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R-loops do not accumulate in transcription-defective *hpr1-101* mutants: implications for the functional role of THO/TREX

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ABSTRACT

To get further insight into the effect that THO/TREX and R-loops have in transcription-associated recombination and transcription, we analyzed the ability to form R-loops of *hpr1-101*, a THO mutation that impairs transcription and mRNP biogenesis without triggering hyper-recombination. Human AID, a cytidine deaminase that acts on ssDNA displaced by RNA-DNA hybrids, strongly induced both hyper-recombination and hyper-mutation in *hpr1-101*, similar to *hpr1Δ* mutants. However, in contrast to *hpr1Δ*, AID-induced mutations in *hpr1-101* occur at similar frequencies in both the transcribed and non-transcribed strands, implying that the enhanced AID action in these mutants is not caused by co-transcriptional R-loops. These results indicate for the first time that THO has a transcriptional function that is not mediated by R-loops, providing a new perspective for the understanding of the coupling of transcription with mRNP biogenesis and export.

INTRODUCTION

In eukaryotic RNA polymerase II (RNAPII) transcription, nascent pre-mRNAs are processed and matured into mRNAs by protein complexes that are loaded onto transcription sites *via* their interaction with the C-terminal domain of RNAPII. Processing and maturation events are coupled with the formation of an export-competent ribonucleoparticle, mRNP, which is actively exported through the nuclear pore complex (NPC) into the cytoplasm (1–4). One key factor in the coupling between transcription and mRNP processing and export is THO, a conserved eukaryotic nuclear complex containing Tho2, Hpr1, Mft1 and Thp2, as first isolated from yeast (5). THO interacts physically and functionally with proteins involved in mRNA export, such as the Sub2 RNA-dependent ATPase, to form a larger complex termed TREX (6,7). THO mutations lead to gene expression defects particularly evident for long and GC-rich DNA sequences, (5) as well as for repeat-containing genes (8). Such defects reflect impairment in transcription elongation as determined both *in vivo* and *in vitro* (5,9,10). In the present view, it is believed that THO participates during transcription elongation in the formation of export-competent mRNPs. Consistent with it, overexpression of the RNA-dependent ATPase Sub2 or the RNA binding protein Tho1 suppresses the transcription and RNA-export defects of THO mutants (6,11,12).

It has been shown that *hpr1Δ* mutants accumulate co-transcriptional R-loop structures in which the nascent RNA forms an RNA-DNA hybrid with the transcribed (T) DNA strand, the non-transcribed (NT) strand remaining single-stranded behind the RNA polymerase II (RNAPII) (13). Such R-loops are linked to transcription defects and transcription-associated recombination (TAR) in THO mutants. The relevance of R-loops in genetic instability has also been shown in class switch recombination (CSR) as well as in depleted vertebrate cells of the ASF/SF2 splicing factor (14,15). R-loops in THO mutants have been confirmed genetically by showing that human AID, a cytidine deaminase that acts preferentially on ssDNA in the V and S-regions of Immunoglobulin (Ig) genes during somatic hypermutation (SHM) and CSR (16), induces both mutation and recombination by acting at the ssDNA that is displaced at the R-loops (17). However, the mechanisms by which R-loops impair transcription and induce TAR are unknown. It has been hypothesized that R-loops may become an obstacle for the next elongating RNAPII, impairing transcription elongation, as well as for the replicative DNA polymerase machinery, yielding to DNA lesions whose repair would require recombination (18–20). Consistent with this view, artificially constructed RNA–DNA hybrids have been shown to reduce transcription elongation efficiency *in vitro* (21). Nonetheless, the possibility that THO could play a more direct role in transcription not mediated by

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R-loops has not been ruled out. In this sense, it is worth noting that R-loops formed in ASF/SF2-depleted DT40 chicken cells do not seem to be linked to transcription impairment (15).

A particularly interesting allele to answer this question is *hpr1-101*, a point mutation that, in contrast to THO null mutations, confers a gene expression defect not accompanied by a significant increase neither in TAR nor in replication-fork progression slowdown (22). Despite the separation of phenotypes of *hpr1-101*, whether R-loops may differentially contribute to the transcription or genetic stability functions of THO mutants has not been elucidated. Here we show that human AID induced both hyper-recombination and hyper-mutation in *hpr1-101*, similar to *hpr1Δ* null mutants. However, in contrast to *hpr1Δ*, AID-induced mutations in *hpr1-101* were found at similar frequencies in both the T and NT strands, implying that the enhanced AID action in these mutants is not caused by co-transcriptional R-loops but by the opening of DNA strands during transcription. These results suggest that, in addition to the impact of R-loops on transcription elongation, THO mutations cause a transcriptional impairment that is independent of R-loops. This result provides a novel perspective in the understanding of the coupling between transcription and mRNP processing and export.

