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Cotranscriptionally Formed DNA:RNA Hybrids Mediate Transcription Elongation Impairment and Transcription-Associated Recombination

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Summary

Genetic instability, a phenomenon relevant for developmentally regulated processes, cancer, and inherited disorders, can be induced by transcription. However, the mechanisms of transcription-associated genetic instability are not yet understood. Analysis of *S. cerevisiae* mutants of THO/TREX, a conserved eukaryotic protein complex functioning at the interface of transcription and mRNA metabolism, has provided evidence that transcription elongation impairment can cause hyperrecombination. Here we show, using *hpr1*Δ mutants, that the nascent mRNA can diminish transcription elongation efficiency and promote recombination. If during transcription the nascent mRNA is self-cleaved by a hammerhead ribozyme, the transcription-defect and hyperrecombination phenotypes of *hpr1*Δ cells are suppressed. Abolishment of hyperrecombination by overexpression of RNase H1 and molecular detection of DNA:RNA hybrids indicate that these are formed cotranscriptionally in *hpr1*Δ cells. These data support a model to explain the connection between recombination, transcription, and mRNA metabolism and provide a new perspective to understanding transcription-associated recombination.

Introduction

Genetic instability is a phenomenon connected to several developmentally regulated processes, cancer, and genetics disorders (Khanna and Jackson, 2001; Moses, 2001). DNA lesions occurring as a consequence of chemical and physical reactions, and errors occurring during DNA replication and repair are the main causes of genetic instability, but transcription is another essential biological process influencing it. Transcription can induce recombination of a DNA sequence, a ubiquitous phenomenon that we will refer to as transcription-associated recombination (TAR) (see Aguilera, 2002). TAR is observed in prokaryotes (Ikeda and Matsumoto, 1979; Vilette et al., 1995) and eukaryotes from yeast (Thomas and Rothstein, 1989; Voelkel-Meiman et al., 1987) to mammals (Nickoloff, 1992), and it is essential to important developmentally regulated processes like class switching (Reaban and Griffin, 1990) and somatic hypermutation of immunoglobulin (Ig) genes (Peters and Storb, 1996).

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Despite the ubiquity and relevance of TAR, its mechanism(s) remains unknown (see Aguilera 2002). It has been proposed as being caused by the transcription-associated changes in chromatin structure or in local DNA supercoiling. Indeed, an increase in recombination caused by mutations affecting DNA supercoiling (Christman et al., 1988) or chromatin (Malagón and Aguilera, 1996; Myung et al. 2003) has been documented, but there is no experimental evidence that the supercoiling and chromatin changes caused by transcription are recombinogenic. Interestingly, it has recently been shown that the collision between RNA polymerase I-mediated transcription and replication increases recombination between the rDNA repeat copies in *S. cerevisiae* (Takeuchi et al., 2003). Finally, in TAR occurring during class switching of Ig genes, the observation that class switch regions form DNA:RNA hybrids (Mizuta et al., 2003; Reaban and Griffin, 1990; Yu et al., 2003) has raised the possibility that DNA:RNA hybrids are involved in initiation of class switch recombination, but this has not been proven.

In the last few years, the analysis of yeast THO hyperrecombination mutants has led to the idea that TAR may be a consequence of transcriptional elongation impairment (Chávez and Aguilera, 1997; Prado et al., 1997). THO is a conserved eukaryotic factor, identified in yeast as a four-protein complex containing Tho2, Hpr1, Mft1, and Thp2 (Chávez et al., 2000). Null mutants of any THO subunits show strong transcription-dependent hyperrecombination phenotypes and transcription elongation impairment (Chávez and Aguilera, 1997; Chávez et al., 2000; Piruat and Aguilera, 1998). As the increased levels of recombination are only observed when there is active transcription elongation through the DNA sequence where recombination occurs, it has been proposed that TAR and transcription elongation impairment are tightly linked in these mutants and are promoted by the same intermediate (see Aguilera, 2002).

One important property of transcription elongation is that it occurs concomitantly with different mRNA metabolic processes, such as 5′-end capping or splicing (Hirose and Manley, 2000; Proudfoot et al., 2002; Orphanides and Reinberg 2002). Further evidence of this connection has come from the functional analysis of THO and other functionally related factors. Thus, THO is present in a larger conserved eukaryotic complex, termed TREX, together with components of the mRNA export machinery such as Sub2/UAP56 and Yra1/ALY (Strasser et al., 2002). Interestingly, *sub2* and *yra1* show gene expression defects and hyperrecombination phenotypes that are similar to those of the THO mutants (Fan et al., 2001; Jimeno et al., 2002). This, together with the observation that mutations in RNA export factors such as *mex67* and *mtr2* (Jimeno et al., 2002) and *thp1* and *sac3* (Gallardo and Aguilera, 2001; Gallardo et al., 2003) have similar gene expression and recombination phenotypes closely resembling those of THO mutants, suggests that THO may also have a role in mRNA metabolism (Jimeno et al., 2002). These results indicate that there are a number of proteins that function at the inter-

face between transcription and mRNA metabolism and affect transcription and genetic instability.

The tight dependency of transcription efficiency and TAR on factors functionally linked to the metabolism of mRNA opens the possibility that the nascent mRNA molecule played a key role in transcription efficiency and TAR. Using a mutant of the THO complex, *hpr1*Δ, we show here that the nascent mRNA mediates transcription impairment and TAR. We provide evidence that DNA:RNA hybrids can be formed behind the elongating RNAPII by the nascent mRNA, negatively affecting transcription efficiency and promoting TAR.

Results

Transcription Elongation Impairment of *hpr1*Δ Is Abolished In Vivo When Nascent mRNAs Are Self-Cleaved by an Artificially Engineered Hammerhead Ribozyme

To test the possibility that the nascent mRNA molecule plays a key role for the efficiency of transcription elongation, we took advantage of the observation that the defect of THO mutants in transcription is stronger for either GC-rich or long transcriptional units driven from a strong promoter. The *hpr1*Δ mutants cannot efficiently express a transcriptional fusion in which a 0.4 kb *lacZ* sequence is inserted at the 3′ UTR of the 1.5 kb long *PHO5* ORF, whereas they do efficiently transcribe this 0.4 kb *lacZ* sequence when fused immediately downstream of the *GAL1* promoter (Chávez et al., 2001). To assay whether it was the *PHO5* nascent mRNA rather than the *PHO5* DNA template that was responsible for the transcription inefficiency in *hpr1*Δ, we placed a hammerhead ribozyme (*Rib*) between the *PHO5* and *lacZ* sequences (*Rib*$^+$ construct) fused under the control of the *GAL1* promoter. Thus, when the hammerhead is transcribed, the nascent transcript is self-cleaved, resulting in two different mRNAs from the same transcriptional unit, one containing the upstream *PHO5* sequence and the other the downstream *Rib-lacZ* sequence (Figure 1). To avoid degradation of the *Rib-lacZ* fragment, the U3 snRNA 5′ fragment was immediately downstream of the ribozyme (Samarsky et al., 1999). The U3 snRNA sequence has the ability to form a secondary structure to which several proteins are bound and protect the transcript from degradation (Samarsky et al., 1999). As a control, we made a second identical construct (*rib*^{m}), except for harboring a single base pair mutation (G to C) at the hammerhead sequence to avoid self-cleavage. In both constructs, RNAPII transcription has to proceed through an identical 2.2 kb DNA sequence. However, whereas the *Rib*$^+$ construct only yields 0.6 kb long transcripts because of efficient ribozyme cleavage, the *rib*^{m} construct gives rise to complete 2.2 kb mRNAs (Figure 1).

