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Topoisomerase 1-dependent deletions initiated by incision at ribonucleotides are biased to the non-transcribed strand of a highly activated reporter

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ABSTRACT

DNA polymerases incorporate ribonucleoside monophosphates (rNMPs) into genomic DNA at a low level and such rNMPs are efficiently removed in an error-free manner by ribonuclease (RNase) H2. In the absence of RNase H2 in budding yeast, persistent rNMPs give rise to short deletions via a mutagenic process initiated by Topoisomerase 1 (Top1). We examined the activity of a 2-bp, rNMP-dependent deletion hotspot [the $(TG)_2$ hotspot] when on the transcribed or non-transcribed strand (TS or NTS, respectively) of a reporter placed in both orientations near a strong origin of replication. Under low-transcription conditions, hotspot activity depended on whether the $(TG)_2$ sequence was part of the newly synthesized leading or lagging strand of replication. In agreement with an earlier study, deletions occurred at a much higher rate when $(TG)_2$ was on the nascent leading strand. Under high-transcription conditions, however, hotspot activity was not dependent on replication direction, but rather on whether the $(TG)_2$ sequence was on the TS or NTS of the reporter. Deletion rates were several orders of magnitude higher when $(TG)_2$ was on the NTS. These results highlight the complex interplay between replication and transcription in regulating Top1-dependent genetic instability.

INTRODUCTION

Ribonucleoside monophosphates (rNMPs) are the most abundant noncanonical nucleotide present in eukaryotic DNA [reviewed in (1)]. During replication, rNMPs can persist as remnants of Okazaki fragment priming or can be directly inserted in place of the corresponding dNMPs. With regard to the latter, rNTP exclusion from the active site pocket of replicative DNA polymerases is efficient but not perfect (2), and rNTP levels in the nucleotide pool are high relative to those of dNTPs (3). The Polϵ leading-strand DNA polymerase of the yeast *Saccharomyces cerevisiae* is more rNMP-permissive than lagging-strand Polδ *in vitro* (4), and most likely *in vivo* as well [reviewed in (5)]. In addition to rNMP incorporation during replication, other types of DNA synthesis, such as damage bypass by rNTP-permissive translesion synthesis DNA polymerases (3,6) or gap-filling during repair/recombination reactions, can introduce rNMPs into DNA. At least some of these reactions likely occur outside of S phase, when ribonucleotide reductase levels are low (7) and rNTP:dNTP ratios are correspondingly elevated. Finally, recent work has shown that RNA transcripts can be used to directly repair DNA double-strand breaks (8), providing another potential source of rNMPs in DNA. If not removed, rNMPs in a DNA template can slow down subsequent DNA synthesis and generate replication stress (9) or can trigger mutagenesis (4).

The heterotrimeric RNase H2 complex is responsible for the removal of one or a few rNMPs embedded in DNA [reviewed in (10)]. In the absence of yeast RNase H2, deletions within short tandem-repeat hotspots accumulate; repeat unit sizes range from 2–5 bp, and hotspots typically contain 2–4 tandem repeats (4,11). rNMP-associated deletions absolutely require the activity of Topoisomerase 1 (Top1) (11), a type IB topoisomerase that transiently forms a covalent, 3′-phosphotyrosyl complex with nicked DNA (12). Top1 is important for removing transcription-associated torsional stress and interacts with the phosphorylated C-terminal domain of RNA polymerase (RNAP) II (13). Accordingly, rNMP-dependent mutagenesis is highly elevated under conditions of robust transcription, and Top1 is the major source of transcription-associated mutagenesis (14,15). When Top1 cleaves at an rNMP embedded in DNA, the 2′-OH of ribose can attack the 3′-phosphotyrosyl linkage with Top1, which releases the enzyme and leaves a nick flanked by a 2′,3′ cyclic phosphate and a 5′-OH (16). We previously proposed that mutagenesis associated with rN-

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MPs requires sequential cleavage by Top1, first at an rNMP to generate a non-ligatable nick and then at an upstream (5′) dNMP, which generates a small gap between the cleavage sites (17). The gap is predicted to be the size of the relevant repeat unit and to be flanked by a 3′-linked Top1 cleavage complex and a 5′-OH. Spontaneous misalignment between repeats could then convert the gap to a nick, thereby facilitating enzyme-mediated ligation. Recent *in vitro* data have provided support for sequential Top1 cleavage during rNMP-dependent deletion formation (18,19).