MATERIALS AND METHODS

Strains and plasmids

We used W303-1A isogenic strains U678-4C (*hpr1Δ::HIS3*), WMK-2A (*mft1Δ::KAN*) (5), WRS52-4B (*rad52Δ::KAN*) (23) and WH101-4A (*hpr1-101*) (22), described previously. Centromeric plasmids pCM184-LAUR containing *lacZ::URA3* under the tet promoter (6), pRS316-LYΔNS containing the *leu2* repeats recombination assay (24), pGAID carrying the human AID ORF under the *GAL1* promoter and pGLG containing the GFP-based recombination system (17) were described previously. pRS425GALRNH1 was constructed by subcloning the SalI-SpeI GALRNH1 fragment from pRS416GALRNH1 (13) into SalI-SpeI digested pRS425.

Recombination and mutation analysis

GFP fluorescence was determined in a FACScalibur (Becton-Dickinson, USA) from 10^6 cells grown in SC overnight and re-suspended in 1 ml H_2O as described (17). For the LYΔNS recombination and LAUR mutation assay, cells were cultured in SC plates, from which six independent colonies were analyzed. Leu+ recombinants were selected on SC-leu and Ura- mutants were selected on SC containing SC + 700 mg/l FOA as previously described (17). *lacZ*$^+$ and *lacZ*$^-$ were distinguished by color on SC + X-gal medium. Median mutation and recombination frequencies were obtained by fluctuation tests as the median value of six independent colonies isolated from SC plates. The final frequency given for each strain and condition is the mean and standard deviation of three to four median values.

RESULTS

Increased mutagenic and recombinogenic activity of AID in *hpr1-101* cells

In order to establish whether the transcription–elongation impairment of THO null mutants is linked to the accumulation of R-loops we determined whether *hpr1-101*, a THO point mutation that impairs transcription but does not trigger hyper-recombination (22), forms R-loops. For this, we analyzed the effect of AID in the direct-repeat recombination assay LYΔNS based on 0.6-kb *leu2* repeats in which transcription has to proceed through a 3.7-kb intervening sequence (24) (Figure 1). Due to the strong hyper-recombination of THO mutants in direct-repeat systems, we did not expect AID to cause a strong increase in recombination, but a significant 4.5-fold AID effect was observed in the *hpr1Δ* allele reaching 98% of recombination frequency. Unexpectedly, although the recombination frequency in *hpr1-101* was lower, AID overexpression also increased this frequency to 79%.

To confirm this result, we used the GFP repeat recombination assay GLG, in which GFP$^+$ recombinants can be directly scored by FACS analysis (17). Strikingly, AID led to a strong synergistic increase in the formation of GFP$^+$ recombinants in *hpr1-101* (Figure 2), similar to what was previously reported for R-loop-forming *mft1Δ* cells (17). These results indicate that, in contrast to wild-type cells, AID can access the DNA very efficiently in *hpr1-101* as well as in THO null mutants inducing a strong hyper-recombination.

To further test the ability of AID to access transcribed DNA in *hpr1-101* we looked at AID induced mutation in