If transcription impairment depends on the DNA template, the two constructs (*rib*^{m} and *Rib*$^+$) should support the same degree of transcription deficiency in *hpr1*Δ mutants. If, however, transcription impairment depends on the nascent mRNA molecule, only transcription of the *rib*^{m} construct (inactive ribozyme) should be impaired, as indeed is shown in Figure 1. In contrast, transcription of the self-cleaved *Rib*$^+$ construct (active ribozyme) was equally efficient in wild-type and *hpr1*Δ cells (Figure 1). Therefore, removal of the upstream 1.5 kb *PHO5* mRNA relieves the defect in transcribing long GC-rich templates caused by *hpr1*Δ. It is worth noticing that, in contrast to what has been suggested recently (Libri et al., 2002; Zenklusen et al., 2002), this result excludes the possibility that the transcription defect observed in THO mutants is a consequence of an exosome-mediated 3′-end degradation of the nascent mRNA.

To confirm that the RNA-length-dependent defects of mRNA accumulation in the *Rib*$^+$ construct were occurring at the elongation step of transcription in *hpr1*Δ cells, we performed run on assays. We determined the efficiency of transcription at three sites: the 5′-end, an internal region, and the 3′-end of the two types of *PHO5-Rib-lacZ* constructs. Transcription along the three sites occurred with similar efficiency in wild-type cells in the two constructs (Figure 2). By contrast, a clear transcription elongation impairment was observed at the transition between the *PHO5* and *lacZ* sequences in the *rib*^{m} construct (inactive ribozyme) in cells lacking Hpr1 (Figure 2). This result is in agreement with our previous observation that the transcription elongation defect in *hpr1*Δ cells is primarily observed at a distance from the promoter (Chávez et al., 2001). Importantly, the elongation impairment in *hpr1*Δ cells was abolished in the *Rib*$^+$ construct (active ribozyme). Therefore, we conclude that the transcriptional elongation defect of *hpr1*Δ cells can be completely suppressed by self-cleavage of the nascent mRNA.

RNase H Activity Is Required for Full Suppression of Transcription Elongation Impairment of *hpr1*Δ Cells Caused by the Ribozyme-Mediated Cleavage of the Nascent mRNA

To shed light on the possible mechanism by which the nascent mRNA is responsible for the reduced elongation efficiency of THO/TREX mutants, we considered the possibility that failure to form fully functional mRNA-hnRNPs (mRNPs) in *hpr1*Δ might allow improper interactions between the RNA and the DNA template upstream of the elongating RNAPII. Such interactions might lead to short stretches of DNA:RNA hybrids. Such hybrids have been proposed as being responsible for inefficient transcription throughout the rDNA region in *E. coli* in *topA* mutants (Hraiky et al., 2000). If DNA:RNA hybrids play a role in the transcription defect of *hpr1*Δ cells, it would be expected that these defects would also be stronger in cells lacking RNases H1 and H2 than in cells expressing both RNases H. Figure 3 shows that wild-type and *rnh1*Δ *rnh2*Δ double mutants have similar kinetics of full-length mRNA formation and that *hpr1*Δ and the *rnh1*Δ *rnh2*Δ *hpr1*Δ triple mutant were similarly impaired in their ability to transcribe the *rib*^{m} construct (inactive ribozyme). In both cases transcription efficiency was clearly below wild-type levels (4-fold) (Figure 3, upper panels). As expected, the kinetics of transcript formation in *hpr1*Δ cells was similar to wild-type and *rnh1*Δ *rnh2*Δ cells in the *Rib*$^+$ construct (active ribozyme) (Figure 3, lower panels). However, the ability of *rnh1*Δ *rnh2*Δ *hpr1*Δ cells to transcribe the *Rib*$^+$ construct was clearly reduced compared to *hpr1*Δ cells. Therefore, the ribozyme-mediated cleavage of the nascent mRNA long 5′ tail completely suppresses the transcription defect

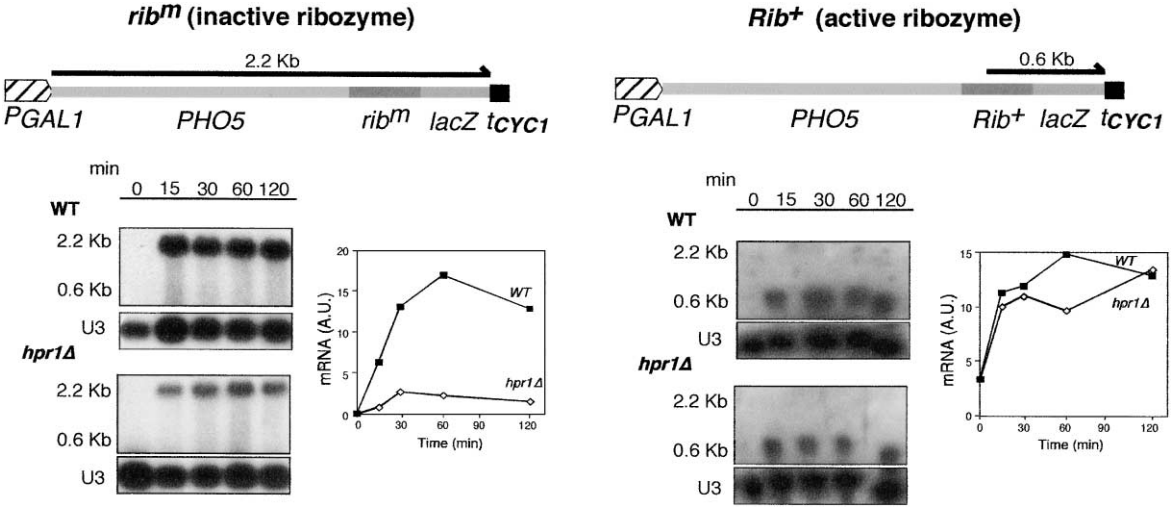

Figure 1. Nascent mRNA Dependency of the Transcription Defects of *hpr1*Δ Cells

Kinetic analyses of transcription activation of the *rib*^{m} and *Rib*$^+$ fusions in W303-1A (wild-type) and U678-4C (*hpr1*Δ) strains. The *PHO5-rib*^{m}-*lacZ* (*rib*^{m}) and *PHO5-Rib*$^+$-*lacZ* (*Rib*$^+$) transcriptional fusions were under the *GAL1* regulable (P_{GAL1}) promoter in centromeric plasmid pRS416. Both fusions contain an active synthetically made 52 bp ribozyme (*Rib*), followed by a 266 bp fragment of the U3 gene to prevent the cleaved RNA from degradation and the 369 bp PvuII 3′-end *lacZ* fragment at the UTR of *PHO5* (position +1405). The *rib*^{m} sequence contains a single base pair mutation (G to C) that inactivates the ribozyme. The *rib*^{m} system (inactive ribozyme) yields a final 2.2 kb *PHO5-rib*^{m}-*lacZ* mRNA, whereas in the *Rib*$^+$ system (active ribozyme) self-cleavage of the *PHO5-Rib*$^+$-*lacZ* mRNA leads to the 0.6 kb *Rib*$^+$-*lacZ* 3′-end transcript. No full 2.2 kb transcript was detected, indicating that the ribozyme sequence cut with full efficiency. Electrophoresis was performed in formaldehyde-agarose gels (*rib*^{m}) or urea-acrylamide gels (*Rib*$^+$) and was hybridized with a U3 probe. All data were normalized with respect to the endogenous U3 signal. The average of two different experiments is plotted.