Understanding the precise mechanism of rNMP-dependent mutagenesis at hotspots requires knowledge of where rNMPs are incorporated into and where Top1 cleaves genomic DNA. Methods that map rNMPs to single nucleotide resolution *in vivo* have been recently described (20–22), but it is difficult to predict where the enzyme nicks DNA given the very weak consensus site for Top1 cleavage *in vitro* [5′-A/T-G/C-T/A-T; (23)]. In addition, cleavage-site mapping *in vitro* is typically done using short, linear DNA fragments and detection generally requires either a suicide substrate or chemical stabilization of the covalent enzyme-DNA intermediate. Which strand of a hotspot is cleaved *in vivo* can be deduced, however, if placement of the hotspot in both orientations near a well-defined origin of replication is accompanied by an orientation-dependent bias for associated deletions. This reflects Top1 cleavage only at rNMPs that are incorporated by the Polϵ leading-strand DNA polymerase; rNMPs incorporated by the Polδ lagging-strand polymerase are not mutagenic (24). It was suggested that the replication-associated bias might reflect a need for Top1 to remove supercoils that accumulate behind the fork during leading-strand synthesis; nicks that naturally accumulate during discontinuous lagging-strand synthesis would prevent similar torsional stress. While it is generally accepted that precatenanes, which are substrates only for type 2 topoisomerases, can form behind the fork (25), the occurrence of leading-strand specific supercoiling is not well established (26).

In the current study, we focus on an rNMP-dependent, 2-bp deletion hotspot whose activity was previously shown to be profoundly affected by the direction of DNA replication (4). To specifically examine the effect of transcription on 2-bp deletions, the hotspot was transplanted into a highly transcribed reporter placed in both orientations next to a well-defined origin of replication. We demonstrate that, in contrast to low-transcription conditions, the direction of DNA replication has little, if any, effect on deletion rates under high-transcription conditions. Instead, mutagenesis under high-transcription conditions is primarily determined by whether the Top1 cleavage site is located on the transcribed versus the non-transcribed strand (TS or NTS, respectively) of the reporter. These data suggest that a transcription-related asymmetry between DNA strands either dictates which strand is cleaved by Top1 and/or determines the genetic outcome of Top1 incision at an rNMP.

MATERIALS AND METHODS

Yeast strains

Strains SJR2259–SJR2262 were derived by transformation of YPH45 [*MATa ura3–52 ade2–101$_{oc}$ lys2–801$_{am}$ trp1Δ1*; (27)], a strain congenic to S288C. Strains SJR2259 and SJR2260 contain a *pLYS-LYS2* construct in both orientations near *ARS306* on chromosome III; in SJR2261 and SJR2262, *pLYS* was replaced by *pTET* [for construction details, see (28)]. Transcription and replication forks move in the same direction in strains designated *LYS2F*; in strains designated *LYS2R*, transcription and replication forks converge. Strains containing the *pLYS-lys2FΔA746NR,(TG)$_2$* and *pTET-lys2FΔA746NR,(TG)$_2$* alleles were constructed via two-step allele replacement following transformation of SJR2259 and SJR2261 (28), respectively, with *Afl*II-digested pSR1030. *pLYS-lys2RΔA746NR,(TG)$_2$-inv* and *pTET-lys2RΔA746NR,(TG)$_2$-inv* strains were constructed via two-step allele replacement following transformation of strains SJR2260 and SJR2262 (28), respectively, with *Afl*II-digested pSR1031. pSR1030 and pSR1031 were constructed by ligating *Bgl*II-digested pSR963 (29) to annealed oligonucleotides 5′-GATCTCCATGGAGGGCACAGTTCAGCC and 5′-GATCGGCTGAACTGTGCCCTCCATGGA; the introduced sequence is from *URA3* and the hotspot is underlined. pSR1030 has the $(TG)_2$ sequence on the TS of *lys2* and pSR1031 has the $(TG)_2$ sequence on the NTS of *lys2*. The *RNH201*, *TOP1* or *RAD14* gene was deleted by one-step allele replacement using PCR-generated deletion cassettes amplified from a plasmid containing an appropriate selective marker. The *pol2-M644L* or *pol2-M644G* allele was introduced by two-step allele replacement using *Age*I-digested p173-pol2-M644L or p173-pol2-M644G (4), respectively. Strains with the *pol3-L612M* allele were constructed via two-step allele replacement using *Hpa*I-digested p170-pol3-L612M (30). Mutant *pol2* and *pol3* alleles were confirmed by sequencing an appropriate genomic DNA fragment. The mating type of SJR2805 (*MATa pTET-lys2F top1*Δ strain) was switched to *MATα* using a pGAL-HO plasmid. This allowed construction of double- and triple-mutant strains by mating, sporulation and tetrad dissection. Supplementary Table S1 provides a complete strain list.