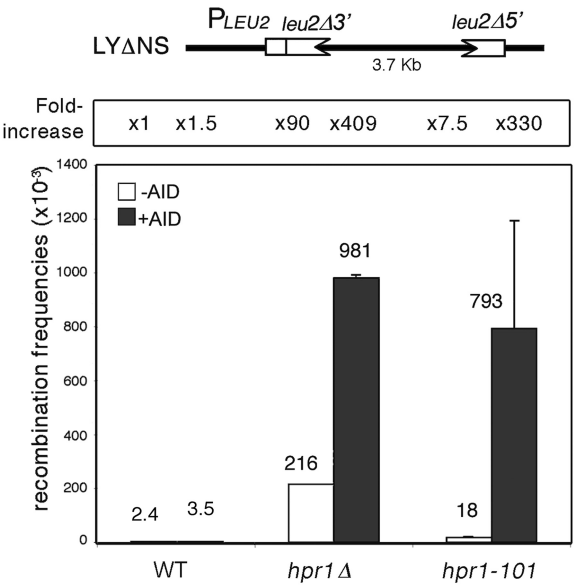


Figure 1. Effect of AID on direct-repeat recombination as detected by Leu$^+$ recombinant colonies. Recombination frequencies were obtained for the LYΔNS direct-repeat recombination system in wild-type, *hpr1Δ* and *hpr1-101* strains with or without AID overexpression. Mean recombination frequency and standard deviation of three to four different fluctuation tests are plotted. A diagram of the system is shown at the top.

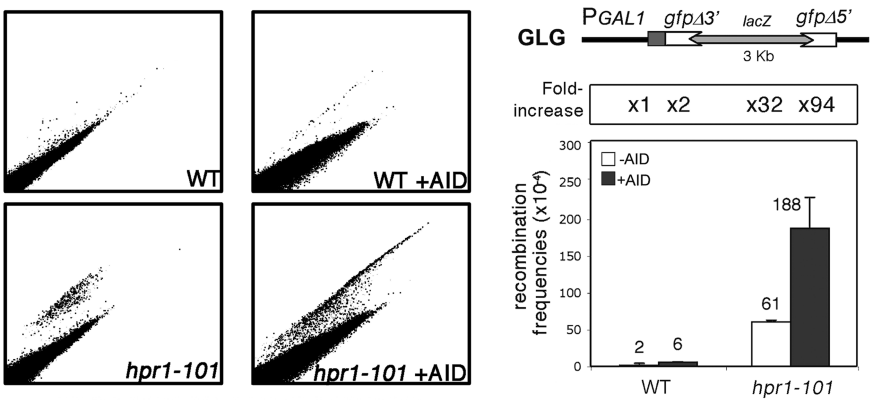
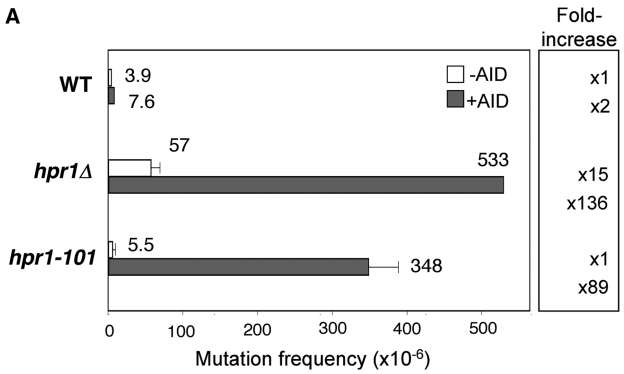


Figure 2. Effect of AID on direct-repeat recombination as detected by FACS. Recombination frequencies were obtained for the GLG direct-repeat recombination system in wild-type and *hpr1-101* strains with or without AID overexpression. *y*-axis, green fluorescence (FL1H); *x*-axis, unspecific fluorescence (FL2H). Mean recombination frequency and standard deviation of three to four different experiments are plotted. A diagram of the system is shown at the top.

hpr1-101 in the LAUR mutation assay, a *lacZ::URA3* translational fusion under control of the regulated *Tet* promoter in which Ura$^-$ mutants can be selected on synthetic complete (SC) medium supplemented with 5-fluoroorotic acid (17). In Figure 3A, we can observe that AID increased the mutation frequency 136-fold in *hpr1Δ*, consistently with what was reported for *mft1Δ* (17). As expected, AID also led to an 89-fold enhanced hyper-mutation in *hpr1-101* (Figure 3A). Therefore, AID action is enhanced in *hpr1-101* mutant, similarly to what occurred in R-loop forming THO null mutants. Nevertheless, we observed a clear difference analyzing the pattern of distribution of the mutations obtained in each mutant. While all mutations obtained in *hpr1Δ* were in *lacZ*, mutations obtained in *hpr1-101* appeared both in *lacZ* and *URA3* (Figure 3B). The abundance of mutations in *lacZ* in THO null mutants was previously proved to be due to the strong hyper-recombination (17). Therefore, the mutation distribution in *hpr1-101* was similar to the wild type rather than to the hyper-recombinant THO null mutants.