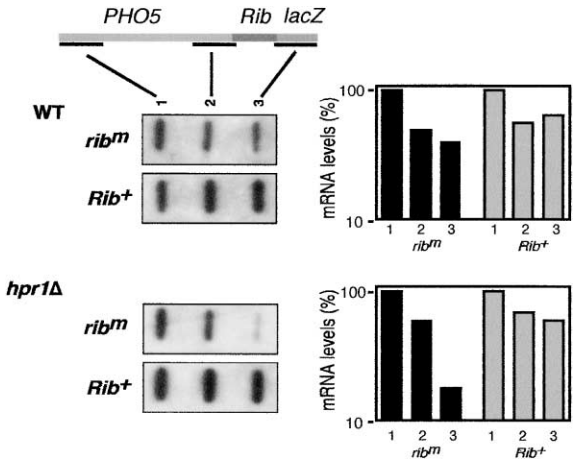

Figure 2. Run On Analysis of Transcription Elongation in the *Rib*$^+$ and *PHO5-rib*^{m}-*lacZ* Fusion Constructs

As probes we used 0.4 kb PCR fragments covering either the 5′ (1) or the 3′ (2) ends of *PHO5* or the 0.4 kb *lacZ* sequence (3). 1 μg of each DNA fragment was immobilized in Hybond-N membranes and hybridized with in vivo α^{32}P-UTP-labeled total RNA extracted from wild-type and *hpr1*Δ strains harboring the *Rib*$^+$ or *rib*^{m} systems. As positive and negative controls, we used the yeast 28 rDNA fragment and genomic DNA from *S. thyphimurium*, respectively (data not shown). Quantification of run on data was normalized with respect to the internal 28S rDNA. In all cases, the percentage of radiolabeled mRNA bound to each probe was calculated with respect to their corresponding levels with probe 1, taken as 100%. Experiments were repeated three times and the results were the same.

of *hpr1*Δ only if the RNases H1 and H2 are active. As RNases H specifically remove DNA:RNA hybrids, the nascent mRNA may have a higher chance of forming DNA:RNA hybrids behind the elongating RNAPII in *hpr1*Δ strains than in wild-type. Such hybrids may be responsible for the reduction in the transcription elongation efficiency caused by mutations of THO/TREX.

Suppression of Transcription-Associated Hyperrecombination by mRNA Self-Cleavage and RNase H Overexpression in *hpr1*Δ Mutants

As the transcription impairment defect caused by mutations of the THO complex is linked to a strong increase in recombination between direct DNA repeats, we expected that suppression of the transcription defect caused by the ribozyme-mediated self-cleavage of the nascent mRNA would also reduce hyperrecombination in *hpr1*Δ mutants. To test this hypothesis we constructed recombination systems in which the *PHO5-Rib*$^+$ fusion fragment—harboring *PHO5*, the U3-protecting sequence, and an active hammerhead ribozyme—was placed between two direct repeats of *leu2* truncated ORFs, all under the transcriptional control of the *GAL1* promoter (system GL-*Rib*$^+$). As control we constructed an identical system but harboring the single-base mutated *rib*^{m} sequence, inactivating the ribozyme (GL-*rib*^{m}) (Figure 4A). Under repressed conditions (glucose), recombination frequencies were similar in wild-type and *hpr1*Δ strains, regardless of whether the recombination system contained the active or the inactive ribozyme (Figure 4B). Under high levels of transcription (galactose), wild-type cells showed the same recombination levels as when transcription was repressed in both GL-*Rib*$^+$ and

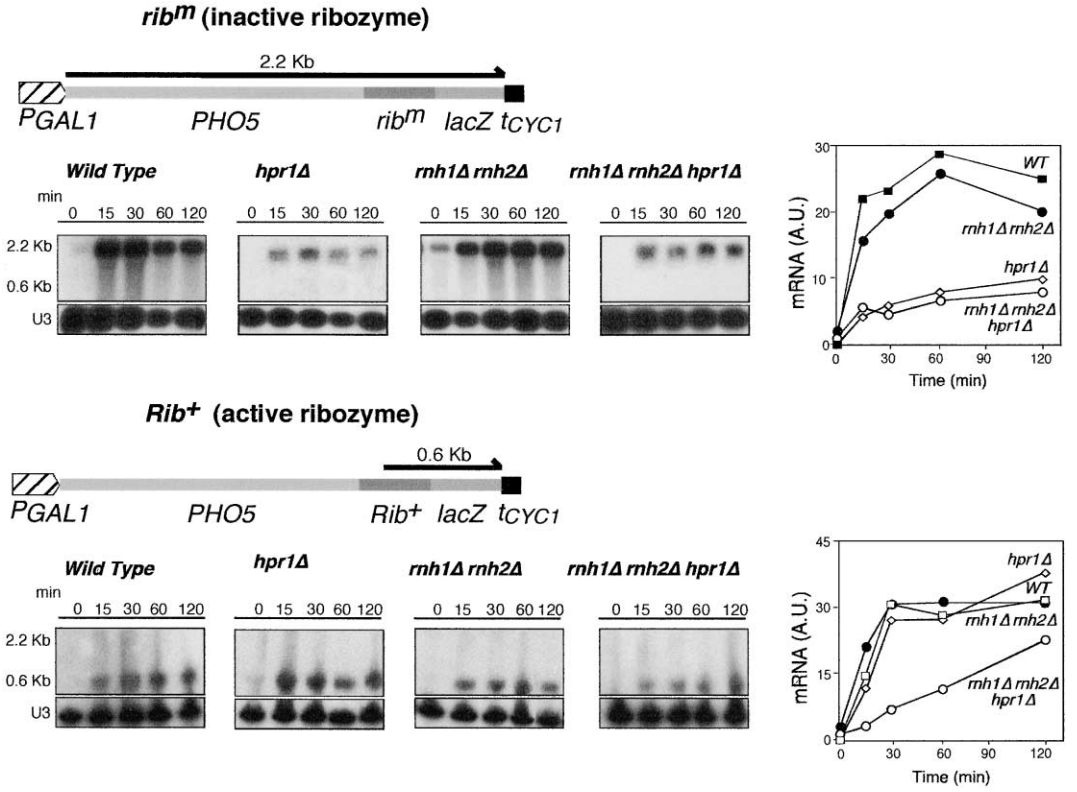

Figure 3. Effect of RNase H on Transcription Elongation Impairment in *hpr1*Δ Cells

The kinetic analysis of transcription activation of the *Rib*$^+$ and *rib*^{m} fusion constructs in strains HRN2-8A (wild-type), HRN2-10A (*hpr1*Δ), HRN2-10B (*rnh1*Δ *rnh2*Δ *hpr1*Δ), and HRN2-10C (*rnh1*Δ *rnh2*Δ) is shown. Details as in Figure 1.

GL-*rib*^{m} systems. Recombination was strongly increased in *hpr1*Δ cells (170-fold above wild-type levels) in GL-*rib*^{m} (inactive ribozyme). However, hyperrecombination was significantly reduced (6-fold) in GL-*Rib*$^+$ (active ribozyme; Figure 4B). In contrast to the relief of the transcription defect of *hpr1*Δ (Figure 1), suppression of hyperrecombination by ribozyme cleavage was thus less dramatic. This might be due to different threshold levels of defective mRNA required for detection of the transcription defect and the hyperrecombination phenotype. It could also be envisioned that short mRNAs stayed at the transcription site after ribozyme cleavage, without effect on elongation of the polymerase-associated RNA, but with a significant effect on recombination.

