

Activation-induced cytidine deaminase action is strongly stimulated by mutations of the THO complex

Belén Gómez-González and Andrés Aguilera*

Departamento de Genética, Facultad de Biología, and Departamento de Biología Molecular, Centro Andaluz de Biología Molecular y Medicina Regenerativa, Consejo Superior de Investigaciones Científicas-Universidad de Sevilla, Avenida Américo Vespucio s/n, 41092 Sevilla, Spain

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Activation-induced cytidine deaminase (AID) is a B cell enzyme essential for Ig somatic hypermutation and class switch recombination. AID acts on ssDNA, and switch regions of Ig genes, a target of AID, form R-loops that contain ssDNA. Nevertheless, how AID action is specifically targeted to particular DNA sequences is not clear. Because mutations altering cotranscriptional messenger ribonucleoprotein (mRNP) formation such as those in THO/TREX in yeast promote R-loops, we investigated whether the cotranscriptional assembly of mRNPs could affect AID targeting. Here we show that AID action is transcription-dependent in yeast and that strong and transcription-dependent hypermutation and hyperrecombination are induced by AID if cells are deprived of THO. In these strains AID-induced mutations occurred preferentially at WRC motifs in the nontranscribed DNA strand. We propose that a suboptimal cotranscriptional mRNP assembly at particular DNA regions could play an important role in Ig diversification and genome dynamics.

hyperrecombination | hypermutation | messenger ribonucleoprotein biogenesis | R-loops

Activation-induced cytidine deaminase (AID) is a specific B cell enzyme believed to be responsible for the initiation of somatic hypermutation (SHM) and class switch recombination (CSR) during B cell differentiation (1–3). Many studies have shown that AID acts directly on DNA (4), the natural target for AID action in the variable and switch (S) regions for SHM and CSR, respectively. The specific mechanisms of AID function are still unclear, but evidence suggests that the preferential target of AID may be ssDNA (5, 6). Thus, *in vitro* experiments have shown that AID deaminates ssDNA and dsDNA that is transcribed (5–7), and transcription has been shown to be required for both SHM and CSR *in vivo* (8, 9).

Studies have shown a strand preference for the action of AID during *in vitro* transcription with AID-induced mutations detected preferentially in the nontranscribed (NT) strand (6, 10–12). Analysis of the products of SHM in B cells, however, reveals that both DNA strands are mutated (13). Other *in vitro* studies have shown that AID can deaminate both strands within regions of supercoiled DNA (14) and that the ssDNA-binding replication protein A is required for deamination of SHM targets (15). All of these findings are consistent with the idea that transient formation of ssDNA during transcription facilitates AID action.

Along the same line, formation of R-loops during transcription of the S regions has been proposed (16). Such R-loops would possibly provide AID with ssDNA substrates at its target region because there the transcribed (T) strand hybridizes with the nascent RNA and the NT strand is present as ssDNA. Because of its high G content, the NT DNA strand at S regions could be stabilized by the formation of parallel four-stranded G quartets (17, 18). Indeed, AID appears to bind specifically to G-loops within transcribed S regions (19) and can deaminate the displaced strand of a transcription-induced R-loop *in vitro* (20).

Interestingly, cotranscriptional messenger ribonucleoprotein (mRNP) biogenesis is an essential step in gene expression that

may influence genetic integrity (21, 22). In the yeast *Saccharomyces cerevisiae*, hyperrecombination can be triggered by transcription in mutants depleted of THO/TREX, a conserved protein complex that functions at the interface between transcription and mRNA export (23, 24). THO is a conserved complex first identified in yeast and formed by Tho2, Hpr1, Mft1, and Thp2 (24). It interacts with the Sub2 RNA-dependent ATPase and the Yra1 RNA-binding protein to form the TREX complex (23). THO has been shown to be required for the transcription of long genes and genes containing either high GC content or multiple internal repeats (25, 26). It is believed that THO controls the cotranscriptional formation of export-competent mRNP during transcription elongation by controlling the assembly of heterogeneous nuclear ribonucleoproteins onto the nascent mRNA (21). Hyperrecombination of THO mutants is particularly evident in long and GC-rich DNA sequences such as the bacterial *lacZ* gene (26). Because hyperrecombination depends on the formation of DNA:RNA hybrids (22), it has been proposed that one function of THO/TREX is to prevent the nascent mRNA from interacting with the template DNA to form R-loops. The observation that the ASF/SF2 splicing factor has a similar role in chicken DT40 and HeLa cells (27) suggests that a number of RNA processing enzymes have an additional role in preventing R-loop formation.