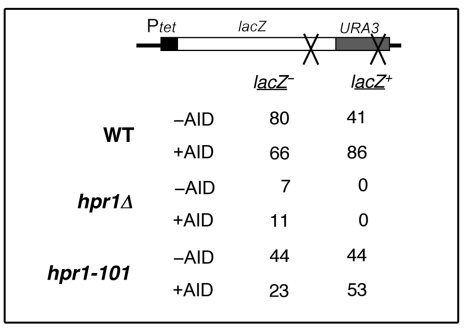


Figure 3. Effect of AID on mutation with the LAUR system. (**A**) Effect of AID on the frequency of Ura$^-$ mutants in the LAUR assay in wild-type, *hpr1Δ* and *hpr1-101* strains. All experiments were performed under high transcription conditions (without doxycycline). Mean mutation frequency and standard deviation of three to four different fluctuation tests are plotted. (**B**) Genetic analysis of spontaneous (−AID) and AID-induced (+AID) mutations. Distribution of *lacZ*$^-$:*lacZ*$^+$ mutations among Ura$^-$ mutants in wild-type, *hpr1Δ* and *hpr1-101* strains. Wild-type data were taken from (17). Ura$^+$ mutants were selected on SC + FOA, whereas *lacZ*$^+$ mutants and *lacZ*$^-$ were scored by color on SC + X-Gal.

AID accesses with similar frequency both the transcribed and non-transcribed DNA strands of a transcribed reporter

We have previously shown that in THO null mutants AID acts preferentially on the NT strand in a 3:1 ratio versus the T strand, consistent with the displacement of the NT strand as ssDNA in R-loops (17). To assess whether R-loops form in *hpr1-101*, we sequenced the mutations that had occurred in *hpr1-101* with or without AID overexpression (Figure 4 and Supplementary Table 1) and analyzed the ratio of mutations in C or G as a measurement of AID targeting to the non-transcribed versus the transcribed strand (NT:T) (Table 1). In the absence of AID, *hpr1-101* yielded a NT:T ratio of mutations of 1:1 (four mutations at C and four mutations at G). Unexpectedly, this ratio was very similar in the presence of AID (0.6:1, 14 at C and 23 at G). The WRC AID-target motif was also mutated at an NT:T ratio 1:1 in the wild type (15 at WRC and 15 at GYW) and 0.6:1 in *hpr1-101* (12 at WRC and 18 at GYW) while it was 5:1 in *mft1Δ* (10 at WRC and 2 at GYW) [Table 1 and (17)]. Therefore, both strands are equally accessible to AID in *hpr1-101*. This is indicative that R-loops are either not formed or