As indicated by the transcription results, nascent mRNAs might form short DNA:RNA hybrids responsible for the induction of TAR in *hpr1*Δ strains. To test this possibility, we overexpressed the structural gene for one of the two major yeast RNases H, *RNH1*, which degrades the RNA in DNA:RNA hybrids (Crouch et al., 2001). We expected that if DNA:RNA hybrids accumulated at a higher frequency in *hpr1*Δ, recombination levels should diminish in GL-*rib*^{m} (inactive ribozyme) and even more drastically in the GL-*Rib*$^+$ system (active ribozyme) in cells overexpressing RNaseH1. As can be seen in Figure 4C, *RNH1* overexpression reduced recombination levels 5-fold in GL-*rib*^{m} and 2.5-fold in GL-*Rib*$^+$ (compare GL-*rib*^{m} and GL-*Rib*$^+$ + or − *RNH1*). However, overexpression of *RNH1* plus ribozyme-mediated self-cleavage of the mRNA decreased hyperrecombination 9-fold (Figure 4C, compare GL-*rib*^{m} − *RNH1* with GL-*rib*$^+$ + *RNH1*). This suggests that either RNase H1 by itself might not be able to efficiently remove all putatively recombinogenic DNA:RNA hybrids formed in *hpr1*Δ cells, unless the nascent mRNA is self-cleaved, or that DNA:RNA hybrids might not be the only recombinogenic structure formed by the nascent mRNA. Next, we reasoned that if the DNA:RNA hybrid levels were the result of an equilibrium between DNA:RNA hybrid formation during transcription and removal by RNase H, deletion of structural *RNH* genes should increase the steady-state levels of DNA:RNA hybrids. Consequently, we repeated all the recombination assays in the *rnh1*Δ *rnh2*Δ genetic backgrounds. Figure 4C shows that the *rnh1*Δ *rnh2*Δ double mutants had wild-type levels of recombination in both the GL-*rib*^{m} (inactive ribozyme) and GL-*Rib*$^+$ (active ribozyme) systems. Significantly, in the absence of RNases H1 and H2, the active ribozyme was not able to suppress the hyperrecombination phenotype of *hpr1*Δ cells. This result is consistent with the idea that short stretches of the nascent ribozyme-cleaved mRNA remain at the site of transcription in the form of DNA:RNA hybrids when RNases H1 and H2 are lacking in *hpr1*Δ cells.

Consistent with this interpretation, when *RNH1* was overexpressed in *rnh1*Δ *rnh2*Δ *hpr1*Δ cells, recombination was reduced 30-fold in GL-*rib*^{m} (inactive ribozyme) and 160-fold in GL-*Rib*$^+$ (active ribozyme) (Figure 4C). Remarkably, in the latter case recombination was re-

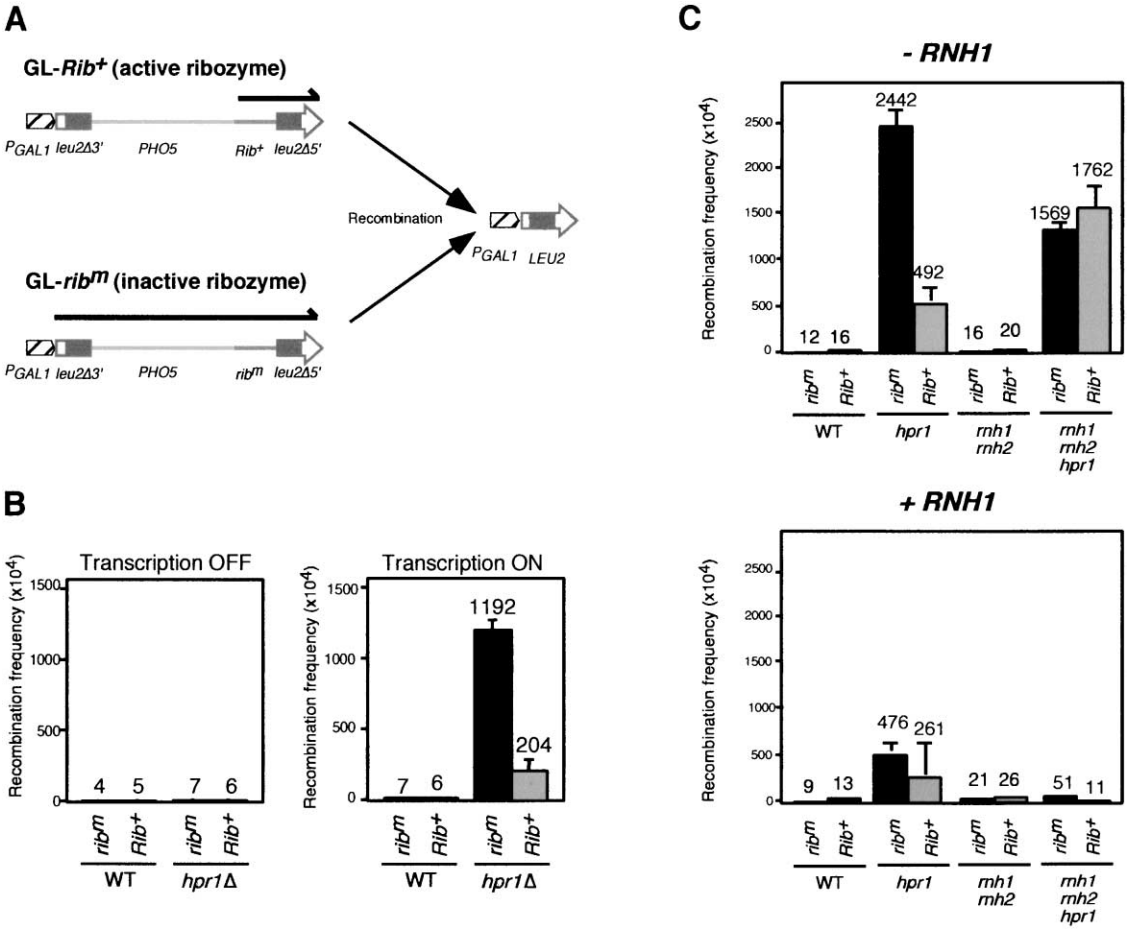

Figure 4. Effect of mRNA in Transcription-Associated Recombination

(A) Direct-repeat recombination systems GL-*Rib*$^+$ and GL-*rib*^{m} containing the *PHO5-Rib*$^+$ or *PHO5-rib*^{m} sequences flanked by two truncated copies of *LEU2* in direct orientation under the *GAL1* promoter. Transcription of both systems is under the control of the inducible P_{GAL} promoter. Recombination initiated in or between the *leu2* repeats (gray-shaded boxes) restores a *LEU2* wild-type allele.

(B) Recombination frequencies under repressed (OFF) and activated conditions (ON).