One important and unresolved question is why AID is targeted at specific DNA regions during B cell differentiation, and we wondered whether this could be because of suboptimal mRNP formation. To test this possibility, we confirmed that AID can induce hypermutation and hyperrecombination at low levels in yeast cells (28, 29) and demonstrated additionally that this instability occurs in a transcription-dependent manner. Using yeast THO mutants as a way to generate suboptimal mRNP formation, we found that both hypermutation and hyperrecombination were strongly and synergistically increased in a transcription-dependent manner, with mutations primarily occurring in the NT strand. We propose that a suboptimal cotranscriptional mRNP assembly at particular DNA regions can regulate AID action, and we suggest that the control of mRNP biogenesis may play an important role in Ig diversification and genome dynamics.

Results

Mutations in the THO Complex Confer a Slight Transcription-Dependent Hypermutation Phenotype. Because THO mutants share phenotypes of recombination-mediated genetic instability

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Abbreviations: AID, activation-induced cytidine deaminase; CSR, class switch recombination; mRNP, messenger ribonucleoprotein; NT, nontranscribed; S, switch; T, transcribed; SC, synthetic complete; SHM, somatic hypermutation; TAM, transcription-associated mutation.

*To whom correspondence should be addressed. E-mail: aguilero@us.es.

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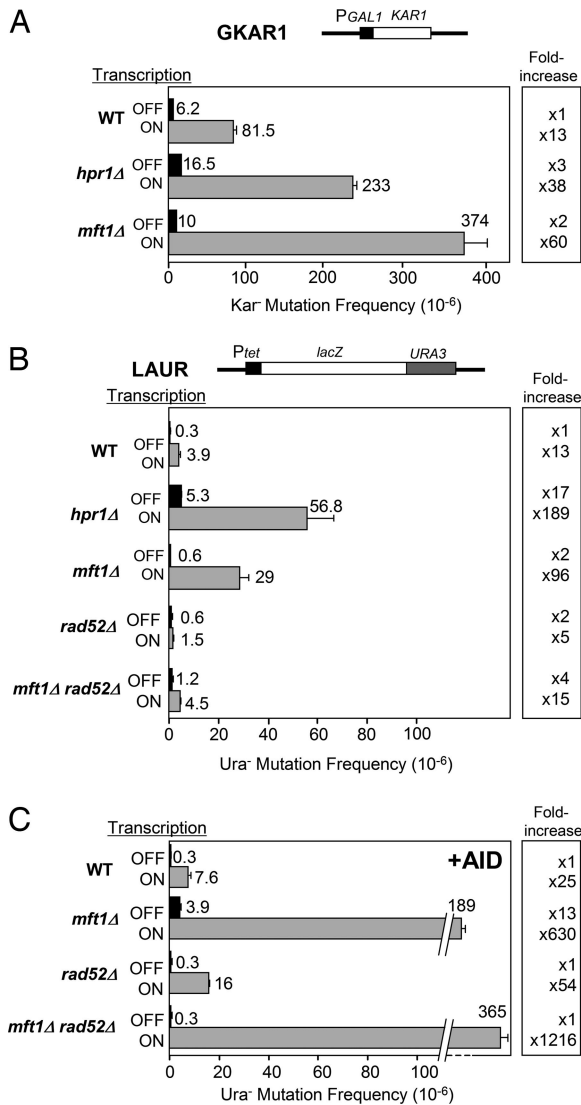


Fig. 1. Effect of transcription and AID on the frequency of mutation in *S. cerevisiae* wild-type and THO mutant strains. (A) Frequency of Kar⁻ mutants obtained in the GKAR1 system. (B) Frequency of Ura⁻ mutants obtained in the LAUR system and Rad52 dependency. (C) Effect of AID on the frequency of Ura⁻ mutants in the LAUR system in wild-type, *mft1Δ*, *rad52Δ*, and *mft1Δ rad52Δ* strains. All experiments were performed under high (ON) and low (OFF) transcription. Diagrams of systems are shown above each graph. Mean mutation frequency and standard deviation of three to four different fluctuation tests are plotted.

associated with transcription, it was important to know whether they showed, in addition to hyperrecombination, a transcription-dependent hypermutation phenotype. Two different plasmid-borne assays were developed for the analysis of forward mutations. The first assay is based on a fusion of the *KAR1* ORF to the regulated *GAL1* promoter (GKAR1 system). Because *KAR1* overexpression is lethal in yeast (30), *kar1* mutations can be selected on galactose medium. The second assay is based on a *lacZ::URA3* translational fusion under control of the regulated *Tet* promoter (LAUR system) (31). In this assay, Ura⁻ mutants are selected on synthetic complete (SC) medium supplemented with 5-fluoroorotic acid, whereas *lacZ*⁻ mutants and *lacZ*⁺ are scored by color on SC plus X-Gal. As can be seen in Fig. 1A and B, the mutation frequencies were increased 13-fold when transcription was high in both systems. Transcription-associated mutation (TAM) was also observed in *rad52Δ* cells although to

