The S-Phase Checkpoint Is Required To Respond to R-Loops Accumulated in THO Mutants*
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Cotranscriptional R-loops are formed in yeast mutants of the THO complex, which functions at the interface between transcription and mRNA export. Despite the relevance of R-loops in transcription-associated recombination, the mechanisms by which they trigger recombination are still elusive. In order to understand how R-loops compromise genome stability, we have analyzed the genetic interaction of THO with 26 genes involved in replication, S-phase checkpoint, DNA repair, and chromatin remodeling. We found a synthetic growth defect in double null mutants of THO and S-phase checkpoint factors, such as the replication factor C- and PCNA-like complexes. Under replicative stress, R-loop-forming THO null mutants require functional S-phase checkpoint functions but not double-strand-break repair functions for survival. Furthermore, R-loop-forming hpr1Δ mutants display replication fork progression impairment at actively transcribed chromosomal regions and trigger Rad53 phosphorylation. We conclude that R-loop-mediated DNA damage activates the S-phase checkpoint, which is required for the cell survival of THO mutants under replicative stress. In light of these results, we propose a model in which R-loop-mediated recombination is explained by template switching.

Mutation and recombination are increased by transcription in bacteria, yeasts, and humans, phenomena referred to as transcription-associated mutation (TAM) and transcription-associated recombination (TAR) (1). The molecular mechanism leading to TAM and TAR is unclear. DNA may become more susceptible to being damaged when it is transcribed, which is consistent with the fact that DNA-damaging agents show a synergistic effect with transcription in the induction of recombination in wild-type yeast (21) or mutation in bacteria (13, 27). Indeed, topological changes (65) or chromatin remodeling associated with transcription (31, 53) may increase the probability of the occurrence of single-stranded DNA (ssDNA), which is chemically less stable than double-stranded DNA (38) and more susceptible to damage.

Nevertheless, the major accessibility of the transcribed DNA may not be sufficient to explain TAR, and it seems likely that there is more than one mechanism by which transcription stimulates recombination. In this regard, TAR is mainly seen when transcription and replication occurs in the S phase and has been related to replication fork impairment when transcription and replication collide (57, 71). The requirements for TAM may be different as convergent replication-transcription does not affect mutation frequency but causes differences in mutation spectrum (33).

A particularly strong TAR has been observed in mutants of THO-Sub2 and THSC complexes (24, 55, 58). THO is a conserved multiprotein complex, containing Hpr1, Mft1, Tho2, and Thp2 in yeast, which functions at the interface between transcription and mRNA export via its interaction with Sub2 in a high-molecular-weight complex termed TREX (15, 67). THO mutant yeast cells showed a replication fork impairment that was linked to this strong TAR phenotype (71). In addition, the cotranscriptional formation of R-loops, in which the nascent mRNA forms an RNA-DNA hybrid with the transcribed strand, is linked to the strong TAR observed in THO mutants (22, 29). This has been confirmed by the specific mutagenic activity of human (activation-induced cytidine deaminase) AID on the transcribed strand of an active gene reporter in THO mutant cells (22). The relevance of R-loops in genetic instability has also been shown in class switch recombination as well as in depleted vertebrate cells of the ASF/SF2 splicing factor (36, 72). It still remains unclear whether the R-loop is responsible for the impairment in replication fork progression.

Different types of obstacles can impair the progression of replication forks, but multiple factors sense replication fork failures or DNA damage produced during the S phase, triggering a checkpoint response to stabilize active replication forks once the obstacle is overcome (reviewed in reference 40). In the absence of a functional checkpoint, many replisome components dissociate, and the replication fork can collapse, leading to the formation of aberrant and potentially toxic DNA structures (reviewed in reference 34). Checkpoint pathways consist of damage signal amplification and transduction cascades though the Mec1/Tel1 kinases that converge in the Rad53/Chk1 effector kinases to coordinate replication with DNA repair and cell cycle progression. Replication forks undergoing unusual or prolonged stress trigger the recruitment of Mec1 as a result of the accumulation of ssDNA-bound replication protein A (RPA) (74). Further, Mec1 activation requires the RPA-mediated recruitment of the PCNA-like complex (Rad17/Mec3/Ddc1) via the Rad24 replication factor C (RFC)-like complex (50, 75). However, two alternative RFC-like complexes formed with either Elg1 or Ctf18 may also work on PCNA (7), although their role is not clear. The three RFC-
like complexes participate in checkpoint signaling as the three of them need to be eliminated from the cell to completely abolish Rad53 phosphorylation after hydroxyurea (HU) or methyl methanesulfonate (MMS) treatment (10, 11, 32). Similarly, Rad53 activation absolutely requires the PCNA-like complex in vivo (9).