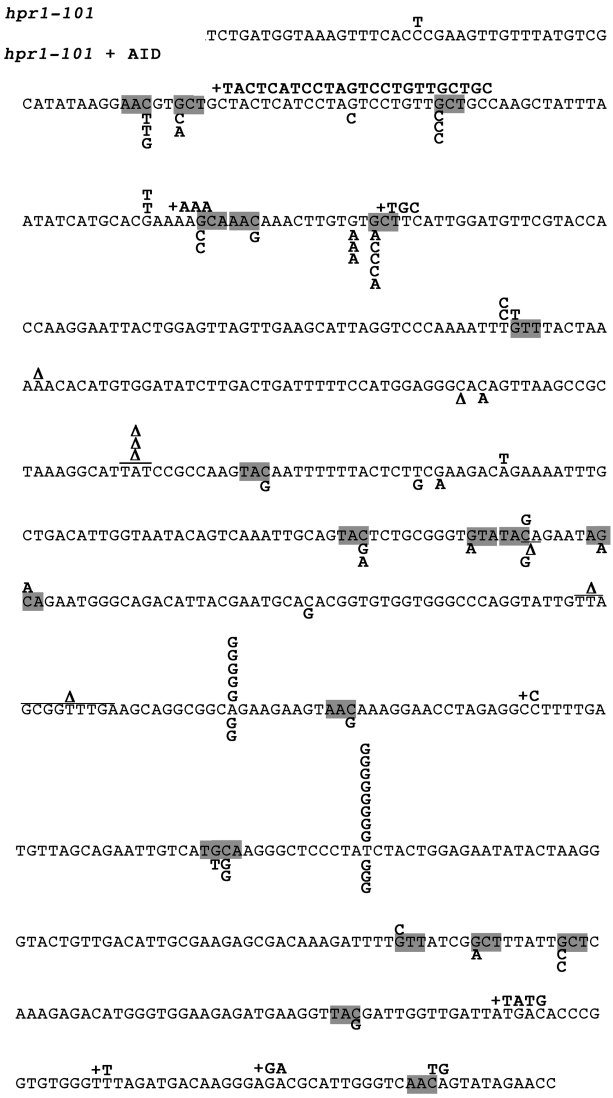


Figure 4. Spectra and distribution of mutations obtained in the LAUR system. DNA sequence of 39 and 45 *lacZ*$^{+}$ Ura^{-} spontaneous (–AID; upper part of the sequence) and AID-induced (+AID; lower part of the sequence) mutations, respectively, in *hpr1-101* cells. Mutations are shown in bold. Symbols: 'plus sign', insertion; 'triangle', deletion. Grey highlighting identifies sites where mutations occurred in the WRC/GYW AID target motif, in which W is A or T, R is A or G and Y is C or T.

formed at a reduced rate in *hpr1-101*. The major accessibility of AID must be explained by the transient opening of the DNA strands produced by the local negative supercoiling generated during transcription. Such a negatively supercoiled region may be larger or last longer in *hpr1-101* due to the transcription impairment.

RNaseH1 overexpression in R-loop-forming THO null mutants mimics the *hpr1-101* mutation

Our results indicate that impairment of mRNP formation and transcription elongation caused by *hpr1-101* is not accompanied by R-loop formation. Since *hpr1-101* is not hyper-recombinant and does not show replication-fork slowdown, it is likely that R-loops are linked to the

Table 1. Spontaneous and AID-induced base substitutions in wild-type (WT) and *hpr1-101* mutant classified according to different sequence features

	WT	WT +AID	*hpr1-101*	*hpr1-101* +AID
Mutations at C	2	16	4	14
Mutations at G	9	21	4	23
Mutations at WRC	1	15	3	12
Mutations at GYW	1	15	2	18
Point mutations	21	46	25	43
Total mutations	31	53	38	45

W is A or T, R is A or G and Y is C or T. Wild-type data were taken from (17) and new sequence results were added.

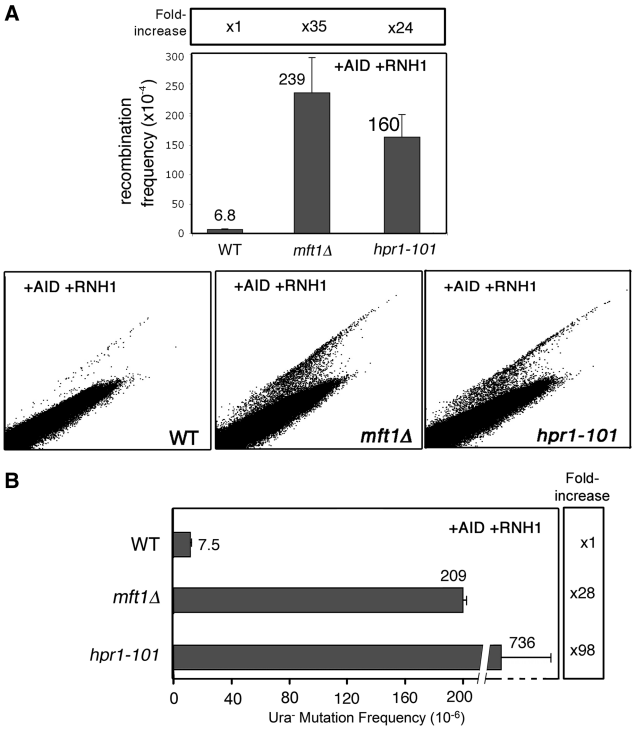


Figure 5. RNH1 overexpression does not suppress AID action in THO mutants. (**A**) Effect of RNH1 overexpression on AID-induced direct-repeat recombination in the GLG system in wild-type, *mft1Δ* and *hpr1-101* strains. Other details as in Figure 2. (**B**) Effect of RNH1 overexpression on AID-induced mutation in the LAUR system in wild-type, *mft1Δ* and *hpr1-101* strains. Other details as in Figure 3.