Transcription		WT		<i>mft1Δ</i>	
		<i>lacZ</i> ⁻	<i>lacZ</i> ⁺	<i>lacZ</i> ⁻	<i>lacZ</i> ⁺
OFF	-AID	50	32	4	0
	+AID	11	9	-	-
ON	-AID	80	41	54	0 *
	+AID	66	86 *	36	0 *

Fig. 2. Genetic analysis of spontaneous (-AID) and AID-induced (+AID) mutations. Distribution of *lacZ*⁻:*lacZ*⁺ mutations among Ura⁻ mutants in wild-type and *mft1Δ* strains is shown. An asterisk indicates a statistically significant difference ($P < 0.05$) with respect to the wild-type value under the same transcription conditions, as determined by contingency χ^2 analysis.

a minor extent (Fig. 1B). The result is in agreement with previous reports indicating that transcription increases mutation in yeast cells (32) and *Escherichia coli* (reviewed in ref. 33).

Next, we used both the LAUR and GKAR1 assays to determine whether different THO mutations stimulated TAM. As seen in Fig. 1A, *mft1Δ* and *hpr1Δ*, which caused an elimination of the THO complex in yeast cells (34), increased the frequency of mutation 2- to 3-fold in GKAR1 at low transcription versus 38- to 60-fold at high transcription levels. Similarly, in the LAUR system, the increase in mutation frequency was significantly stronger for high transcription (96- to 189-fold) than for low transcription (2- to 17-fold) as assayed in both the *mft1Δ* and *hpr1Δ* backgrounds (Fig. 1B). Therefore, THO mutants share a transcription-dependent hypermutation phenotype that is not as strong as its hyperrecombination phenotype observed in several direct-repeat assays (26). Because all null mutations of the genes encoding the different THO subunits lead to deprivation of THO (34) and the same phenotypes (24), we decided to continue our analysis with *mft1Δ*, which, in contrast to *hpr1Δ*, has little impact on growth.

To gain insight into the molecular basis of mutations caused by THO mutations, we performed a genetic analysis of the pattern of mutations caused by *mft1Δ*. As expected from the fact that transcription through *lacZ* is the most sensitive step in THO mutants (26), every Ura⁻ mutant was also *lacZ*⁻ in all conditions tested, implying that all mutations mapped within *lacZ* (54 in high transcription and four in low transcription) (Fig. 2), whereas, in the wild type, 34–40% of the Ura⁻ mutations were *lacZ*⁺ (*lacZ*⁻:*lacZ*⁺ ratios of 50:32 and 80:41 in low and high transcription, respectively) (Fig. 2); that is, they occurred within *URA3*. The *mft1Δ* mutations must, therefore, be either frameshifts or premature stop codons within *lacZ* or DNA rearrangements covering *lacZ* and *URA3*. Given the strong transcription-dependent hyperrecombination phenotype of THO mutants, it was important to determine whether *lacZ*⁻ Ura⁻ colonies in the *mft1Δ* background arose by recombination-dependent rearrangements, which are known to be Rad52-dependent. The spontaneous mutation frequency under high transcription in the *rad52Δ* strain was 2-fold lower than in the wild type (Fig. 1B), although it has been reported that TAM increases in *rad52Δ* cells (32, 35), indicating that some of the TAM events observed in the wild type may arise by a Rad52-dependent pathway in the LAUR construct. The *rad52Δ* mutation causes a decline in the mutation frequency of *mft1Δ* cells of 6.5-fold (from 2.9×10^{-5} to 4.5×10^{-6}), indicating that most of TAM events in *mft1Δ* occur by RAD52-dependent rearrangements rather than by point mutations (Fig. 1B). In contrast to wild-type cells in which none of eight analyzed Ura⁻ mutants contained detectable rearrangements, 62% of the mutants (23 of 37) contained rearrangements in *mft1Δ* [supporting information (SI) Fig. 5]. Thus, the absence of THO stimulates Rad52-independent mutations 2- to 3-fold (from 0.6×10^{-6} to 1.2×10^{-6} and from 1.5×10^{-6} to 4.5×10^{-6} in low and high transcription, respectively) (Fig. 1B). Indeed,

50% of *Ura*⁻ mutations are *lacZ*⁺ in the *mft1Δ rad52Δ* mutant (7:7 *lacZ*⁻:*lacZ*⁺ ratio; data not shown), consistent with the conclusion that THO mutants also stimulate point mutations, although slightly, in a transcription-dependent manner.