Two alternative checkpoint pathways function during the S phase to converge in Rad53 phosphorylation and subsequent checkpoint activation: the intra-S DNA damage and the replication checkpoints (reviewed in reference 52), which differ in their mediator proteins. Whereas Rad9 mediates the general DNA damage checkpoint all over the cell cycle, Mrc1 is part of the replication apparatus and is specific to the replication checkpoint (5, 54).

In order to get further insights into the mechanisms of TAR and its connection with R-loop formation and DNA replication impairment, we have analyzed the genetic interaction of THO with a selection of 26 genes involved in replication, S-phase checkpoint, DNA repair, and chromatin remodeling. We found a synthetic growth defect in double null mutants of THO and checkpoint factors, such as the RFC-like and PCNA-like complexes. In addition, we found that under replicative stress, hyperrecombinant THO null mutants require functional S-phase checkpoints for survival, whereas this is not the case for the nonhyperrecombinant hpr1-101 (hpr1-L586P) THO point mutant. Our results show a link between cotranscriptionally formed R-loops and replication fork progression impairment in chromosome regions. We conclude that R-loop-mediated DNA damage activates the S-phase checkpoint, which is required for survival under replicative stress. These results fit with a model in which R-loops would be bypassed by inter- or intrachromosomal template switching that would lead to deletions between DNA repeats, the major type of recombination event observed in THO mutants.

**MATERIALS AND METHODS**

**Strains and plasmids.** All yeast strains used in the experiments are described in Table S1 in the supplemental material. The centromeric plasmids pRS316-LY1NS, containing the recombination system based on the two lid2 repeats (58), pWJ1344 containing tagged RAD52-YFP (where YFP is the yellow fluorescent protein gene) (39), and pAO138 containing the mrc1AQ allele (54) were described previously. pYGWRNH1, containing the RNH1 gene inserted in the BstXI restriction site of pYGW1 (Research Genetics), was kindly provided by R. Crouch.

**Detection of Rad52-YFP.** Rad52-YFP foci from mid-log-phase cells transformed with plasmid pWJ1344 were visualized with a Leica DC 350F microscope as previously described (39).

**Cell cycle synchronization and flow cytometry.** The bar1Δ cells were used for cell cycle synchronization to prevent adaptation to α-factor. Cells were arrested in the G1 stage with 5 μg/ml α-factor mating pheromone and were released into SC medium to allow synchronous progression into the S phase. At the time of α-factor release, 130 mM HU was added where indicated. Approximately 10^7 cells were collected at each of the indicated time points postrelease from α-factor arrest and processed for flow-cytometric analysis. The samples were processed as described previously (17). Cell cycle distribution was analyzed using a FACs-Calibur system (Becton-Dickinson).

**Rad53 immunoblotting.** Total protein extracts were prepared using trichloroacetic acid precipitation (18). Similar quantities of whole-cell protein extract per well were electrophoresed on an 8% sodium dodecyl sulfate-polyacrylamide gel and run according to standard procedures. After electrophoresis, the gels were run and blotted onto nitrocellulose membranes (GE Healthcare, Amersham), and proteins were detected using rabbit polyclonal JD148 or mouse monoclonal ELZE1 α-Rad53 antibodies, kindly provided by J. Diffley and M. Foiani, respectively, with similar results.

**2-D gel electrophoresis.** Wild-type, hpr1Δ and hpr1-101 (bar1Δ) cells were arrested with α-factor and released into minimal medium containing the corresponding HU concentrations for 30 min prior to DNA extraction. DNA extraction was performed with the cetyltrimethylammonium bromide method, and neutral-neutral 2-D gel electrophoresis was performed as described previously (37).

**Miscellanea.** Yeast methodology, ^32^P-labeled DNA probes, and Southern blotting were performed following standard procedures. Rich medium YPAD was made of 1% yeast extract, 2% peptone, and 2% glucose (dextrose) supplemented with 10 mg/ml adenine.