(C) TAR frequencies of wild-type, *hpr1*Δ, *rnh1*Δ *rnh2*Δ, and *rnh1*Δ *rnh2*Δ *hpr1*Δ cells containing the recombination systems GL-*Rib*$^+$ (black bars) and GL-*rib*^{m} (gray bars) are shown. All experiments were performed in 2% galactose (+) to allow expression of the direct repeats. Lack (−*RNH1*) or overpresence (+ *RNH1*) of RNase H1 in the cells was achieved with either empty vector p416-GAL1 or the multicopy plasmid pGAL-RNH1 carrying *RNH1* under the *GAL1* promoter, respectively. Recombination frequencies were determined as the median value of six independent cultures as previously described (Chávez and Aguilera, 1997). The average median value of two to four experiments plus standard deviations is plotted.

duced to wild-type levels (1.1×10^{-3}). Therefore, the joint action of the ribozyme and overexpressed RNase H1 completely abolished hyperrecombination in *hpr1*Δ cells. The incomplete abolishment of hyperrecombination by the joint action of overexpressed RNase H1 and ribozyme-mediated mRNA self-cleavage, unless RNase H2 was removed from the cells, suggests that RNase H2 interferes with RNase H1. This is in agreement with the observation that *rnh1*Δ mutants show higher levels of RNase H2 (Arudchandran et al., 2000).

Molecular Detection of DNA:RNA Hybrids in *hpr1*Δ Mutants

Our previous results indicate that in *hpr1*Δ mutants the nascent mRNA is able to form DNA:RNA hybrids. To directly demonstrate the existence of such hybrids, we isolated nucleic acids using a DNA isolation method under RNase-free conditions from wild-type and *hpr1*Δ strains harboring the *Rib*$^+$ or the *rib*^{m} constructs. For detection and quantification of the different RNA species, nucleic acids were then treated with different nucleases as indicated. First, samples were treated with RNase A, which degrades free RNA and has no activity on RNA forming DNA:RNA hybrids, with or without RNase H, which specifically degrades the RNA strand of DNA:RNA hybrids. After that, DNA was eliminated by treatment with DNase I. Thus, three different nucleic acid species were detected (Figure 5A): total DNA (samples treated with RNase A and RNase H), total RNA (samples treated with DNase I), and RNA forming DNA:RNA hybrids (samples treated with RNase A and DNase I). Dot-blot hybridization with α^{32}P-labeled PCR probes from the 5′, middle-part, and 3′ ends of the *PHO5-Rib-lacZ* constructs allowed us to quantify the amount of free RNA and of

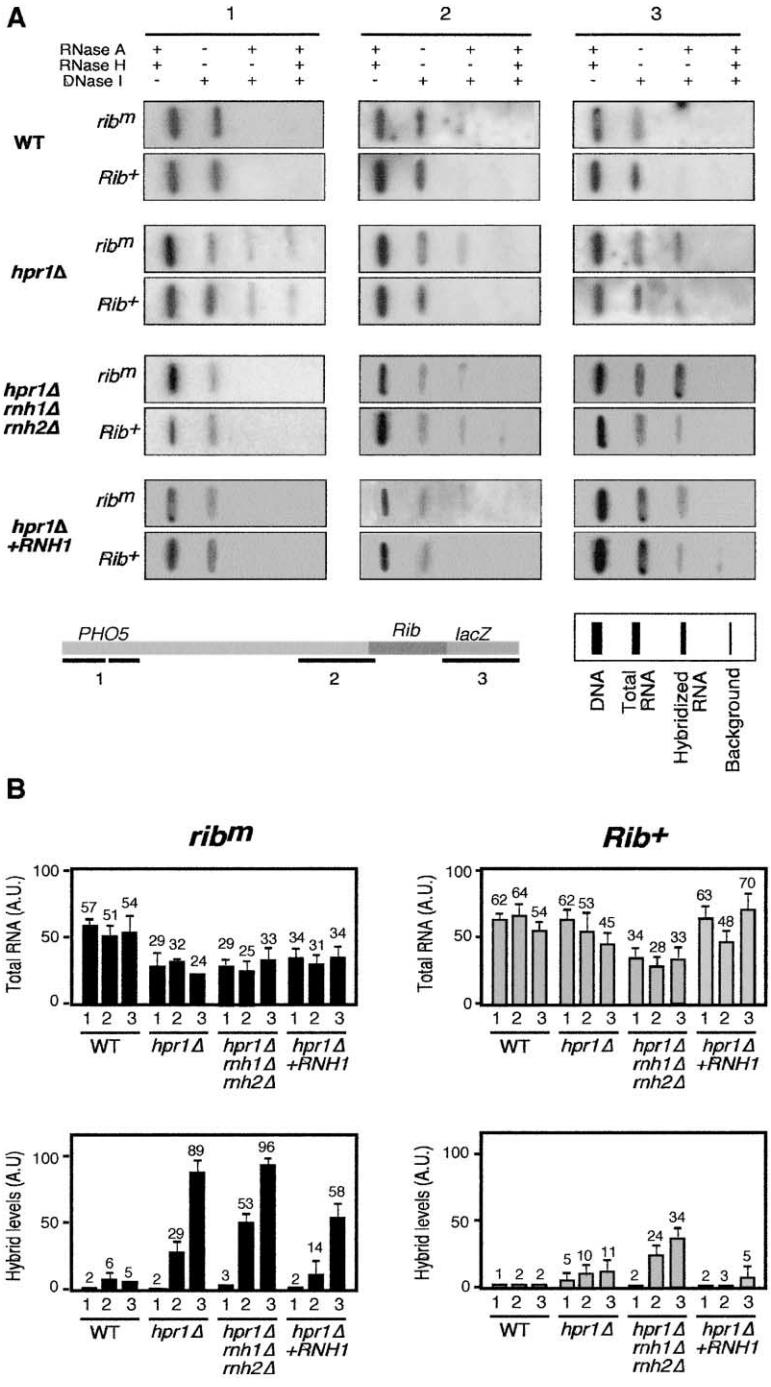

Figure 5. Molecular Detection of DNA:RNA Hybrids

(A) Hybridization results of the nucleic acids isolated from wild-type, *hpr1*Δ, and *hpr1*Δ *rnh1*Δ *rnh2*Δ cells as well as *hpr1*Δ cells overexpressing *RNH1*. All strains harbored the *PHO5-Rib*$^+$*-lacZ* (*Rib*$^+$) or *PHO5-rib*^{m}*-lacZ* (*rib*^{m}) fusion constructs. Total nucleic acids were treated with DNase I, RNase A, or RNase H, as indicated, to remove DNA, free RNA, or RNA hybridized with DNA, respectively, and immobilized in Hybond-N membranes. PCR fragments of the 5′ or the 3′ ends of *PHO5* or the 0.4 kb *lacZ* sequence covering the 5′-end (1), middle-part (2), and 3′-end (3) of the fusion construct used as DNA probes are indicated. A schematic diagram of the bands analyzed and their biological meaning is shown.

(B) Quantification analyses of the total RNA and the RNA present in the form of DNA:RNA hybrid referred to the DNA and total RNA concentrations, respectively. The mean and standard deviations of two independent experiments are plotted.

RNA forming DNA:RNA hybrids along the fusion construct.

In wild-type cells total RNA was produced at similar levels regardless of whether the system was *Rib*$^+$ or *rib*^{m} (Figure 5B, top panels). However, in *hpr1*Δ mutants the RNA levels of the *rib*^{m} system (inactive ribozyme) were clearly below wild-type levels, whereas in *Rib*$^+$ (active ribozyme) they were the same as in the wild-type. The results are thus consistent with those of Figures 1 and 2, which indicated by Northern kinetics and run on analysis that *hpr1*Δ cells did not properly transcribe the *PHO5-Rib-lacZ* construct unless the message was self-cleaved by an active ribozyme. As expected, in *hpr1*Δ *rnh1*Δ *rnh2*Δ triple mutants the RNA levels of the *rib*^{m} construct were similar to those of *hpr1*Δ, and the RNA levels of the *Rib*$^+$ construct were lower than in wild-type and *hpr1*Δ. On the other hand, overexpression of *RNH1* in *hpr1*Δ strains had little effect on the total amount of RNA (Figure 5B, top panels).