AID-Induced Mutation Is Transcription-Dependent in Yeast. Taking advantage of the LAUR assay, we studied the ability of AID to induce mutation and whether this ability depended on transcription in wild-type cells. As can be seen in Fig. 1 *B* and *C*, AID expression in wild-type yeast did not lead to hypermutation at low transcription, but caused a 2-fold increase at high transcription (from 3.9×10^{-6} to 7.6×10^{-6}). Consistent with a lack of an AID effect on mutation frequency under low transcription in the wild type, the ratio of *lacZ*⁻:*lacZ*⁺ among *Ura*⁻ mutants was similar regardless of whether cells expressed AID (11:9 versus 50:32) (Fig. 2). However, a significant change ($P < 0.05$) in this ratio was observed under high transcription if AID was expressed (66:86 versus 80:41), indicating that the pattern of mutations was changed by AID in a transcription-dependent manner. Therefore, AID induces a weak transcriptional-dependent increase in the frequency of mutation and a change in the pattern of mutations.

AID-Induced Mutation Is Strongly Stimulated by THO Mutations. THO mutations may create local regions that are susceptible to forming recombinogenic and mutagenic intermediates in a transcription-dependent manner. To determine whether such regions would be more susceptible to AID action, we tested the effect of AID on the frequency and pattern of mutations in *mft1Δ* cells. As can be seen in Fig. 1*C*, in *mft1Δ* cells the effect of AID on the frequency of mutations was greater than in the wild type, leading to a 13-fold increase in mutations at low transcription that went up to 630-fold at high transcription (Fig. 1*C*). Under low and high transcription, AID stimulated the mutation frequency 6.5 times (from 0.6×10^{-6} to 3.9×10^{-6} and from 2.9×10^{-5} to 1.9×10^{-4} , respectively) in *mft1Δ* cells. Importantly, this hypermutator effect of AID was not dependent on Rad52. AID expression further increased *Ura*⁻ mutations 81-fold (from 4.5×10^{-6} to 3.7×10^{-4}) in *mft1Δ rad52Δ* cells under high transcription (Fig. 1 *B* and *C*), indicating that they are not due to rearrangements but most likely point mutations.

THO mutations enhance AID action presumably by creating a local structure in the transcribed DNA region that works as a target for AID. Therefore, we would expect that nonframeshift point mutations mapping at *lacZ* and conferring a *lacZ*⁻ but not *Ura*⁻ phenotype would occur at a high frequency in *mft1Δ* but not in wild-type cells expressing AID. As expected, we were not able to detect *lacZ*⁻ *Ura*⁺ mutants from 5,922 colonies in wild-type cells expressing AID (frequency $< 10^{-3}$), whereas in *mft1Δ* expressing AID we counted 47 *lacZ*⁻ *Ura*⁺ mutants of 4,692 colonies (frequency of 1×10^{-2}) (SI Table 2). This result indicates that the frequency of *Ura*⁻ AID-induced mutations in *mft1Δ* was underestimated in the LAUR system and indicates a strong effect of THO mutations on AID action. Therefore, transcription and AID result in a strong hypermutator phenotype in *mft1Δ* cells.

AID Mutates Preferentially the NT Strand in *mft1Δ* but Shows No Strand Preference in the Wild Type. AID acts preferentially at WRC (GYW in the opposite strand) motifs (11) that are part of the WRCY motifs (RGYW in the opposite strand) found as hotspots of SHM (36) (where W is A or T, R is A or G, and Y is C or T). As R-loops in THO mutants (19) leave ssDNA opposite the DNA:RNA hybrid, one prediction is that most of the AID-induced mutations in *mft1Δ* cells should be at WRC motifs and in the NT strand. Therefore, we sequenced spontaneous and AID-induced mutations in wild-type and *mft1Δ* cells. As shown in Fig. 2, all mutants in *mft1Δ* cells were *lacZ*⁻ *Ura*⁻, which can

Table 1. Spontaneous (–AID) and AID-induced (+AID) base substitutions in the wild type (*lacZ*⁺ *Ura*⁻) and *mft1Δ* mutants (*lacZ*⁻ *Ura*⁺) classified according to different sequence features

Sequence features	WT	WT + AID	<i>mft1Δ</i> + AID
Mutations at C	0	16	16
Mutations at G	7	21	5
Mutations at WRC	0	15	10
Mutations at GYW	1	15	2
Transversions	11	35	13
Transitions	0	11	9
Point mutations	11	46	22
Total mutations	19	53	24

DNA sequences are shown in SI Fig. 6 *A* and *B*.