**RESULTS**

**Genetic interactions of THO with S-phase checkpoint factors.** In order to gain insight into the molecular basis of TAR and its connection with replication, we have investigated whether or not genetic interactions exist between the genes encoding the THO complex and 26 genes involved in nuclear DNA processes. For this purpose, we crossed an hpr1Δ strain with the 28 mutants listed in Fig. 1. These include mutations in genes with a function in checkpoints (RAD24, ELG1, CTF18, DDC1, MEC3, RAD17, RAD9, MRC1, MEC1, RAD53, and Sic1), replication (CDC44, CDC6, PR11, POL30, CDC2, DNA2, DIA2, and RFA1), DNA repair (MRE11, SSR2, SGS1, MUS81, RAD52, and POL32), and chromatin remodeling (SPT16). The results are summarized in Fig. 1 and Fig. S1 in the supplemental material.

A tetrad analysis of the diploids revealed a synthetic growth defect in the germination of the hpr1Δ rad24Δ, hpr1Δ ddc1Δ, hpr1Δ mec3Δ, hpr1Δ mec1Δ, and hpr1Δ rad35Δ double mutants (Fig. 2A). All these genes are involved in the S-phase checkpoint response. Briefly, Rad24, together with the replication proteins Rfc2 to -5, forms an RFC-like clamp loader that loads the PCNA-like complex Rad17-Mec3-Ddc1 onto DNA (reviewed in reference 68). This RFC/PCNA-like complex acts as a DNA damage sensor participating in the recruitment of Mec1, which is responsible for the phosphorylation of effector kinases, such as Rad53 (68). As can be seen in Fig. 2A, the strongest growth defect was observed in hpr1Δ rad24Δ cells. To confirm that the genetic interactions of RAD24 with HPR1 can be extended to other members of the THO complex, we crossed rad2Δ4 Δ cells with mfl1Δ cells. A synthetic growth defect was also observed for mfl1Δ rad24Δ double mutants (Fig. 2A). Thus, we conclude that the S-phase checkpoint response is required for proper growth of THO mutants.

THO mutants are slightly sensitive to drugs that produce replicative stress, such as HU at a concentration of 200 mM (see below), which may indicate a replication defect in hpr1Δ cells. To determine whether the genetic interaction between THO and DNA damage checkpoint proteins was caused by such a replicative defect, we assayed the sensitivity of double mutants to HU or MMS. The results are summarized in Fig. 1 and in Fig. S1 in the supplemental material. The effect of the
mutations in the main checkpoint kinases Mec1 and Rad53 on 
*hpr1Δ* survival was not tested because they confer a severe 
sensitivity to DNA damage even at low HU and MMS concen-
trations. Nevertheless, and consistent with its synthetic growth 
defect, the *hpr1Δ rad24Δ* and *mft1Δ rad24Δ* double mutants 
were more sensitive than the *hpr1Δ, mft1Δ*, and *rad24Δ* single 
mutants to both HU and MMS (Fig. 1 and 2B; see also Fig. S1 
in the supplemental material). In addition, double mutants of 
*hpr1Δ* with the PCNA-like factor mutations *ddc1Δ, mec3Δ*, and 
*rad17Δ*, with mutations in two other RFC-like factors, Elg1 
and Ctf18, or with mutations in the Rad9 and Mrc1 checkpoint 
mediators, were highly sensitive to HU and MMS (Fig. 1; see 
also Fig. S1 in the supplemental material). These results sug-

ggest that *hpr1Δ* mutants accumulate lesions under replicative 
stress that are sensed by both S-phase checkpoints.

By contrast, mutations in the canonical replication factors, 
such as RFC1 (*cdc44-8*) or PCNA (*pol30-52*) as well as *cdc6-1*, 
*cdd2-3*, and *raf1-11*, did not cause such a lethal effect in the 
*hpr1Δ* background (Fig. 1 and 2B). Nevertheless, it is worth 
noting that we also found an increase in HU and MMS sensi-