Mutation spectra and rates

All growth of yeast was at 30°C. To determine the Lys$^+$ reversion rate, independent cultures were started either by inoculating 2×10^5 cells from an overnight starter culture or by inoculating each culture with an independent colony. Cultures were grown in YEPGE (1% yeast extract, 2% Bacto-peptone, 250 μg/ml adenine hemisulfate supplemented with 2% glycerol and 2% ethanol) until saturated. Appropriate dilutions were plated on YEPD (1% yeast extract, 2% Bacto-peptone, 250 μg/ml adenine hemisulfate supplemented with 2% dextrose) to determine the total number of viable cells and on synthetic complete dextrose medium lacking lysine (SCD-Lys) to determine the total number of revertants in each culture. Mutation rates were calculated using the method of the median (31) and 95% confidence intervals were determined as described previously (32).

Independent Lys$^+$ revertants were selected on SCD-Lys following non-selective growth in YEPGE medium. A portion of *lys2* containing the $(TG)_2$

hotspot was PCR-amplified using primers LYSWINF (5-GCCTCATGATAGTTTTTCTAACAAATACG) and LYSWINR (5′-CCCATCACACATACCATCAAATCCAC) and the product was sequenced by the Duke University DNA Analysis Facility, Eurofins MWG Operon or Eton Bioscience INC. The rate of 2-bp deletions was calculated by multiplying the total reversion rate by the proportion of 2-bp deletions in the corresponding mutation spectrum.

RNH1 overexpression

Yeast strains were transformed with an empty *URA3-CEN* vector or with the same plasmid containing a *pGAL-RNH1* construct (33). Following the selection of Ura$^+$ transformants, cultures were started directly from individual colonies. Growth was in SC-Ura supplemented with 2% galactose to induce expression of *RNH1*. Cells were plated onto SCD-Ura to determine total cell number or on SCD-Ura-Lys plates to determine the number of revertants. Independent revertants were sequenced as described above and the 2-bp deletion frequency was similarly calculated.

RESULTS

The rNMP-dependent hotspot used in the current study was initially detected when analyzing *URA3* forward mutations isolated in an *rnh201*Δ strain expressing an rNTP-permissive form of Polϵ [(*pol2-M644G* allele; (4)]. The hotspot was referred to as CACA because this sequence is on the *URA3* coding strand, the strand that has the same sequence as the mRNA and hence is the non-transcribed strand (NTS); the complementary TGTG sequence is on the noncoding/transcribed strand (TS). A striking feature of the rNMP-dependent CACA hotspot was its strong dependence on the direction of DNA replication through *URA3*, which had been inserted in both orientations relative to *ARS306* on chromosome III. In Ori1, CACA was on the nascent leading strand and synthesized by Polϵ; in Ori2, TGTG was synthesized by Polϵ. Here, we refer to Ori1 and Ori2 as SAME and OPPO, respectively, to indicate the direction of replication fork movement relative to that of the transcription machinery (Figure 1). In the *URA3* system, hotspot activity was estimated to be ∼100-fold greater in Ori2/OPPO than in Ori1/SAME. This indicates that Top1-dependent deletions arise mostly, if not exclusively, when Polϵ synthesizes and inserts rNMPs into the TGTG-containing strand. The replication-related bias for rNMP-dependent deletions was initially reported in a *pol2-M644G* background, but a similar bias occurs in a *POL2* background (34). Because it is the TGTG-containing strand where the deletion intermediate is generated, we will hereafter refer to this site as the $(TG)_2$ hotspot to reflect the strand cleaved by Top1 to initiate mutagenesis.