be explained if the *Ura*⁻ phenotype results from frameshift mutations, stop codons, or rearrangements occurring at *lacZ*. This view is consistent with the fact that transcription through *lacZ* is the most sensitive step in THO mutants (26). Therefore, because point mutations at *URA3* are not detected and *lacZ*⁻ *Ura*⁻ mutants represent a biased class of mutations at *lacZ*, we decided to sequence the *lacZ* sequence of the *lacZ*⁻ *Ura*⁺ mutants that arose in *mft1Δ* cells expressing AID under high transcription conditions. The *URA3* ORF from 19 and 53 independent *lacZ*⁺ *Ura*⁻ mutants obtained from the wild type with and without AID overexpression, respectively, was also sequenced (SI Fig. 6 *A* and *B*). Results are summarized in Table 1 and SI Table 3. In wild-type cells not expressing AID <60% (11 of 19) were point mutations and only 9% of these (1 of 11) were within the WRC/GYW target motif of AID. In contrast, in the wild type and *mft1Δ* expressing AID, the percentage of point mutations increased to 86% (46 of 53) and 91% (22 of 24), respectively. Of these, 65% (30 of 46) and 54% (12 of 22), respectively, occurred at the WRC/GYW motif. Altogether, the data indicate that AID has a preferential function as a cytosine deaminase at the WRC/GYW motif, consistent with the preferential target of AID deamination observed *in vitro* (11).

As AID deaminates C within ssDNA, mutations occurring at C should reflect the action of AID directly on the NT strand, whereas mutations at G reflect the action of AID on C in the T strand. Therefore, we analyzed the NT:T ratio of AID-induced mutations in both strains. Whereas in the wild type expressing AID the NT:T ratio of mutations was 0.75:1 (16:21), this ratio increased significantly ($P < 0.05$) to 3:1 (16:5) in *mft1Δ*. Importantly, from these mutations, the WRC AID-target motif was mutated at an NT:T ratio of 1:1 in the wild type (15:15) and 5:1 in *mft1Δ* (10:2). The lack of strand preference of the mutation pattern produced by AID in the wild type was not observed previously (29) and is consistent with the transient opening of DNA strands by transcription presumably promoted by negative supercoiling. By contrast, the putative impairment of mRNP biogenesis caused by *mft1Δ* promotes a more stable formation of ssDNA on the NT strand and makes it an appropriate target for the action of human AID. The transversions:transitions ratio also decreased from 11:0 in the wild type to 35:11 and 13:9 in wild-type and *mft1Δ* cells expressing AID, respectively (Table 1). The increase in transitions is consistent with the direct action of AID in deaminating C to U, which leads to a C-to-T transition (G to A in the opposite strand). Consistently, the changes produced by AID were more evident for C (or G) with a strong bias for C in *mft1Δ* cells (Table 1).

AID-Induced Recombination Is Transcription-Dependent and Strongly Stimulated by THO Mutations in Yeast Cells. Finally, AID has also been shown to contribute to recombination in yeast (28, 29). This increase reflects the ability of AID to, in a direct or an indirect

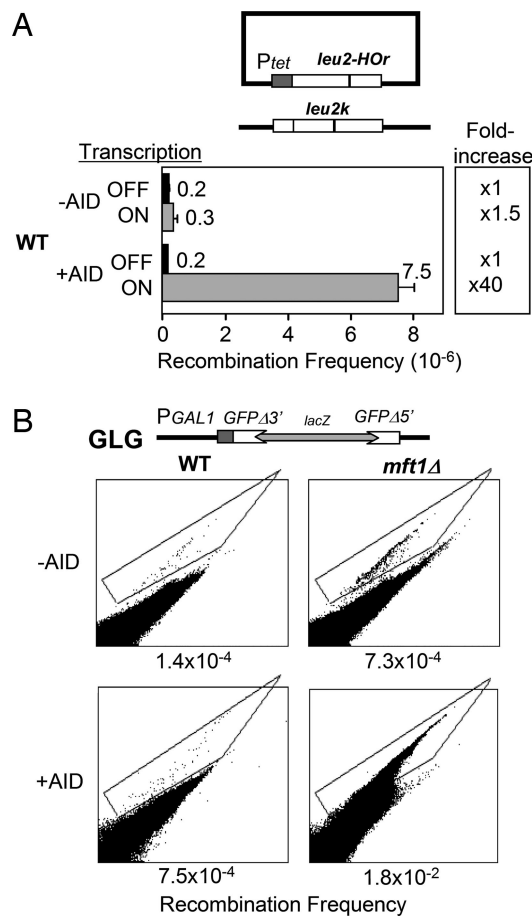


Fig. 3. Effect of AID on transcription-associated recombination. (A) Spontaneous (–AID) and AID-induced (+AID) frequency of gene conversion under high (ON) and low (OFF) transcription between a plasmid *leu2-r* and a chromosomal *leu2-k* allele in a wild-type strain. (B) Effect of AID expression on direct-repeat recombination in the GLG system. The frequency of GFP⁺ recombinants (signals inside the box) is indicated. *y* axis, green fluorescence (FL1H); *x* axis, unspecific fluorescence (FL2H). A diagram of each system is shown on the top of each panel.