![FIG. 1. Genetic interactions of THO mutations.](http://mcb.asm.org/)}
tivity with replication or repair mutations, such as those in the primase and Dna2 helicase alleles previously described as sensitive to DNA-damaging agents (pri1-M4, pri1-2, dna2-1, and dna2-2) (14, 19, 45), and the deletion of the Dia2 ubiquitin ligase required to overcome replication impairment (12), the Sgs1 DNA helicase involved in DSB repair, and the Pol32 subunit of Pol δ involved in break-induced replication (43). Since both primase and Dna2 helicase are involved in lagging-strand synthesis, we tested whether the genetic interaction was also observed in leading-strand replication defective mutants. For this, we analyzed the effect of depleting the leading-strand polymerase Pol ε (POL2) using a POL2 gene under the tet promoter. As can be seen in Fig. S1 in the supplemental material, whereas the depletion of POL3 (Pol γ/H9254), used as a control, had very little effect on the growth of hpr1Δ cells (consistent with the normal growth of the hpr1Δ cdc2-3 double mutant), the depletion of POL2 (Pol ε) causes a growth defect in hpr1Δ cells. Therefore, the synthetic growth defect is not specific for lagging replication mutants.

The S-phase checkpoint requirement of THO mutants is R-loop dependent. Since it has been shown that THO mutants accumulate R-loops (22, 29), we assessed whether the requirement for the S-phase checkpoint factors and the replication/repair proteins of THO null mutants were dependent on the cotranscriptional formation of R-loops. For this, we analyzed the hpr1-101 (hpr1-L586P) point mutant, which is impaired in transcription elongation and export without showing a hyper-recombinant phenotype (30) or R-loop formation (23). As can be seen in Fig. 2C, the hpr1-101 rad24Δ, hpr1-101 elg1Δ, and hpr1-101 ctf18Δ double mutants grew as well as their respective single mutants in the presence of HU or MMS. This result indicates that the requirement of a functional S-phase checkpoint for the cell survival of THO mutants under replicative stress is caused by R-loop formation.

Double mutants of THO and checkpoint factors show increased genetic instability. The HU sensitivity observed in hpr1Δ mutants defective in S-phase checkpoints can result from unrepaired DNA lesions, which in turn can lead to an increase in genetic instability. To test this, we investigated genome instability in single and double mutants by measuring the following three different events: the percentage of cells that showed Rad52 foci, the frequency of recombination between direct repeats, and the rate of GCRs.

As can be seen in Fig. 3A, hpr1Δ leads to an increase in the percentage of cells with Rad52 foci, as previously reported (71). rad24Δ increased Rad52 foci threefold. Interestingly, Rad52 foci were further increased in hpr1Δ rad24Δ cells (Fig. 3A). Similarly, we measured Rad52 foci in mutants in the other RFC-like factor genes. An increase in the elg1Δ and ctf18Δ single mutants was previously reported (6). Interestingly, this increase was even higher in the hpr1Δ elg1Δ double mutant.
although less evident in the case of the hpr1Δ ctf18Δ double mutant (see Fig. S2A in the supplemental material). Adding HU to the cultures slightly increased the percentage of Rad52 foci in all cases (see Fig. S2A in the supplemental material).

Recombination was measured with the LYΔNS system, a plasmid-based 0.6-kb leu2 direct-repeat recombination assay in which transcription has to proceed through a 3.7-kb-long intervening sequence located between the repeats (58). A significant 2.3-fold increase of Leu+ recombinants could be seen in hpr1Δ rad24Δ and mft1Δ rad24Δ cells with respect to the single mutants (Fig. 3B; see Fig. S2B in the supplemental material), implying that the occurrence of recombinogenic DNA lesions is higher in the absence of the S-phase checkpoint.

Finally, we measured the rate of GCRs occurring in the nonessential portion of the left arm of chromosome V, which has the nonessential gene HXT13 located distal to the CAN1 gene, replaced by URA3 (61). GCR events, detected as CFU in SC-5-fluoroorotic acid–L-canavanine, were fivefold higher in rad24Δ cells than in the wild type, as previously reported (49), whereas hpr1Δ increased the GCR frequency sevenfold (from $1.2 \times 10^{-9}$ to $6.1 \times 10^{-9}$ [Fig. 3C]). Therefore, the genetic-instability phenotype associated with THO depletion is not only manifested as hyperrecombination in DNA repeat systems but also in the form of GCRs, although to a much lesser extent (63). The hpr1Δ rad24Δ double mutant further showed GCR levels 10-fold above wild-type levels (Fig. 3C).