Figure 5B (low panels) shows that the RNA present in the form of DNA:RNA hybrids, detected in the sample treated with DNase I and RNase A but absent in the sample treated with RNase H, was strongly accumulated in the *rib*^{m} (inactive ribozyme) but very weakly in the *Rib*$^+$

(active ribozyme) construct in *hpr1*Δ (compare *hpr1*Δ in Figure 5B, lower panels). No accumulation was observed in the wild-type. Accumulation of DNA:RNA hybrids in *hpr1*Δ was position dependent. Thus, hybrids were undetectable at the 5′-end of the *PHO5-rib^m-lacZ* construct but were 5- and 15-fold above wild-type levels at the middle-part and 3′-end of the gene, respectively. As expected, in the sample also treated with RNase H, this signal disappeared. This is consistent with the idea that the longer the nascent mRNA, the higher the probability of forming DNA:RNA hybrids and the possibility that hybrids are formed preferentially close behind the elongating RNAPII. The results confirm at the molecular level the existence of DNA:RNA hybrids responsible for the transcription elongation impairment and hyperrecombination.

This observation, together with the results of Figures 3 and 4, predicts that DNA:RNA hybrids should accumulate at higher levels in *hpr1*Δ *rnh1*Δ *rnh2*Δ triple mutants but at lower levels in *hpr1*Δ strains overexpressing *RNH1*. As can be seen in Figure 5B (bottom panels), DNA:RNA hybrids in the *rib^m* (inactive ribozyme) construct in *hpr1*Δ *rnh1*Δ *rnh2*Δ mutants were accumulated at the middle-part and 3′-end of the gene at levels 1.8- and 1.2-fold above *hpr1*Δ levels, respectively. For the *Rib$^+$* (active ribozyme) construct, these values were 2.2- and 3-fold, respectively, consistent with the observed defect in transcription (Figure 3). Importantly, overexpression of *RNH1* in *hpr1*Δ cells reduced the amount of hybrids observed (Figure 5B, bottom panels). For *rib^m*, the hybrids detected at the middle-part and 3′-end of the gene in cells overexpressing *RNH1* were reduced to 27% and 61% of the *hpr1*Δ levels, respectively. For the *Rib$^+$* system (active ribozyme), overexpression of *RNH1* leads to DNA:RNA levels that were 33% and 43% of the *hpr1*Δ levels. Therefore, the accentuated hyperrecombination and transcriptional impairment of *Rib$^+$* in *hpr1*Δ *rnh1*Δ *rnh2*Δ and the suppression of the hyperrecombination phenotype caused by *RNH1* overexpression in *hpr1*Δ cells correlated with the levels of DNA:RNA hybrids detected in each case.

Discussion

Using a *hpr1*Δ mutant defective in the THO complex, we show that the nascent mRNA can diminish transcription elongation efficiency and promote genetic instability. Molecular analysis provides evidence that DNA:RNA hybrids can be formed in vivo behind the RNAPII in these mutants, impairing RNAPII elongation and promoting TAR. We propose a molecular mechanism to explain the tight link between the transcription and recombination phenotypes of mutants affected in mRNA biogenesis, such as *hpr1*Δ.

The transcription defect of *hpr1*Δ mutants is mainly observed for long and GC-rich DNA sequences driven from a strong promoter (Chávez et al., 2001). Here we show that the requirement of the THO complex for proper transcription elongation is not related to the length of the transcribed DNA itself, but to the length of the nascent mRNA. If a 2.2 kb long ORF contains a hammerhead ribozyme 1.5 kb downstream of the translation start, ribozyme-mediated self-cleavage of the nascent mRNA allows the RNAPII to elongate transcription with wild-type efficiency (Figure 1). Therefore, THO/TREX may not be required for RNAPII processivity through long DNA sequences. Instead, it impedes long nascent mRNAs from forming intermediates that could diminish transcription elongation. Here we show that DNA:RNA hybrids are one such intermediate. RNases H are required for full suppression of the transcription elongation defect of *hpr1*Δ caused by the ribozyme-mediated cleavage of the nascent mRNA (Figure 3), suggesting that DNA:RNA hybrids need to be removed for complete abolishment of the transcription defect. Consistent with this conclusion, and in contrast to wild-type cells, a significant fraction of the total *PHO5-Rib-lacZ* nascent mRNA in *hpr1*Δ cells form RNase H-sensitive DNA:RNA hybrid stretches (Figure 5), providing evidence that DNA:RNA hybrids can be formed in eukaryotes in vivo. Such hybrids accumulate at the 3′-end of the gene, that is, the region in which a transcription elongation pausing or stalling is detected by run on analysis (Figure 2). As expected, the DNA:RNA hybrids accumulate at higher levels in *hpr1*Δ *rnh1*Δ *rnh2*Δ mutants and at lower levels in *hpr1*Δ mutants overexpressing RNase H1 (Figure 5B). These results suggest that DNA:RNA hybrids may be responsible of the transcriptional elongation impairment. Interestingly, we have not been able to detect suppression of the expression defect of *hpr1* mutants in the *rib^m* system by overexpressing *RNH1*, as determined by run on and Northern analyses (data not shown). It is possible that the DNA:RNA hybrids are degraded by the overexpressed RNase H1, therefore impeding detection of a significant increase in the mRNA levels, in particular at the 3′-end. However, it is also likely that, in addition to DNA:RNA hybrids, abnormal "nascent mRNP-RNAPII-DNA" ternary complexes could be formed and cause transcription elongation impairment.

Independent studies in *E. coli* have provided evidence that RNA molecules can form DNA:RNA hybrids and impair transcription in *topA*Δ mutants (see Drolet et al., 2003). Thus, RNase H partially complements the growth defects and rRNA transcription defects of *topA*Δ mutants (Hraiky et al., 2000). Severe growth inhibition in the absence of DNA topoisomerase I correlates with transcription-induced negative supercoiling (Masse and Drolet, 1999), which may facilitate the formation of DNA:RNA hybrids. Topoisomerase I likely inhibits RNA-DNA hybrid formation in *E. coli* by its capacity to relax transcription-induced negative supercoiling (Masse and Drolet, 1999). During transcription, it is likely that the local accumulation of negatively supercoiled DNA behind the advancing RNAPII in eukaryotes has the potential to increase DNA denaturing and the formation of stretches of ssDNA. One possibility is that this ssDNA could be more susceptible to pair with the nascent mRNA in mutants of proteins involved in mRNP biogenesis, such as *hpr1*Δ. This would be consistent with the observation that DNA:RNA hybrids are formed at the 3′-end of long transcripts but not at the 5′-end in *hpr1*Δ mutants (Figure 5). Interestingly, THO mutants are extremely sick in combination with mutations in topoisomerase structural genes (Aguilera and Klein 1990; Sadoff et al., 1995). In addition, GC-rich DNA sequences such as *lacZ* do not position nucleosomes (Chávez et al.,