The replication bias of the $(TG)_2$ hotspot is recapitulated in a frameshift-reversion assay under low-transcription conditions

We previously demonstrated that small (∼20 bp) DNA fragments containing Top1-dependent, 2-bp deletion hotspots are fully functional when transferred into a *LYS2*-based, frameshift-reversion assay that detects 2-bp deletions (15).

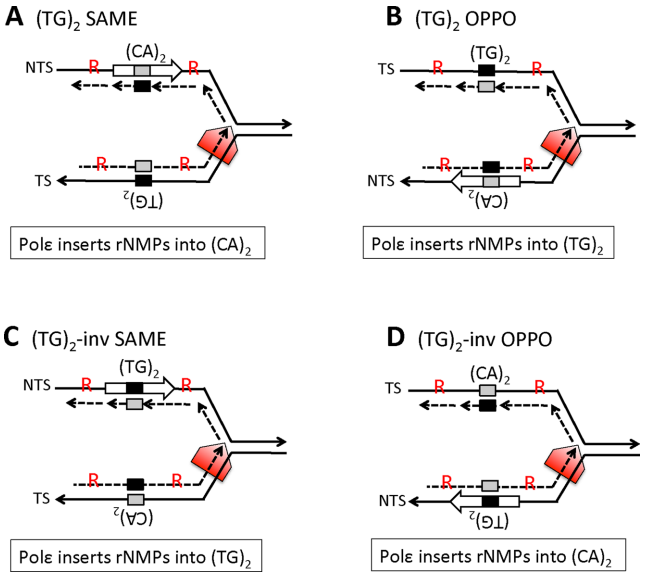


Figure 1. *lys2* reporter constructs containing the $(TG)_2$ hotspot adjacent to *ARS306*. The white arrow corresponds to the coding strand of the *lys2* reporter; $(CA)_2$ and $(TG)_2$ sequences are indicated as gray and black boxes, respectively. Solid lines correspond to template strands for replication and dotted lines to newly synthesized DNA; arrowheads correspond to 3′ ends. Polϵ is indicated by a red pentagon; the rNMPs inserted into nascent DNA by Polϵ are indicated by red R's.

To focus specifically on the $(TG)_2$ hotspot, a hotspot-containing fragment was introduced into a *lys2* reporter inserted in both the SAME and OPPO orientations near *ARS306* on chromosome III (28). In initial experiments, the reporter was under the control of its endogenous promoter (*pLYS*), which promotes a low level of transcription. No 2-bp deletions were seen in either orientation when RNase H2 was functional (Supplementary Table S2), but were readily detected in the absence of the catalytic subunit (*rnh201*Δ; Figure 2A). All subsequent experiments were performed in an *rnh201*Δ background. Although the total rate of Lys$^+$ revertants was only 2-fold higher in the OPPO than in the SAME orientation, the proportion of mutants with a 2-bp deletion in the $(TG)_2$ hotspot increased from 10% in the SAME to 67% in the OPPO orientation (6/61 and 36/54, respectively). The rate of deletions at the $(TG)_2$ hotspot was obtained by multiplying the total Lys$^+$ rate by the proportion of hotspot deletions in the corresponding spectrum; the 2-bp deletion rate was thus 16-fold higher in the OPPO than in the SAME orientation (Figure 2A). No hotspot activity was observed in the *rnh201*Δ *top1*Δ OPPO strain (Supplementary Table S2), confirming that rNMP-dependent deletions are also Top1 dependent.