hyper-recombination and replication fork slowdown that occurs in THO null mutants (13). However, the fact that AID efficiently acts in *hpr1-101* mutants, which do not form co-transcriptional RNA-DNA hybrids, opens the possibility that R-loop removal in *hpr1Δ* cells may not affect the action of AID either. We therefore reasoned that overexpression of RNase H1, which specifically remove RNA-DNA hybrids and has been shown to partially suppress *hpr1Δ* hyper-recombination (13), might not suppress AID-induced recombination in THO null mutants. To test this, we studied the effect of RNase H1 overexpression in *mft1Δ* in the GLG recombination and LAUR mutation system in the presence of AID. As shown in Figure 5, overexpression of RNH1 did not suppress

AID-induced recombination or AID-induced mutation in *mft1Δ* or *hpr1-101*. Altogether, these results imply that the transcription defect of *hpr1Δ*, even though it can be exacerbated by R-loop formation (13), is a direct consequence of the lack of THO and is independent on R-loop formation, consistent with the fact that *hpr1-101* impairs transcription without forming R-loops.

DISCUSSION

We provide genetic evidence that co-transcriptional R-loops are either not formed or formed at a reduced rate in the *hpr1-101* point mutant, which, in contrast to *hpr1Δ*, is impaired in transcription but does not lead to a strong increase in TAR or to a defect in replication fork progression. AID is able to act on both DNA strands of a transcribed sequence in *hpr1-101* mutant, indicating that R-loops are not stably formed in this THO point mutant. The fact that *hpr1-101* mutants show a transcription defect but no hyper-recombination nor replication slowdown suggests that whereas the two latter phenotypes of THO mutants are linked to R-loop formation, as previously reported (13,22), the transcription impairment also occurs in the absence of R-loop formation.

In mRNP biogenesis mutants, such as THO null mutants, a co-transcriptional R-loop is formed, in which the mRNA hybridizes with the complementary transcribed strand, displacing the other strand that remains as ssDNA (13) (Figure 6). Short and unstable, transient R-loops may sporadically occur during transcription in wild-type cells but R-loops are accumulated in THO null mutants. This is presumably favored by the accumulation of negative supercoiling associated with transcription. Indeed, *in vitro* transcription experiments have revealed that R-loop formation is more extensive on hypernegatively supercoiled templates (25). One possibility could be that THO deficiency in *hpr1-101* causes retention of the elongating RNA polymerase favoring the persistence of negative supercoiling. In this sense it is interesting to note that THO mutants are extremely sick in combination with topoisomerase mutations (26). As it can be seen in Figure 6, the NT strand in the form of ssDNA is more susceptible to be deaminated by AID leading to the strong hyper-mutation and hyper-recombination (17). Interestingly, recent *in vitro* transcription experiments have shown that AID treatment of R-loops, caused by arresting the elongation complexes by a quick removal of NTPs, results in an increase in revertants with multiple clustered mutations (27). Long and unusually stable R-loops are involved in the priming of DNA replication in certain bacterial plasmids as well as in the mitochondrial genome of eukaryotic cells from yeasts to humans (28,29). These bacterial RNA–DNA hybrids involved in replication initiation have been suggested as interfering with replication fork progression (30). Similarly, in THO null-mutants, the formation of R-loops correlates with replication fork progression impairment as seen by 2D-gel analysis (18). Replication disturbance would thus yield recombination-mediated repair, consistent with the strong TAR phenotype of THO null mutants.