In summary, genetic instability, measured as the percentage of cells with Rad52 foci, recombination, or GCRs, was higher in THO mutants if they were deficient in the S-phase checkpoint, suggesting that recombinogenic DNA lesions or DNA-damage-like structures that are sensed by the S-phase checkpoint accumulate in THO null mutants.

**R-loop-mediated DNA damage is sensed by Mad2 at G2/M.** RFC- and PCNA-like complexes are checkpoint sensors that act in the DNA damage checkpoint throughout the cell cycle. To assess whether the synthetic growth defect of double mutants of hpr1Δ with mutations in those complexes is S phase dependent, we determined the pattern of the cell cycle progression in G1-synchronized wild-type, hpr1Δ, rad24Δ, and hpr1Δ rad24Δ cells released in the presence and absence of HU (Fig. 4A). In the absence of replication stress (Fig. 4A, top panel), wild-type and rad24Δ cells behave similarly (both reach G2 in 2 h), while hpr1Δ cells display a slightly slower S-phase progression (they reach G2 in 4 h). Instead, cycle progression is impaired in hpr1Δ rad24Δ cells, and S and G2 cells accumulate in this case.

More interestingly, in the presence of HU (Fig. 4A, bottom panel), hpr1Δ cells showed a similar but again slightly slower cell cycle progression pattern than the wild type. HU caused rad24Δ cells to arrest in the S phase, implying that rad24Δ leads to failures of DNA synthesis in the presence of HU. Indeed, cells do not reach G2 even 5 h after release. We are unsure whether this is due to the processing of damage so that it now requires longer for repair and replication resumption or to an adaptation phenomenon. A strong accumulation of S-phase cells is still observed in the hpr1Δ rad24Δ double mutant, but a significant portion of the cell population can complete the S phase under HU treatment and continued progression to G2. This suggests an S-phase checkpoint bypass that requires further analysis to understand its molecular nature. Similar
results of S-phase arrest bypass were obtained in the hpr1Δ elg1Δ, hpr1Δ ctf18Δ, hpr1Δ dia2Δ, and hpr1Δ dna2-2 double mutants (see Fig. S3 in the supplemental material). Therefore, the R-loop or a derivate DNA lesion seems to arrest cells in G2 if the S-phase checkpoint is defective.

This G2 arrest suggested that the strong HU sensitivity might be due to the mitotic checkpoint. In G2, the spindle assembly checkpoint, which requires the Mad1 and Mad2 proteins, delays the onset of anaphase in cells with defects in the mitotic spindle assembly (48). Interestingly, the spindle assembly checkpoint has also been suggested as protecting cells from defects in DNA replication, as shown by the fact that mad2Δ relieved the G2/M arrest caused by mutations in replication proteins (20), as well as that caused by DSBs in rad24Δ cells (8). Consequently, we decided to remove the mitotic spindle checkpoint gene MADD2 in the hpr1Δ rad24Δ double mutant and test its HU sensitivity. As can be seen in Fig. 4B, mad2Δ suppressed the strong HU sensitivity of the hpr1Δ rad24Δ double mutant. Importantly, this suppression is not specific for hpr1Δ rad24Δ; it was also observed in the case of hpr1Δ elg1Δ. Consistently, we observed that the HU sensitivity of the hpr1Δ rad24Δ double mutant did not kill the cells. These resumed growth after removal of the replicative stress conditions (see Fig. S4 in the supplemental material), further indicating that R-loop-dependent G2/M arrest is mediated by a mitotic checkpoint. Nevertheless, it is worth noting that the hpr1Δ rad24Δ elg1Δ double mutant is very sick after several generations (data not shown) and accumulates a large amount of DNA damage as deduced from the strong increase in Rad52 foci formation (Fig. 4C).

Accumulation of phosphorylated Rad53 in hpr1Δ cells. During the S phase, DNA damage checkpoints require a threshold level of DNA damage for their activation (62). Consequently, we assayed whether the DNA lesions generated in hpr1Δ cells were sufficient to activate the checkpoint, which can be seen by a mobility shift of phosphorylated versus unphosphorylated Rad53 in Western blots. As shown in Fig. 5, spontaneous Rad53 phosphorylation could be detected in hpr1Δ cells. Notably, Rad53 phosphorylation could not be detected in the hpr1-101 mutant, implying that it is mediated by the R-loop. This indicates that DNA-damage-like structures are formed in THO mutants and are transduced into Rad53 phosphorylation.

