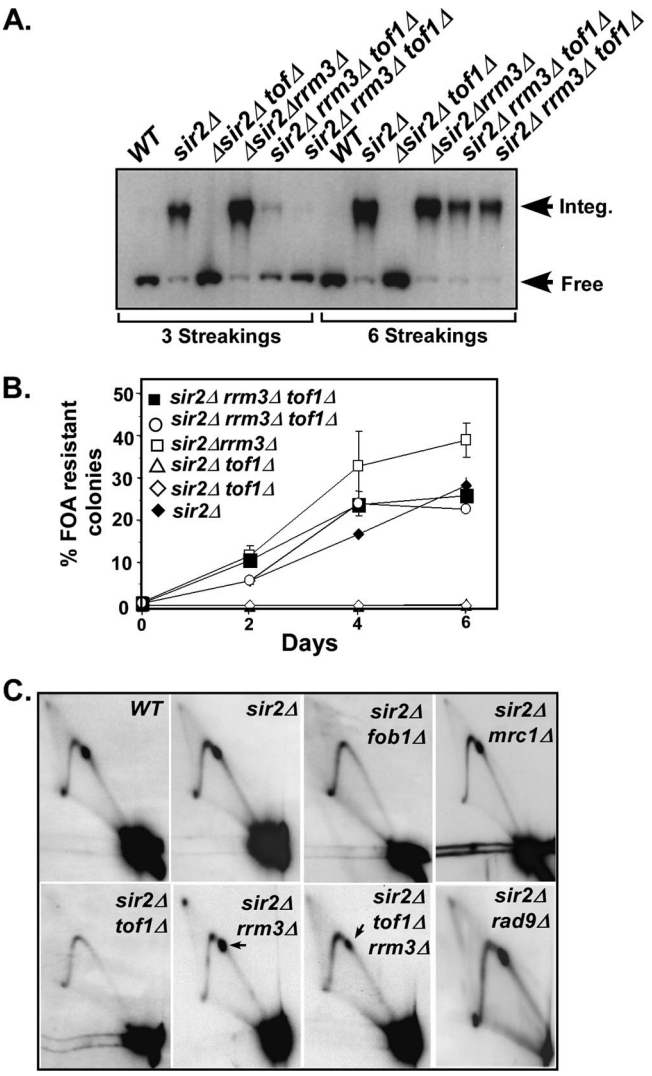

FIG. 2. Contribution of the interplay between *TOF1* and *RRM3* to plasmid integration and excision. (A) Autoradiogram of a representative Southern blot showing the effect of *tof1*Δ *rrm3*Δ double deletions on plasmid integration (pBB-Hyg) in comparison with the single deletions, all in a common *sir2*Δ background after three and six serial streakings and growth in the selective medium. (B) Effects of single and double deletions (*tof1*Δ, *rrm3*Δ, and *tof1*Δ *rrm3*Δ) on pBB3NTS (*URA3*) plasmid excision. (C) Autoradiograms of 2D gels showing replication fork arrest at *Ter* in different strains; the arrows show the termination spots generated by fork arrest at the two closely spaced *Ter1* and *Ter2* sites. WT, wild type.

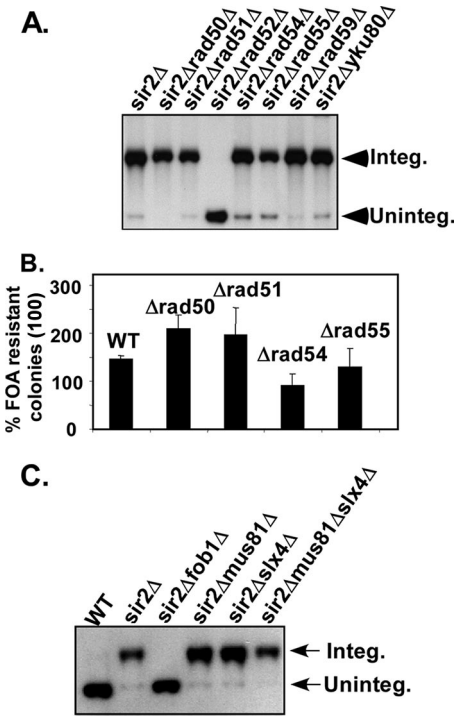

FIG. 3. Impact of deletions of genes involved in HR and NHEJ on plasmid integration and excision. (A) Autoradiogram of a representative Southern blot of nicked DNA showing the status of intracellular plasmid DNA pBB3NTS (*URA3*) in isogenic strains containing various deletions. The lanes are self-explanatory. (B) Plasmid excision kinetics showing the impact of various deletions of recombination genes on the excision of pBB3NTS (*URA3*). (C) Effect of deletions of two structure-specific endonucleases (*slx4*Δ, *mus81*Δ, and *slx4*Δ *mus81*Δ) on plasmid pBB3NTS (*URA3*) integration; the lanes are self-explanatory. WT, wild type.