In addition to reversing the leading- and lagging-strand templates by inverting the orientation of the entire *lys2* reporter relative to *ARS306*, we also inverted only the hotspot-containing fragment [$(TG)_2$-inv alleles] within the SAME and OPPO reporters. As illustrated in Figure 1, $(TG)_2$-inv SAME is equivalent to $(TG)_2$ OPPO in terms of the $(TG)_2$-containing strand being synthesized by Polϵ during leading-strand synthesis; in $(TG)_2$-inv OPPO and $(TG)_2$ SAME, the complementary $(CA)_2$ sequence is synthesized by Polϵ. Consistent with results obtained with the

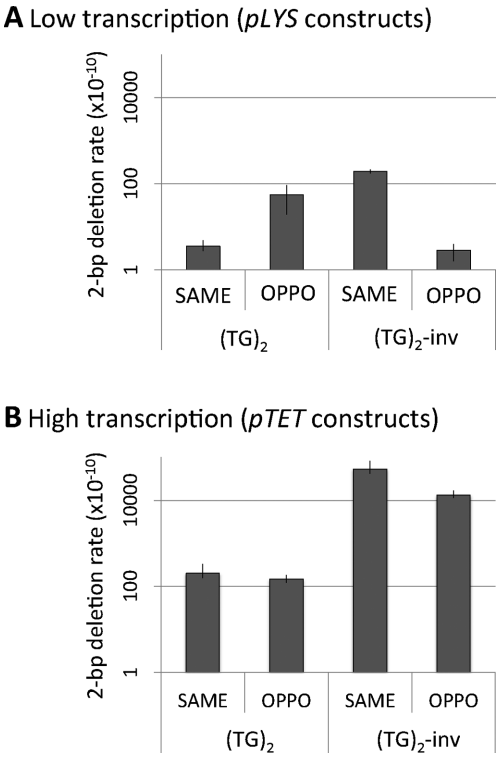


Figure 2. Effects of transcription on 2-bp deletions in the $(TG)_2$ hotspot. All experiments were performed in an *rnh201*Δ background. Under low-transcription conditions (panel A), deletion rates are high when $(TG)_2$ is on the nascent leading strand of replication. Under high-transcription conditions (panel B), deletion rates are high when $(TG)_2$ is on the NTS of the reporter.

$(TG)_2$ constructs, the rate of 2-bp deletions at the hotspot was 67-fold higher for $(TG)_2$-inv when it was in the SAME than when it was in the OPPO orientation (Figure 2A). This was evident both in terms of the overall Lys^+ reversion rate (6-fold difference) and in the proportion of mutants at the hotspot (31/43 and 3/47 for the SAME and OPPO, respectively). Finally, introduction of the rNTP-restrictive *pol2-M644L* allele into $(TG)_2$-inv SAME and $(TG)_2$ OPPO strains reduced 2-bp deletions approximately 50-fold (Supplementary Table S1), confirming that the relevant rNMP was introduced by Polε during leading-strand synthesis. Although these experiments were designed to recapitulate the earlier replication-related observations using our specific reporter, it is important to note that, in addition to switching leading- and lagging-strand specificities, inversion of just the hotspot-containing fragment also moves the $(TG)_2$ sequence from the TS to the NTS of the reporter.

High transcription eliminates the replication-associated bias at the $(TG)_2$ hotspot

The effect of transcription on deletions at the $(TG)_2$ hotspot was examined by placing the four *lys2* alleles shown in Figure 1 under control of the highly active *TET* promoter (*pTET*). Previous analyses demonstrated that the direction of replication through *LYS2* has little, if any, effect on *pTET* activity (28). As observed under low-transcription conditions (*pLYS* constructs), 2-bp deletions were highly