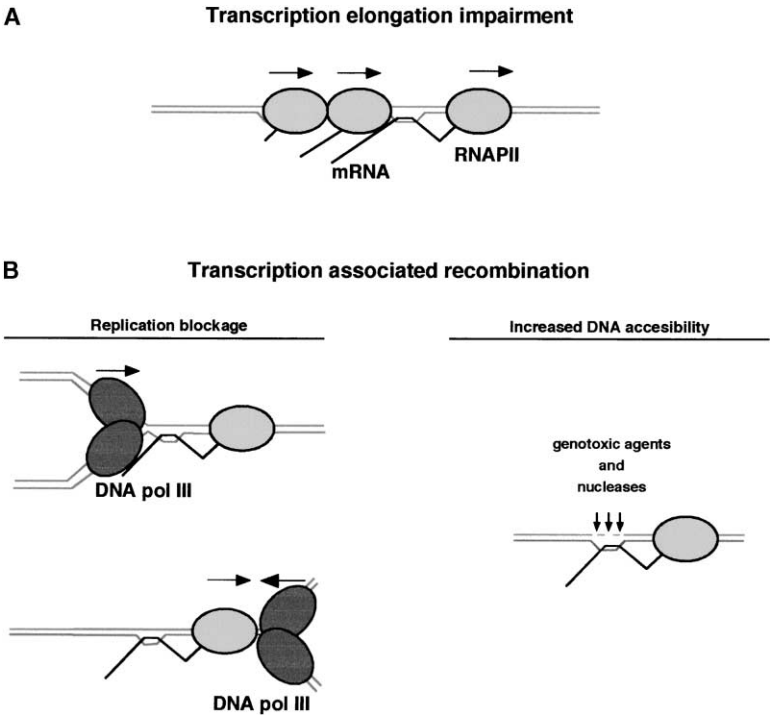

Figure 6. Model to Explain Formation of DNA:RNA Hybrids in THO Mutants and How the Hybrids Could Impair Transcription Elongation and Cause TAR

(A) In the absence of proteins functioning at the interface between transcription and mRNA metabolism, such as *hpr1*Δ, mRNP biogenesis does not occur properly. The nascent mRNA can form stretches of DNA:RNA hybrids that may be an obstacle for the next elongating RNAPII, impairing transcription elongation.

(B) TAR and other forms of genetic instability, such as plasmid and chromosome loss, could be explained by either a possible stalling of the replicating DNA polymerase when encountering the DNA:RNA hybrid (top left) or a blocked RNAPII (bottom left) or by a major susceptibility of ssDNA stretches to DNA damage agents or to nucleases (right).

2001). This, together with the synthetic lethality of *hpr1*Δ mutants in cells with histone imbalance (Fan and Klein, 1994; Zhu et al., 1995), opens the possibility that the nascent mRNA could have a higher chance of forming DNA:RNA hybrid stretches in hypernegatively supercoiled regions lacking an organized chromatin structure.

DNA:RNA hybrids formed in *hpr1*Δ mutants could become roadblocks for the next elongating RNAPII (Figure 6A). This has been proposed as an explanation for rRNA transcription defects in *E. coli topA* mutants (Hraiky et al., 2000). As a consequence, transcription elongation would be diminished. This is consistent with the idea that THO works at the interface between transcription and mRNA metabolism (Jimeno et al., 2002) and with the observations that a His(6)-tagged THO complex purified from yeast associates with RNA in vitro (Jimeno et al., 2002) and that THO components associate with actively transcribed chromatin (Strasser et al., 2002; Zenklusen et al., 2002).

Consistent with the tight link between transcription defect and hyperrecombination in *hpr1*Δ mutants, the nascent mRNA also mediates transcription elongation-dependent hyperrecombination. Direct repeats that are transcribed from an external promoter showed a significant reduction of hyperrecombination when the resulting transcript is self-cleaved by an active ribozyme (Figure 4). In addition, overexpression of RNase H1 reduces the hyperrecombination phenotype partially in *hpr1*Δ and completely in *hpr1*Δ *rnh1*Δ *rnh2*Δ backgrounds (Figure 4). Therefore, we conclude that the DNA:RNA hybrids detected in *hpr1*Δ cells (Figure 5) mediate TAR. This is particularly relevant because there is molecular evidence that the class-switching regions of the Ig genes, in which recombination occurs tightly linked to transcription, form persistent DNA:RNA hybrids after transcription, and these hybrids have been proposed to trigger class-switch recombination (Mizuta et al., 2003; Reaban and Griffin, 1990; Yu et al., 2003). Nevertheless, the observation that only the joint action of overexpressed RNase H1 and the self-cleavage hammerhead ribozyme cause complete suppression of hyperrecombination in *hpr1*Δ mutants also opens the possibility that, in addition to DNA:RNA hybrids, other structures such as abnormal mRNP-RNAPII-DNA ternary complexes could also mediate hyperrecombination.

Two scenarios can be viewed as possibilities to explain the mechanism by which the nascent mRNA mediates hyperrecombination and genetic instability (Figure 6B). In the first scenario (Figure 6B, left), the DNA:RNA hybrids (top) or the blocked mRNP-RNAPII-DNA ternary complex (bottom) could halt the replication fork (see Aguilera, 2002). This would lead to a replication fork collapse, which could subsequently generate recombinogenic DNA breaks or replication fork reversal. The requirement of recombination functions for the resumption of arrested replication forks has been shown in *E. coli* (McGlynn and Lloyd, 2000; Seigneur et al., 1998). Furthermore, it has been shown that transcription by either bacterial or phage T7 RNA polymerase of d(G)n:d(C)n repeats causes replication fork blockage (Krasilnikova et al., 1998). Interestingly, Ig class-switch regions are GC-rich (Nikaido et al., 1981), and the transcription defect and hyperrecombination phenotypes of *hpr1*Δ mutants are more accentuated in GC-rich DNA sequences (Chávez et al., 2001).

In a second scenario (Figure 6B, right), short ssDNA stretches putatively displaced by DNA:RNA hybrids could be more susceptible to attack by DNA damaging agents or nucleases (Figure 6B, top), which could yield recombinogenic DNA breaks. The observation that transcription induces mutagenicity caused by different DNA

damaging agents such as ICR-170 in *E. coli* (Herman and Dworkin, 1971) or 4-NQO- and MMS-induced recombination in yeast (García-Rubio et al., 2003) is consistent with this view and with the observation that *rnh1*Δ *rnh2*Δ double mutants show increased sensitivity to hydroxyurea, caffeine, and ethyl methanesulphonate (Arudchandran et al., 2000). In this context, it is worth noticing that the nucleotide excision repair endonucleases XPF-ERCC1 and XPG can cleave the displaced ssDNA of DNA:RNA hybrids artificially formed at the Ig class-switch regions in vitro (Tian and Alt, 2000).

In summary, our observation that the nascent mRNA can play a key role in modulating and connecting transcription, mRNA metabolism, and genetic instability sheds light on the mechanisms of transcription elongation and their connection to mRNA metabolism and TAR. The conservation of THO/TREX and other functionally related complexes in yeast and humans suggests that the phenomena described here may be relevant to all eukaryotes. Indeed, it may be particularly important in mammals, in which transcription units can be up to several hundred kilobases long. Our results show that DNA:RNA hybrids are, at least, one type of intermediate that can diminish transcription elongation efficiency and promote TAR. In addition, we cannot discard the possibility that the nascent mRNA also formed abnormal nascent mRNP-RNAPII-DNA ternary complexes that could negatively affect transcription elongation and promote TAR in *hpr1*Δ mutants. The relevance of TAR in developmentally regulated processes such as the transcription-dependent class switching of Ig genes adds biological interest to these phenomena. Our results provide new perspectives in understanding transcription elongation and TAR in eukaryotes.