bination, probably at a later step following fork arrest (Fig. 1F). A possible mechanistic explanation of these observations is presented in the Discussion. Deletion of *MEC1* and *RAD53* did not have any visible impact on fork arrest at *Ter* (data not shown).

Impact of deletions of the genes of the *RAD52* epistasis group on recombination at *Ter*. Are plasmid integration and excision at *Ter* caused by HR or by NHEJ? To address this question, we investigated the dependence of plasmid integration on the gene products of the *RAD52* epistasis group needed for HR and on *yKU80*, which encodes a DNA end binding protein needed for NHEJ. We measured the frequency of plasmid integration in the different genotypes described below and discovered that plasmid integration was absolutely dependent on *RAD52*. We made sure that loss of plasmid integration in *rad52*Δ cells was not due to loss of fork arrest (data not shown). Within the limits of the resolution of the plasmid integration assay, we could not detect any effect of the deletion of *RAD50* or *yKU80* on plasmid integration, leading to the conclusion that NHEJ did not play a significant role in plasmid integration at *Ter* (Fig. 3A). Individual deletions of *RAD51*, *RAD54*, *RAD55*, and *RAD59* in the common *sir2*Δ background did not reveal any significant impact on the frequency of plasmid integration after three cycles of serial streaking on selective medium (Fig. 3A). We also performed plasmid excision assays in the same genetic backgrounds mentioned above and discovered that plasmid excision did not require *RAD51*, *RAD50*, *RAD54*, and *RAD55* (Fig. 3B).

We performed 2D gel analyses of replication intermediates from each of the strains containing the deletions of the recombination genes and observed no difference in the extent of fork arrest at *Ter* in any of the deletion derivatives, in comparison with the strain containing the single *sir2*Δ deletion (data not shown).

Recombination initiation at *Ter* does not require *SLX4*. Which gene product(s) catalyzed the double-strand (DS) break at *Ter* to initiate the recombination process? Previous work has revealed that in the absence of Sgs1 helicase, which resolves Holliday junctions and removes "chicken foot" structures caused by fork reversal, two classes of structure-specific nucleases (and a third group of genes encoding ubiquitin ligase) are essential for resolution and processing of replication intermediates in the rDNA and for survival of budding yeast (20). The first class of structure-specific endonucleases encoded by the *SLX1*/*SLX4* genes preferentially cuts at DNA forks. The second class consists of the endonuclease gene *MUS81* and its partner *MMS4* (20). In fission yeast the homolog of the *SLX1*/*SLX4* complex is the principal nuclease that acts at natural replication termini of rDNA to promote recombination (12). Are *SLX4* and/or *MUS81* also involved in initiation of recombination at *Ter* of budding yeast when *SGS1* is present? In order to address this question, we performed a quantitative plasmid integration assay as described above in the appropriate genotypic backgrounds (Fig. 3C). The results showed that after three cycles of serial streaking on selective plates, plasmid integration was not detectably reduced in the *mus81*Δ, *slx4*Δ, or *mus81*Δ *slx4*Δ strains (in the common *sir2*Δ background). There was little if any free plasmid present in the autoradiograms, suggesting that deletions of *slx4* and *mus81* did not reduce the rate of plasmid integration. The data are consistent with the interpretation that neither *SLX1*/*SLX4* nor *MUS81*/*MMS4* appeared to be essential for initiation of recombination at the *Ter* sites of *Saccharomyces cerevisiae*.