way, induce the formation of DNA double-strand breaks, which in yeast are preferentially repaired by recombination, whereas in human cells they are repaired by nonhomologous end-joining. Indeed nonhomologous end-joining is believed to be the major mechanism that leads to class switching in B cells (see ref. 37). We expected that AID should also increase recombination in a transcription-dependent manner in wild-type yeast and much more strongly in *mft1Δ*. For this purpose we used a plasmid–chromosome recombination assay between a CEN-plasmid *leu2-r* under control of the regulated *Tet* promoter and a chromosomal *leu2-k* mutation (38) in the wild type. As seen in Fig. 3A, AID did not change the frequency of gene conversion of *leu2-r* at low transcription. However, it caused a 40-fold increase at high transcription. Thus, like the mutagenic activity, the hyperrecombinogenic activity of AID is transcription-dependent in yeast. These findings are consistent with the view that both AID-induced mutation and recombination are the output products of a common intermediate.

Because direct-repeat recombinants are the type of event primarily increased by THO mutations (24), a direct-repeat recombination assay was developed for the analysis of deletions to assess whether THO mutations also strengthen AID-induced recombination. The assay is based on two truncated GFP repeats, which are under control of the *GAL1* promoter and

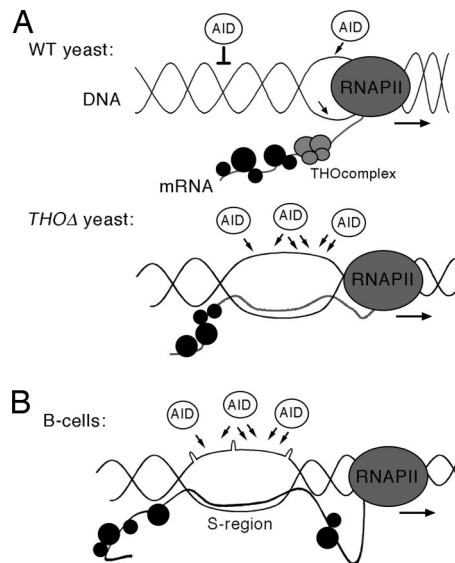


Fig. 4. A model to explain stimulation of genome instability by AID in yeast THO mutants compared with the S regions in B cells. (A) Transcription and mRNP formation mediated by THO in wild-type and THO-depleted yeast cells. In wild-type yeast, AID is able to act on the DNA when it is transcribed. Negative supercoiling behind the elongating RNA polymerase II (RNAPII) allows a weak AID action. In THO-depleted yeast, a cotranscriptional R-loop can be formed, allowing AID to act on the nontranscribed ssDNA, causing strong hypermutation and hyperrecombination. (B) A putative example of transcription and mRNP formation in the S region of Ig genes in human B cells. The G-rich S region could be refractory to a number of RNA-binding proteins, therefore forming a suboptimal mRNP at that particular region. Nascent RNA S regions could lead to local cotranscriptional R-loops or G-loops that would allow AID action.

interrupted by a *lacZ* sequence. GFP⁺ recombinants are scored directly by FACS. As shown in Fig. 3B, *mft1Δ* and AID independently produced a 6-fold increase in GFP⁺ deletions, whereas the combination of *mft1Δ* and AID expression caused a strong synergistic 100-fold increase. Therefore, we conclude that impairment of mRNP formation caused by THO mutations enhances the action of AID in a transcription-dependent manner, resulting in a strong induction of both point mutations and direct-repeat recombination.

Discussion

In this work we show that AID can function in yeast THO mutants similar to that in human B cells. In wild-type yeast not only can AID induce hypermutation and hyperrecombination, as it was previously shown, but, importantly, this induction depends on transcription. We show that such transcription-dependent hypermutation and hyperrecombination effects are weak in the wild type but strongly and synergistically enhanced in mutants of the THO complex. The synergistic effect of AID and THO mutations is also demonstrated at the molecular level by showing that most AID mutations occurred preferentially on the NT DNA strand. Because THO mutants are affected in mRNP biogenesis (21, 39), our results suggest that AID action can be enhanced by suboptimal mRNP formation. These results open the possibility that a suboptimal formation of the mRNP would enhance the probability of the nascent mRNA to form a DNA:RNA hybrid, leaving the NT strand as single-stranded. Such a single-stranded region would be stabilized by the G-loops in S regions, so that the final structure promotes AID targeting (Fig. 4).

For this study we first characterized new *in vivo* assays for the analysis of forward mutations under the control of transcription-

regulated promoters in yeast, with which we have been able to show that transcription significantly enhances mutation, consistent with a previous result (32), in which this stimulation was accompanied by a change in the mutation spectrum (40). TAM has also been shown in bacteria with both spontaneous and damage-induced mutations (reviewed in ref. 33).