Experimental Procedures

Strains and Plasmids

Wild-type (W303-1A) and *hpr1*Δ*::HIS3* (U678-1C) yeast strains have been reported previously (Piruat and Aguilera, 1998). *rnh1*Δ and *rnh2*Δ simple mutants obtained from EUROSCARF and *hpr1*Δ*HIS3* (U678-1C) were crossed to obtain wild-type (HRN2-8A), *hpr1*Δ*::HIS3* (HRN2-10A), *hpr1*Δ*::HIS3 rnh1*Δ *rnh2*Δ (HRN2-10B), and *rnh1*Δ *rnh2*Δ (HRN2-10C) congenic derivatives.

The *PHO5-rib*^{m}*-lacZ* (*rib*^{m}) and *PHO5-Rib*$^+$*-lacZ* (*Rib*$^+$) transcriptional fusions were placed under the *GAL1* regulable promoter in centromeric plasmid pRS416. Both fusions contain a PCR fragment with an inactive or active, respectively, synthetically made 52 bp ribozyme (*Rib*) (Samarsky et al., 1999) followed by a 266 bp fragment of the U3 gene. These PCR fragments were obtained using primers 5′-GGAAAAAAGGGATCCACTTGTCAGACTGCC-3′ and 5′-TGTCG GATCCCCTGTCACCGGATGTGTTTTCCGGTCTGATGAGTCCGTG AGGACGAAACAGGAATCCAACTTGGTTGATG-3′ (*Rib*$^+$) or 5′-GGA AAAAAGGGATCCACTTGTCAGACTGCC-3′ and 5′-TGTCGGATCC CCTGTCACCGGATGTGTTTTCCGGTCTCATGAGTCCGTGAGGAC GAAACAGGAATCCAACTTGGTTGATG-3′ (*rib*^{m}) and were cloned at the UTR of *PHO5* (position +1405). The U3 snRNA sequence has the ability to form a secondary structure to which several proteins bind, protecting the transcript from degradation (Samarsky et al., 1999). The 369 bp PvuII 3′-end *lacZ* fragment was subcloned immediately after the *Rib* sequence. The GL-*Rib*$^+$ and GL-*rib*^{m} systems were constructed cloning the 1.7 kb PstI-PstI fragment, containing the *PHO5* and *Rib* sequences, of *PHO5-Rib*$^+$*-lacZ* and *PHO5-rib*^{m}*-lacZ*, respectively, in the plasmid pRS314-GLB (Prado et al., 1997).

Plasmid pGAL:RNH1, with the *RNH1* gene under the *GAL1* promoter, was a gift from R.J. Crouch (Bethesda, MD). Expression vector p416-GAL1, harboring the *GAL1* promoter upstream of a multiple cloning site, has been described (Mumberg et al., 1995).

RNA Analyses

RNA was prepared and analyzed by Northern as described (Chávez and Aguilera, 1997). RNA electrophoresis was performed in formaldehyde-agarose gels (*rib*^{m}) or urea-acrylamide gels (*Rib*$^+$) and hybridized with a U3 probe. All data were normalized with respect to the endogenous U3 signal. Run on analyses were performed as previously described (Chávez and Aguilera, 1997). As probes we used 0.4 kb PCR fragments covering either the 5′- (1) or the 3′- (2) ends of *PHO5* or the 0.4 kb *lacZ* sequence (3), obtained using primers 5′-CTGTTGTTTATTCAATTTTAGCCGC-3′ and 5′-TTCATTTCACCAG TGTATGGGTTC-3′ (1), 5′-GTTCCATTCATGGGCAACACTTTC-3′ and 5′-CTCTGGGGTACAAAGGTTATGGGAC-3′ (2), or 5′-GGTGGGTAC AAATGGCAGTCTGAC-3′ and 5′-AGCTGGAATTCCGCCGATACT GAC-3′ (3). 1 μg of each DNA probe was immobilized in Hybond-N membranes. Filters were hybridized with in vivo α^{32}P-UTP-labeled total RNA extracted from wild-type and *hpr1*Δ strains harboring the *Rib*$^+$ or *rib*^{m} systems. In all cases, the percentage of radiolabeled mRNA bound to each probe was referred with respect to their probe 1 corresponding levels, taken as 100%.

Recombination Analyses

Recombination frequencies were determined as the median value of six independent cultures obtained from independent colonies isolated in synthetic medium containing either 2% glucose (repressed) or 2% galactose (activated) as previously described (Prado et al., 1997). Recombinants were scored as Leu$^+$ colonies on synthetic medium containing 2% galactose and lacking leucine.

In Vivo Detection of DNA:RNA Hybrids

DNA and RNA were isolated using a modification of a DNA isolation method (Wellinger et al. 2003), with all solutions pretreated with DEPC to avoid RNase activity. Yeast cells were cultured in 500 ml of SC-2% glycerol/lactate medium to an $O.D_{600}$ of 0.8. Then galactose was added to a final concentration of 2%, and cells were cultured for 2 hr at 30°C. Cells were collected by centrifugation and washed twice. The cell pellet was resuspended in 2.5 ml of 1 M Sorbitol, 10 mM EDTA (pH 8.0), and 0.1% β-mercapto-ethanol and treated with 5 mg Zymoliase 100T for 45 min at 37°C. The sample was centrifuged for 5 min, washed with H_2O, and resuspended in 625 μl H_2O. Then, 625 μl of solution I (2% w/v CTAB, 1.4 M NaCl, 100 mM Tris-HCl [pH 7.6], 25 mM EDTA [pH 8.0]) with 1.25 mg proteinase K was added. After 15 min at 37°C, proteins were removed by double extraction with 750 μl chloroform/isoamylalcohol (24:1). Afterwards, 3 ml of solution II (1% w/v CTAB, 50 mM Tris-HCl [pH 7.6], 10 mM EDTA) was added, and the sample was centrifuged for 20 min. The pellet was resuspended in 500 μl of 1.4 M NaCl, 10 mM Tris-HCl (pH 7.6), and 1 mM EDTA (pH 8.0), precipitated with 500 μl isopropanol, washed with 70% ethanol, and resuspended in 300 μl RNase buffer (10 mM Tris-HCl pH [8.0], 10 mM $MgCl_2$, 100 mM NaCl). The mix was divided in three parts (total RNA, hybridized RNA, and background samples) and treated for 1 hr 30 min at 37°C with 5 units RNase H (Roche) and/or 15 μg RNase A (Roche) as indicated. 1 μl of the sample treated with both RNases H and A was stored as a DNA sample. RNases were removed in two steps with 100 μl acidic phenol and 100 μl chloroform. Nucleic acids were precipitated with NaCl and ethanol for 30 min at −20°C, washed with 70% ethanol, and resuspended in 100 μl DNase I buffer (10 mM $MgCl_2$, 50 mM Tris-HCl [pH 7.5]). The mix was incubated with 100 units of RNase-free DNase I (Roche) for 1 hr at 37°C. RNA samples were denatured by incubation with 300 μl of 65% v/v formamide, 21% v/v formaldehyde, 14% MOPS for 1 hr at 50°C. DNA samples were denatured by adding 1 ml 6×SSC and boiling for 10 min. All samples were immobilized in Hybond-N membranes and hybridized at 50°C with α^{32}P-labeled PCR probes as used in run on analyses.

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