Several checkpoint genes including *TOF1* and *CSM3* inhibited recombination at the HOT1 site. The HOT1 site consists of two *cis*-acting elements, namely, E (the enhancer) and I (initiator). HOT1 recombination is stimulated by RNA polymerase I that binds to the I element, and it also depends on Fob1 binding to the *Ter* site present in the E element, where the Fob1-*Ter* complex serves to activate RNA polymerase I. However, HOT1 activity does not depend on fork arrest (45). These observations provided us with an opportunity to investigate the impact of checkpoint proteins and the Rad52 epistasis group proteins on recombination that was dependent on the Fob1-*Ter* complex but was not triggered by replication termination (47). We investigated whether *TOF1* and *CSM3*, along with other checkpoint genes belonging to mediator, sensor, and effector classes, also modulated *HOT1* activity. We constructed strains of the appropriate genotypes that also included the *HOT1* locus placed in chromosome III (Table 1). Recombination was measured by the excision of a *URA3* reporter by induction of illegitimate recombination by *HOT1* between two flanking *his4* sequences (Fig. 4A). We scored the appearance of *ura3* colonies on 5-FOA plates as a function of increasing cumulative periods of prior growth in nonselective medium.

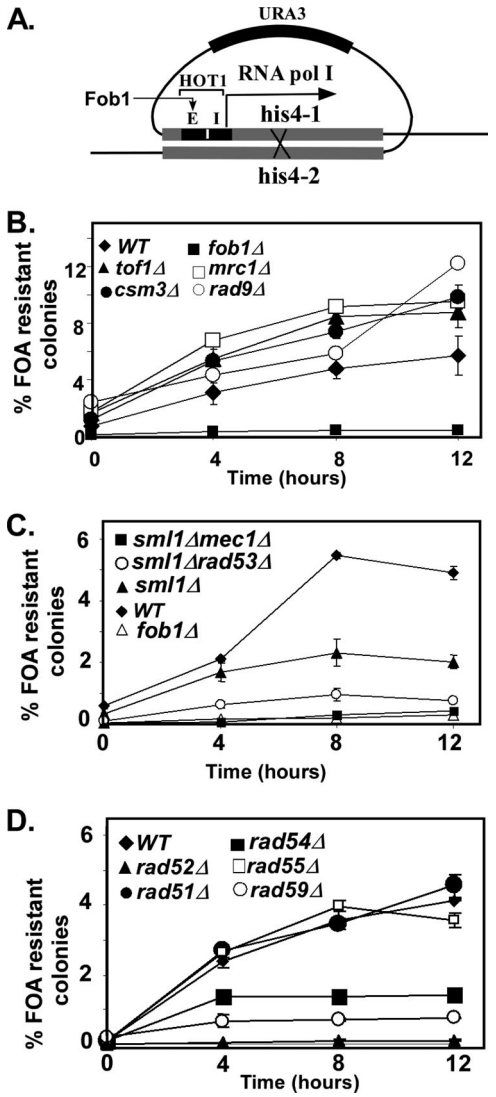

FIG. 4. Impact of deletions of genes encoding checkpoint proteins and recombination proteins on *HOT1* activity. (A) Schematic diagram showing the HOT1 assay; the E elements (containing the Fob1 binding site) and the I elements are shown; recombination between the flanking *his4* genes excises *URA3*, which has no *ARS* and therefore is eliminated from the cells. (B) HOT1 activity in the absence of checkpoint adapter proteins. (C) HOT1 activity in the absence of *MEC1* and *RAD53*. (D) HOT1 activity in different strains with individual deletions of the various members of the *RAD52* epistasis group. WT, wild type.

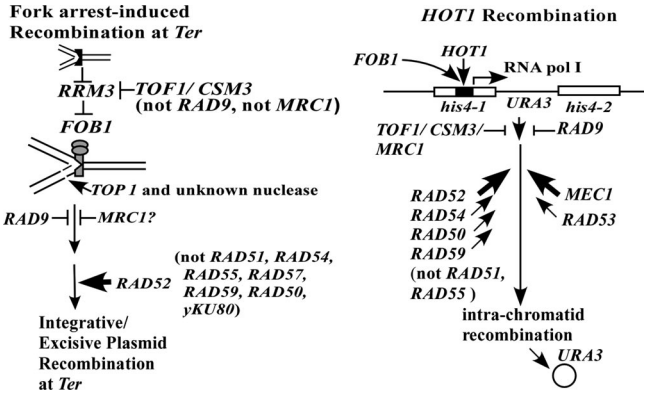

FIG. 5. Schematic diagram that summarizes the data on the impact of various checkpoint proteins and members of the *RAD52* epistasis group on plasmid integration and excision at *Ter* and on *HOT1* activity. The heavy arrows at *RAD52* and lighter ones at *RAD54* and *RAD59* indicate that *RAD52* was absolutely essential for both plasmid integration and HOT1 activity; the latter genes played a stimulatory role in *HOT1* recombination. The question mark next to *MRC1* in the plasmid integration/excision pathway indicates that the inhibitory effect was rather modest compared with that of the wild type. Previous work has shown that *RAD50* was partially required for HOT1 activity. The heavy arrow next to *MEC1* indicates that this gene makes a significant positive contribution to HOT1 activity.