Rad53 phosphorylation is also observed in the absence of the checkpoint mediators Rad9 and Mrc1; this is in hpr1Δ rad9Δ and in hpr1Δ mrc1AQ cells, in which mrc1AQ is a specific replication checkpoint-defective allele not affected in rep-
Chromosomal replication fork progression is impaired in THO mutants. Replication forks have previously been shown to slow down in an artificial plasmid-borne lacZ sequence under the control of the GAL1 promoter in yeast THO mutant transformants (71). However, apart from the observation that hpr1Δ mutants are sensitive to high doses of replicative stress, such as 200 mM HU (Fig. 6A), no evidence exists yet about replication impairment in the endogenous chromosomes. Since our genetic-interaction analysis of the S-phase checkpoint suggests that DNA damage may be accumulated during replication in untransformed cells, we assayed replication fork progression through an endogenous chromosomal region and in the presence of HU in order to amplify all replication-related phenotypes of THO mutants. For this, we selected a
highly and constitutively transcribed long DNA region that was located near an early and active ARS. To identify a sequence with these characteristics in the yeast genome, we did a search of ARS activity in the OriDB DNA replication origin database (51) and of mRNA levels in the genome-wide expression database (28). Thus, we chose to analyze replication fork progression through the SPF1 (YEL031w) gene. SPF1 is a highly transcribed, 3,647-bp-long opening frame (28) that is located on chromosome V near the active and early replication origin ARS508 (51).

To analyze replication forks, DNA was isolated from wild-type, hpr1Δ, and hpr1-101 cells treated with 40 mM HU for 30 min after release from G1 arrest induced with α-factor and subjected to 2-D gel electrophoresis. We analyzed replication intermediates in the 3.9-kb PvuII and the 4.3-kb PstI fragments harboring ARS508 and part of the SPF1 gene (Fig. 6B). As bidirectional fork progression should occur at similar rates, transitions from bubble to simple-Y arcs should take place when about 50% of the fragment is replicated. Therefore, we expected to observe the majority of the Y-shaped intermediates appearing along the descending simple-Y arc, in which the length of the newly replicated fragment exceeded the length of the nonreplicated one. As can be seen in Fig. 6C, the intensity of the Y arc is similar to that of the wild type.

Nevertheless, long Y-shaped molecules tended to accumulate toward the end of the descending simple-Y arc in hpr1Δ cells (indicated by an arrow) as a result of the progression of the replication forks away from the restriction fragment containing ARS508. This is consistent with a slowdown or pausing of replication forks traveling upstream of the SPF1 gene, indicating that replication fork progression through the SPF1 gene was affected. This implies that chromosomal replication is impaired in THO null mutants in the presence of replicative stress and transcription. Such an evident accumulation of long Y-shaped molecules was not observed in the hpr1-101 mutant, consistent with the fact that hpr1-101 is not sensitive to HU (Fig. 6A) and with previous 2-D gel analysis of replication forks in a plasmid (30). To further visualize the replication fork slowdown, we analyzed the progression of passive replication forks throughout the SPF1 gene of the ClaI-SpeI restriction fragment in the presence of high doses of HU in both the hpr1Δ and hpr1-101 mutants compared to that of the wild type (Fig. 6D). Whereas wild-type and hpr1-101 cells had replicated the SPF1 gene 120 min after α-factor release, replication intermediates stayed longer in hpr1Δ cells, consistently with a slowdown of fork progression.

Since the replication slowdown observed for THO mutants was shown to be dependent on both transcription and RNA in a plasmid (71) and it seems to depend on R-loop formation, we reasoned that the overexpression of RNase H1, which specifically removes RNA-DNA hybrids and partially suppresses hpr1Δ hyperrecombination (29), might also suppress the chromosomal replication defects of THO null mutants. As it can be seen in Fig. 6E, RNase H1 overexpression could partially suppress hpr1Δ sensitivity to HU. Altogether, these observations reveal that the transcriptional formation of R-loops contributes to chromosomal replication impairment in THO null mutants.