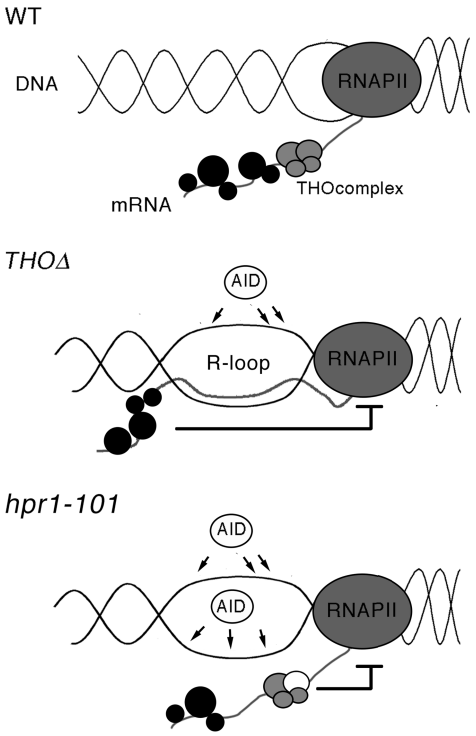


Figure 6. Transcription-elongation impairment in THO mutants. In THO null mutants, a co-transcriptional R-loop can be formed. The R-loop allows AID to act on the non-transcribed ssDNA causing strong AID-induced TAM and TAR. In *hpr1-101*, the nascent mRNA is packed into an mRNP, although not properly, causing transcription impairment. Negative supercoiling is accumulated behind the RNAPII allowing the accumulation of ssDNA in both DNA strands, which enhances AID action. A putative mRNP-biogenesis checkpoint would sense the export-incompetent sub-optimal mRNPs and in response would slowdown or halt transcription.

In *hpr1-101*, co-transcriptional R-loops are not stably formed. The THO complex is present, stable and recruited to chromatin in *hpr1-101* cells (22) (Figure 6). Nevertheless, *hpr1-101* may still cause retention of the elongating RNA polymerase and an upstream accumulation of negative supercoiling, thus enhancing the accessibility of both DNA strands to AID and leading to the strong AID-induced hyper-mutation and hyper-recombination (Figure 6). The conclusions from this result might be extrapolated to the molecular mechanisms leading to CSR and SHM. We previously proposed a role of defective mRNP biogenesis formation in the initiation of CSR and SHM, in which sub-optimal mRNP biogenesis could be leading to a better accessibility of AID to the transcribed S or V-regions, respectively (17). This could be due to the formation of R-loops, as it occurs in yeast THO null mutants, in which the NT strand is maintained single-stranded thus favoring AID action to initiate both SHM and CSR. In fact, R-loops are formed in S-regions (14,31). However, the analysis of the products of SHM reveals that both DNA strands must be mutated *in vivo* (32,33). Here we present a yeast model in which defective mRNP

biogenesis enhances the action of AID independent of R-loops. AID mutates both strands in this yeast model, which is consistent with the pattern of AID action in B-cells (Figure 6). We found that AID induced a high number of mutations not explained by direct replication of an U:G mismatch, which would lead to C to T and G to A transitions (82% in wt and 72% in *hpr1-101*; Supplementary Table 1). Interestingly, this is similar to the pattern observed in SHM, in which 60% and 75% of mutations are neither C to T nor G to A in human and mice, respectively (see (34) and references therein). This suggests that AID-induced mutations in yeast would occur by an initial cytosine deamination leading to a U:G mismatch that is later processed by aberrant/error prone repair processes involving Ung and Msh proteins, as it is believed for SHM in B cells (34).

Notably, transcription is impaired in *hpr1-101* as it is in THO null mutants (22), possibly due to the negative supercoiling transiently accumulated upstream of the elongating RNA polymerase II, which enhances the accessibility of both DNA strands to AID, leading to strong AID-induced hyper-mutation and hyper-recombination (Figure 3). Several hypotheses have been suggested as explanations of the effect of R-loop formation in transcription. The most extended one is that an R-loop could constitute a roadblock for the next RNA polymerase, so that the overall levels of transcription elongation would be reduced (3,19,20). Consistent with this, we have shown that artificially constructed RNA–DNA hybrids reduce the efficiency of transcription elongation *in vitro* (21). It seems, therefore, that R-loops by themselves can impair the progression of RNA polymerases, as it also seems to be the case for DNA polymerases (35). In the case of THO null mutants, the transcription-elongation impairment can be due in part to R-loop formation (13), but the extent of the impact of the R-loop, or a more direct role of THO in transcription, was unclear. The results of this study imply that Hpr1 also has a role in the control of transcription that is not mediated by R-loops. This, indeed, may explain why RNase HI overexpression does not apparently suppress the transcription defect of THO null mutants despite reducing the amount of R-loops (13). It is likely that an mRNP-biogenesis checkpoint might exist that senses export-incompetent sub-optimal mRNPs to impair transcription and to facilitate its degradation by the nuclear exosome. These results provide a new and intriguing perspective on the functional role of THO in transcription elongation and the coupling between transcription and mRNA processing. Further analysis of the effect of THO and related mRNP biogenesis and export factors on transcription elongation should, therefore, contribute to clarify the different mechanisms by which transcription and mRNP biogenesis and export are coupled.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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