The data are consistent with the conclusion that, in contrast to the absolute requirement of *TOF1* and *CSM3* genes for integrative recombination and plasmid excision at an active *Ter* site, these genes suppressed *HOT1* activity. The strains containing *rad9*Δ and *mrc1*Δ also showed various degrees of derepression of *HOT1* activity (Fig. 4B). The data suggested that Tof1 and Csm3 proteins might be protecting a DNA-protein complex from being processed and channeled into the *HOT1*-mediated intrachromatid recombination pathway. Deletion of the *FOB1* gene served as a negative control and, as expected, completely eliminated HOT1 activity.

We further investigated the possible modulatory effects of the sensor kinase Mec1 and the effector kinase Rad53 on HOT1 by constructing the appropriate strains and measuring HOT1 activity as described above (Fig. 4A). Both the *mec1*Δ and the *rad53*Δ strains showed a significant decrease in HOT1 activity, suggesting that the sensor and the effector kinases stimulated transcription-dependent recombination triggered by the binding of Fob1 to the *Ter* site embedded in the enhancer (E) element (Fig. 4A and C). It should be noted that it was necessary to construct double *sml1*Δ *mec1*Δ and *sml1*Δ *rad53*Δ deletions because, without the removal of the *SML1* gene, which is a repressor of ribonucleotide nucleotide reductase, deletions of either *MEC1* or *RAD53* cause cell lethality (51). The data also showed a lesser reduction of HOT1 activity upon individual deletion of the *SML1* gene (Fig. 4C).

***HOT1* activity and the *RAD52* epistasis group.** It was already known that *HOT1* activity was partially dependent on *RAD50* and completely on *RAD52* (50). In order to investigate the impacts of the other members of the *RAD52* epistasis group, we constructed appropriate strains containing individual deletions of the genes (Table 1) and measured HOT1 activity by quantifying the emergence of 5-FOA-resistant colonies as a function of prior growth in nonselective medium. The data confirmed the conclusion that HOT1 activity was completely dependent on the *RAD52* gene and further revealed that it was partially dependent on *RAD54* and *RAD59* but not on *RAD51* and *RAD55* (Fig. 4D). Therefore, the HOT1 recombination pathway appears to be different in this regard from the fork arrest-dependent recombination pathway that results in plasmid integration/excision at *Ter*. A summary of the results is shown schematically in Fig. 5.

DISCUSSION

Efficient synthesis of macromolecular components of high abundance such as rRNA in eukaryotes is facilitated by the

existence of multiple copies of the template DNA that are present in tandemly repeated copies in the rDNA cluster(s). In some organisms, as many as several thousand copies of rDNA repeats are maintained, sometimes on several different chromosomes (4). However, the maintenance of numerous tandem copies of the same sequence poses special challenges to the organism, not the least of which is prevention of excessive recombination mediated by the replication terminator proteins (e.g., Fob1) that might cause disassembly of the array into extrachromosomal rDNA circles, genome instability, and perhaps even premature cellular aging (37).

This work provides new insights into the control of initiation and progression of recombination at Fob1-*Ter* complexes and shows that the checkpoint mediator protein Tof1 and its binding partner Csm3, which are the homologs of the mammalian timeless (*TIM*) and timeless-interacting protein partner (*TIPIN*) and the fission yeast *SWI1* and *SWI3*, respectively (4), promote recombination at *Ter* by preserving fork arrest by counteracting the activity of Rrm3 helicase/sweepase. The sweepase tends to promote fork progression past the Fob1-*Ter* complex in the absence of Tof1 or Csm3 (31). It should be noted that Mrc1, which together with Tof1 and Csm3 forms a fork protection complex (21), is not involved in termination of replication and consequently was not required for initiating recombination at *Ter*. Rather, Mrc1 showed a modest inhibitory effect on this recombination as indicated by the plasmid excision assay. The protein is known to be involved in sister chromatid cohesion (49). Since Sir2 inhibits plasmid integration (5), our experiments were carried out in a *sir2*Δ background in which derepression of the *E-pro* bidirectional promoter causes transcription-mediated removal of some of the cohesin from the *Ter* region, thereby at least partially removing a major barrier to intrachromatid recombination (24). Perhaps in the *mrc1*Δ strain the removal of residual cohesin from the *Ter* region caused a correspondingly modest stimulation of plasmid excision.