Mutants of the THO complex have a strong transcription-dependent hyperrecombination phenotype, as shown in direct-repeat systems (41, 42). Although recombination is also enhanced by transcription, the mechanisms underlying transcription-associated recombination may be different from those of TAM. Thus, impairment of replication progression by transcription may be a critical factor triggering transcription-associated recombination (43, 44), but this is not obvious for TAM. Our study reveals that THO mutations lead to a low hypermutator phenotype in the LAUR system, which is based on a highly expressed *lacZ* gene (Fig. 1). It is important to note that the gene expression defect and hyperrecombination phenotypes of THO mutants have been shown to be particularly strong at GC-rich DNA sequences such as *lacZ* (26). The *mft1Δ*-induced mutations have a clearly different pattern from those obtained in wild-type cells. In *mft1Δ* cells all mutations occurred within the *lacZ* gene of the *lacZ::URA3* fusion, whereas in the wild type 40% of the mutations occurred at *URA3*. Interestingly, mutations originate at the same DNA region, *lacZ*, where hyperrecombination is strongly stimulated (41). Indeed, hypermutation associated with THO mutations is partially dependent on Rad52, and part of the hypermutation events observed in the LAUR assay are caused by Rad52-dependent DNA rearrangements (see Fig. 1B), therefore being a consequence of the strong hyperrecombination phenotype of these mutants. This result indicates that the recombinogenic structures generated in THO mutants may not be mutagenic by themselves.

Our study provides an appropriate system for the analysis of hypermutation caused by AID. *In vitro* and *in vivo* experiments in *E. coli* have shown the transcription dependency of AID deamination and hypermutation, respectively (5–8, 12). A hyperrecombination and hypermutation effect of AID was also observed in yeast (28), but whether this was transcription-dependent was not determined. In this work we show that hypermutation and hyperrecombination caused by AID in yeast are also transcription-dependent, confirming the hypothesis that the action of AID requires transcription of the target sequence (4, 6, 12). This is shown not only by an increase in the frequency of mutations but by the fact that most of these mutations occur at the AID-preferred DNA motif WRC (see Table 1). Importantly, the AID effect observed in yeast is still low, which suggests that there must be additional factors in human B cells responsible for the specific action of AID on its target DNA sequences. Indeed, our mutation spectrum analysis did not reveal a substantial number of A-T mutations (SI Table 3), which typically compose half of the mutational spectrum of SHM (13), consistent with the view that additional factors (e.g., mismatch repair, error-prone polymerases, chromatin structure, etc.) are regulated in B cells to mediate SHM (45).

The slight AID action observed in wild-type yeast may be explained by the prediction that the negatively supercoiled DNA putatively accumulated behind an elongating RNA polymerase can promote transient formation of ssDNA (14, 46), which is the preferential target of AID (5, 6). The results indicating that AID-induced mutations in wild-type yeast occurred at a 1:1 ratio in the NT:T strands would be consistent with this view, implying that both strands have equal probability of becoming single-stranded and, therefore, to be accessed by AID. Previous data in yeast have revealed a different NT:T strand ratio of mutations in the *CAN1* gene (29). It is likely, therefore, that the pattern of mutations caused by AID in yeast may not be unique but dependent on a number of parameters that can include nucle-

otide sequence, chromatin structure, levels of expression, etc. Further investigations using different genes as mutation targets may be required to obtain a more complete view of AID action in yeast. Nevertheless, our results clearly show that a high impact of AID action in yeast is observed only when mRNP biogenesis is compromised by THO mutations. We detected a 5:1 bias in favor of the NT strand, a result that can be explained only if a more stable nontranscribed ssDNA structure is formed and simultaneously the T strand is protected from AID action. Because THO mutations have previously been shown to induce cotranscriptional R-loop formation, as determined in the same GC-rich *lacZ* gene used in this study (22), and S regions also form R-loops (16), formation of R-loops may be a natural way of stabilizing ssDNA for AID activity and of protecting the T strand in the form of the DNA:RNA hybrid. Our data in yeast show, therefore, that the strong AID effect on hypermutation in different THO-depleted yeast cells is consistent with the formation of R-loops during transcription (22, 34). Interestingly, recent reports have shown that AID interacts *in vitro* with the elongation complex (47). Because RNA polymerase II progression is reduced in THO mutants (23), we cannot discard that, in addition, AID action in THO mutants could be strengthened by a longer time in which AID could interact with the elongation complex, therefore increasing its time to act on DNA.