DISCUSSION

Our analysis of the genetic interaction of THO with genes involved in replication, S-phase checkpoint, DNA repair, and chromatin remodeling revealed a synthetic growth defect in double null mutants of THO and S-phase checkpoint factors, such as the RFC-like and PCNA-like complexes. Under replicative stress, hyperrecombinant THO null mutants require a functional S-phase checkpoint for survival that is linked to the formation of R-loops. Thus, R-loop-forming hpr1Δ mutants activate the phosphorylation of the checkpoint effector kinase Rad53. This is accompanied by replication fork progression impairment at transcriptionally active endogenous chromosomal regions. Altogether, our results indicate that R-loops mediate the formation of a replication-dependent DNA damage structure that is sensed by the S-phase checkpoint, which becomes essential for the cell survival of THO mutants under replicative stress.

Double deficiencies of THO together with checkpoint factors, including S-phase checkpoint sensors such as the RFC-like factor Rad24, PCNA-like components Mec3 and Ddc1, and the checkpoint kinases Mec1 and Rad53, showed a synthetic growth defect, while double mutants of hpr1Δ with mutations in canonical RFC and PCNA (cdc44-8 and pol30-52, respectively) grew normally (Fig. 1 and 2). The synthetic growth defect caused by THO null mutations in combination with mutations in the S-phase checkpoint factors is exacerbated in the presence of replicative stress, as tested by sensitivity to HU. Interestingly, this was not occurring with hpr1-101 (hpr1-L586P), a nonhyperrecombinant mutation that does not form R-loops (23), implying that the cotranscriptional formation of R-loops is needed for the S-phase checkpoint requirement. Consistently, Rad53 phosphorylation could be detected in hpr1Δ cells (Fig. 5) and hpr1Δ increases the levels of the RNR3 ribonucleotide reductase (N. Proudfoot, personal communication), an indicator of the existence of a checkpoint response. These results suggest that the replication-born damaged DNA structures formed in hpr1Δ cells are sufficient to activate the DNA damage checkpoint. We cannot disregard that the nontranscribed single-stranded DNA in the R-loop could be covered by RPA and sensed by the checkpoints, although we have no evidence for this possibility yet.

The primary event allowing checkpoint proteins to protect stalled forks appears to be replication fork stabilization (69), and many replication proteins are targeted by the checkpoint machinery, probably to stabilize the replisome fork association when forks stall (41). This is, for example, the case of the replicative primase, and indeed, mutations in the replicative primase (pri1-M4, pri1-2) also gave rise to HU sensitivity in hpr1Δ. Similarly, the requirement of ubiquitin-ligase Dia2 for the survival of hpr1Δ cells under replication stress might be explained by the essential role of Dia2 for the stabilization of replication forks through regions of damaged DNA (12). Therefore, it seems that R-loop-forming THO mutants need the genes involved in replication fork stabilization after DNA damage for survival. This is consistent with the replication fork impairment of THO mutants observed in the endogenous chromosome (Fig. 6) and an artificial plasmid (71) as well as the higher genetic instability observed in the absence of a
functional S-phase checkpoint (Fig. 3). The hyperrecombination of THO mutants is still observed (and further increased) in checkpoint-defective cells, consistent with the recent observation that the replication checkpoint does not affect homologous recombination at replication forks, but it impedes DSB repair (4). It would certainly be interesting to decipher the putative roles of the S-phase checkpoint to overcome replication fork stalling in addition to its role in replisome stabilization. Notably, Hpr1 has been recently identified as a Mecl target (46, 64), raising the intriguing possibility that THO could have a direct role in replication fork progression through transcribed DNA.

The S-phase checkpoint requires a threshold level of DNA damage for activation so that cells can tolerate a certain level of fork-associated ssDNA without leading to cell-cycle arrest. Later in the cell cycle, a threshold level is not required anymore, and the persistence of DSBs or regions of unreplicated DNA in the G2 phase is enough to prevent progression into mitosis (62). The observation that hpr1Δ rad24Δ cells tend to arrest in G2 suggests that this arrest must be the cause of their HU and MMS sensitivity. Supporting this, HU sensitivity is reversible in these mutants and can be suppressed by the deletion of the mitotic checkpoint gene MAD2, implying that a Mad2-mediated checkpoint detected the damaged replicative structure generated in the double mutants in THO and checkpoint factor cells under replicative stress (Fig. 4B).