The present work also showed that *RAD9*, a mediator of the DNA damage checkpoint pathway (48), is also a negative regulator of recombination as revealed by the plasmid excision experiments. *RAD9* is also known to be an inhibitor of recombination at a hot spot located at or near a tRNA gene in budding yeast (1). Although the mechanistic details of *RAD9*-mediated inhibition of recombination are hitherto unknown, like *TOF1* and *MRC1*, *RAD9* could be promoting retention of residual cohesion at *Ter* or by another mechanism that prevents the termination complex from being processed as a recombination intermediate.

Most recombination events are believed to be initiated by a DS break on DNA (39), although there is some evidence that recombination and restart of some stalled forks could be effected without a DS break by template switching and generation of a recombination intermediate (T. Carr, personal communication). What might be catalyzing a DS break at stalled forks at *Ter*? Our work shows that neither the structure-specific endonucleases Slx1 and Slx4 nor the Mus81/Mms4 complex was involved in the process. However, there is a report that the homologous Slx1 and Slx4 of *S. pombe* seem to be needed for this function in fission yeast (12). We have recently discovered that topoisomerase I is one of the enzymes that enhances recombination, probably by generating the DS breaks at *Ter* (B. K. Mohanty and D. Bastia, unpublished data).

It is interesting that the transcription-catalyzed, Fob1-dependent but fork arrest-independent HOT1 recombination was inhibited by Tof1 and Csm3. It is possible that in addition to their absolute requirement for initiating recombination at *Ter*, Tof1 and Csm3 could also be serving as inhibitors of recombination by promoting cohesin assembly around *Ter* at a step(s) following fork arrest. If so, such an effect would be masked from detection due to the absolute requirement for these two proteins at the recombination initiation step. In summary, observations presented here support the conclusion that, depending on whether recombination was initiated by fork arrest at *Ter* or by RNA polymerase I-catalyzed transcription at the *HOT1* locus, the Tof1-Csm3 complex acted as a positive or negative regulator of recombination, respectively.

Neither plasmid integration/excision at *Ter* nor HOT1 activity required the homology search protein Rad51, but both were absolutely dependent on the DNA strand-annealing protein Rad52 (33). However, HOT1 activity but not recombination at *Ter* seemed to be stimulated by Rad59 (strand-annealing protein) and Rad54 (motor protein), indicating additional differences between these two modes of HR provoked by Fob1. Our results showing the dispensability of *RAD51* for recombination at *Ter* are not inconsistent with the observation that recombination intermediates accumulate in a DNA polymerase α mutant of budding yeast in a Rad51-independent mode (52) and that recombination at the *Ter* sites of fission yeast rDNA is also Rad51 independent (12). The impact of the various checkpoint proteins and the Rad52 group of proteins on recombination at *Ter* and at *HOT1* is summarized in Fig. 5.

It is interesting that Mec1 significantly enhanced HOT1 activity. This checkpoint sensor kinase is known to activate HR, as measured by a gap-filling assay, by phosphorylating Rad55, which is a facilitator of the homology search protein Rad51 (3). During meiosis, the sensor promotes recombination by phosphorylating the Hop1 protein (10). Neither the recombination at an active *Ter* site nor HOT1 activity, as reported in this work, required Rad55 or Rad51. Therefore, it is reasonable to predict that the sensor kinase Mec1 is likely to have a different target(s) in the Fob1-dependent HOT1 recombination pathways.

In conclusion, despite the paucity of detailed mechanistic biochemistry of checkpoint pathways, the molecular biological and genetic analyses carried out in this work have provided new insights into how recombination at replication termini is modulated by checkpoint proteins. Since *Ter* sites are conserved in the intergenic spacer regions of rDNA from yeast to humans (4), some of the conclusions reached here should have general significance.

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