AID deamination seems to trigger the intermediates leading to class switch recombination in B cells, and some evidence suggests that class switching occurs by nonhomologous end-joining (37). Consequently, it is believed that AID deamination is the first event of a process ending in double-strand breaks. Whether this putative double-strand break is performed directly by AID or is formed after replication is a question yet to be addressed. Because double-strand breaks in yeast are preferentially repaired by recombination, it was expected that AID would affect recombination similarly to mutation. It was previously reported that AID induces recombination in yeast (28, 29). In this study we show that AID is able to increase the frequency of gene conversion only under high transcription (Fig. 3A), and, using a direct-repeat recombination assay, which allows us to detect hyperrecombination in THO mutants, we have been able to see a strong and synergistic effect of AID and THO mutations on recombination (Fig. 3B). Therefore, in yeast cells THO mutations enhance the transcription-dependent AID effect, whether detected as hypermutation or hyperrecombination.

In summary, our data clearly show that yeast THO mutants can be used as a model *in vivo* system for the study of AID action. Not only are hyperrecombination and hypermutation observed as a consequence of AID, but they occur in a transcription-dependent manner. More importantly, the pattern of mutations fits the previously shown pattern caused by AID *in vitro* (11), mutations occurring preferentially at the WRC motif. However, the pattern observed *in vitro*, in which mutations are preferentially found at the NT strand (6, 11, 29), is seen only in THO mutants. Therefore, our results open the possibility that mRNP biogenesis controlled AID action. It would be interesting to know whether the high G content of the S-region mRNA in B cells might limit its capacity to be assembled in an optimal mRNP, as THO mutations in general do in yeast, thereby increasing the reactivity of the nascent RNA with the DNA template (see Fig. 4). Yeast THO mutants can, therefore, be an excellent tool in deciphering the molecular mechanisms by which AID can induce both hypermutation and hyperrecombination.

Materials and Methods

Strains and Plasmids. We used W303-1A isogenic strains WMK-2A (*mft1Δ::KAN*), U678-1C (*hpr1Δ::HIS3*), WRS52-4B (*rad52Δ::KAN*) [described previously (24)], WMR52-1D and WMR52-4D (*rad52ΔKAN mft1Δ::KAN*) (obtained in this study), and the BY derivative BER08-64A (*his3::leu2-k*) (38).

Centromeric plasmids pMR260 (30) carrying *KARI* under control of the *GALI* promoter, pCM184-LAUR (31) containing *lacZ::URA3* under the *tet* promoter, and pCM184-L2HOr used for the plasmid–chromosome recombination assay (38) were described previously. Centromeric plasmids p414GALAI and p413GALAI carrying the human AID ORF under the *GALI* promoter were obtained by PCR amplification of AID from pGAID (6) using primers 5'-CTCTGGACGAAATTCATG-GACAGCCTCTTC-3' and 5'-CCTGGAAGCTCGAGT-CAAAGCTCCAAAGTA-3' and cloning into the EcoRI-XhoI-digested pRS414GAL and pRS413GAL (38), respectively.

Centromeric plasmid pGLG containing the GFP direct-repeat construct was obtained by amplifying GFP Δ 3' and GFP Δ 5' fragments from pUG34 and pUG23 plasmids (48) by PCR using primers 5'-ACTAGTGCCATGATGTAAACATTG-3', 5'-AATACAGGGTCGTCAGAT-3', 5'-TTAAAGCCTTC-GAGCGTCGGGCCCTTCT-3', and 5'-ACTGGTTCGACTCCCAATTTTGGTTGAAT-3' and subcloning into the SpeI-XbaI and ApaI-SalI sites of pRS413GAL, respectively. The *lacZ* ORF was subcloned at a BamHI between the GFP repeats.

Mutation and Recombination Analysis. For the GKAR1 mutation assay, cells were cultured overnight in SC medium containing 2% glycerol-lactate as a carbon source. Afterward, the culture was split in two, one with 2% glucose (transcription OFF) and the other with 2% galactose (transcription ON), and cultured for another 9 h before mutant selection. For the LAUR mutation

assay and the plasmid–chromosome recombination assay cells were cultured in SC plates with (transcription OFF) or without (transcription ON) 5 μ g/ml doxycycline, from which independent colonies were obtained for the mutation or recombination analyses. Kar⁻ and Ura⁻ mutants were selected on SC containing 2% galactose and SC plus 5-fluoroorotic acid, respectively, and Leu⁺ recombinants on SC-Leu plates. *lacZ*⁺ and *lacZ*⁻ were distinguished by color on SC plus 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.

Miscellanea. Yeast methodology, α -³²P-labeled DNA probes, and Southern and Northern blots were performed by following standard procedures. GFP fluorescence was determined in a FACSCalibur (Becton Dickinson, San Jose, CA) from 10⁶ cells grown in SC overnight and resuspended in 1 ml of H₂O.

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