Taken as a whole, our data suggest that THO mutants accumulate damaged DNA structures during replication that are sensed by the S-phase checkpoints. The emerging questions are, what are these replication-born damaged DNA structures, and how are they repaired? We propose that these structures could be ssDNA gaps, since double mutants harboring null mutations of a THO gene and a DSB repair gene are viable, even in the presence of replicative stress. Instead, a number of mutants that have been either proposed or demonstrated to accumulate DSBs are not viable when harboring null mutations in DSB repair genes. This is the case with rad27Δ or srs2Δ cells harboring mutations in the genes encoding the MRX complex (70). Cotranscriptionally formed RNA-DNA hybrids in THO mutants may cause replication fork stalling on either the leading or lagging strand with the concomitant accumulation of ssDNA that can be covered by RPA and sensed by the S-phase checkpoints (Fig. 7). Presumably, these RNA-DNA hybrids would be efficiently removed or bypassed by a still-unknown mechanism that may involve ribonucleases or some putative DNA-RNA helicases. Alternatively, in codirectional collisions, the replisome could use the RNA strand of the R-loop as a primer and displace the RNAPII, as it has been recently shown for in vitro leading-strand replication in Escherichia coli (56). This would also lead to the accumulation of ssDNA behind the fork that would need to be filled by postreplicative repair. However, when this stalling occurs within a DNA-direct repeat region, the hybrid could be bypassed by recombination, thus explaining the strong hyperrecombination between direct repeats in THO mutants.

It is generally assumed that mitotic recombination is initiated by DSBs, but it has not been discarded that other lesions, such as ssDNA gaps, could initiate homologous recombination. Indeed, there is evidence supporting that spontaneous mitotic recombination, such as that occurring at stalled replication forks, may not necessarily be initiated by DSBs (16, 35, 42). Based on the genetic requirements of hpr1Δ viability in the presence of replicative stress, including S-phase checkpoint factors but not DSB repair factors, as discussed above, it would be possible that the hyperrecombination of THO mutants is not primarily initiated by DSBs. In favor of this idea is the fact that the hyperrecombination of THO mutants is almost restricted to direct repeat recombination (2, 59) or that chromosomal rearrangements are poorly stimulated (5- to 10-fold [Fig. 3]) compared to the increase in direct repeat recombination of 3 orders of magnitude. Our study opens the intriguing possibility that R-loops may not be sufficient for DSB formation, which would explain why R-loop-mediated class switching in B cells requires AID as an essential player in the formation of DSBs (44), and indicates that the genomic instability caused by R-loops is mediated by replication.

We know from bacterial studies that homologous recombination is critical for replication fork restart after its blockage.
and collapse. However, homologous recombination is also involved in lesion bypass (reviewed in reference 26). In the latter case, it has been speculated that homologous recombination can be initiated by template switching between the nascent DNA strands, which would be stimulated by the ssDNA gaps that arise as a consequence of replication fork stalling (see reference 25). Therefore, it seems plausible that the RNA-DNA hybrid occurring within DNA repeats could be bypassed by a recombination-mediated replication process involving inter- or intramolecular template switching. Indeed, such a kind of template-switching model at replication forks has been suggested in mammals to explain the bypass of the DNA polymerase over the transcription machinery (60).

Interestingly, the \textit{hpr1Δ dna2-1, hpr1Δ dna2-2}, and \textit{hpr1Δ sgs1Δ} double mutants grow poorly under replication stress. This indicates that S resection may be required to allow recombination-mediated template switching, since Dna2 has recently been shown to have a role in DSB resection together with Sgs1 (73). Another certainly interesting observation was the stronger HU sensitivity of the \textit{hpr1Δ pol32Δ} double mutant. Pol32 is the only nonessential subunit of Pol \(\delta\). In addition to its role in Okazaki fragment maturation, Pol32 is involved in replication restart via the recombination-mediated replication mechanism break-induced replication (43, 47). Pol32 could be required for recombination-mediated replication between DNA repeats. Alternatively, Pol32 could be required for replication through a DNA template hybridized with RNA. Interestingly, in vitro studies have shown that RNA-DNA hybrids are displaced by Pol \(\delta\) at higher efficiency than DNA duplexes and that Pol32 is required to extend short flaps into very long flaps (66). Either way, our results open the possibility that Pol32 is required to extend short flaps into very long DNA repeats. Inter- or intramolecular template switching. Indeed, such a kind of template switching model at replication forks has been suggested in mammals to explain the bypass of the DNA polymerase over the transcription machinery (60).

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REFERENCES