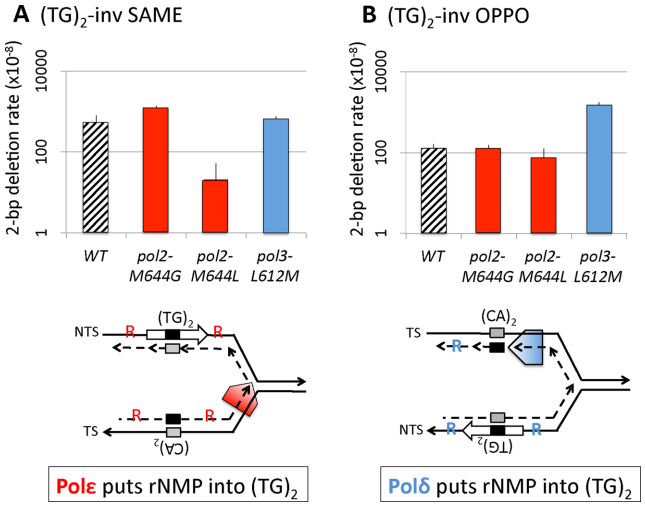


Figure 3. Effects of DNA polymerases with altered rNTP permissiveness on 2-bp deletions. Details of the substrate cartoons are provided in the Figure 1 legend. Polε and Polδ are indicated by the red and blue pentagons, respectively, and their inserted rNMPs (R's) are similarly color-coded.

elevated in an *rnh201*Δ background (Supplementary Table S3), confirming rNMP dependence; all subsequent experiments were done in an *rnh201*Δ background. Our expectation was that, under high-transcription conditions, the distinctive replication bias for 2-bp deletions would be maintained and elevated transcription thus would have a similar stimulatory effect on 2-bp deletions within each construct. In contrast to this prediction, however, the replication-associated bias observed for the $(TG)_2$ allele was completely eliminated and that for the $(TG)_2$-inv allele was reduced 10-fold under high-transcription conditions (Figure 2B). The net result was that the effect of high transcription varied three orders of magnitude among the four constructs, from only 3-fold for $(TG)_2$ OPPO to 4600-fold for $(TG)_2$-inv OPPO. Importantly, for either direction of replication through the *pTET-lys2* reporter, deletion rates in the $(TG)_2$-inv constructs were much higher than in the corresponding $(TG)_2$ constructs. The transcription-associated mutation bias thus correlated with whether the site of Top1 cleavage [$(TG)_2$] was located on the TS or on the NTS of the reporter, with the rate being several orders of magnitude higher when $(TG)_2$ was on the NTS.

Mutagenic rNMPs on the NTS of $(TG)_2$-inv constructs are inserted by Polδ as well as Polε

The data in Figure 2B demonstrate that highly elevated transcription eclipses the replication-associated bias in hotspot activity observed under low-transcription conditions. This suggests that, in contrast to low-transcription conditions where only rNMPs inserted by Polε are mutagenic, the Polδ lagging-strand polymerase can be the primary source of mutagenic rNMPs under high-transcription conditions. We confirmed this for the $(TG)_2$-inv alleles using mutant forms of Polε and Polδ that incorporate either fewer or more rNMPs than the WT polymerases (Figure 3). The catalytic subunit of Polε is encoded by *POL2*; the *pol2-M644G* and *pol2-M644L* alleles render the en-

zyme more and less rNTP permissive, respectively, than WT during leading-strand synthesis (3). The catalytic subunit of Polδ is encoded by *POL3*; the *pol3-L612M* allele renders Polδ more rNMP-permissive than WT and is associated with increased rNMP-incorporation during lagging-strand synthesis (24). In experiments with the $(TG)_2$-inv SAME reporter (Top1 cleavage site on the NTS/leading strand), where Polε is expected to incorporate rNMPs into the $(TG)_2$-containing strand, 2-bp deletions were elevated ∼2-fold in the rNMP-permissive *pol2-M644G* background and reduced ∼50-fold in the rNMP-restrictive *pol2-M644L* background (Figure 3A). There was no significant increase in the deletion rate in the rNMP-permissive *pol3-L612M* background. For the $(TG)_2$-inv OPPO allele (Top1 cleavage site on the NTS/lagging strand), however, there was a 13-fold increase in the rate of 2-bp deletions in the rNMP-permissive *pol3-L612M* background, while altering the rNMP permissiveness of the Polε leading-strand polymerase had no significant effect on mutagenesis (Figure 3B). This confirms that the mutation-initiating rNMPs were inserted into the $(TG)_2$-containing NTS strand of the $(TG)_2$-inv OPPO construct by the Polδ lagging-strand polymerase. Thus, in contrast to the strong, Polε-specific bias observed under low-transcription conditions, hotspot activity is strongly biased to the NTS under high-transcription conditions and either Polδ or Polε can be the primary source of the mutagenic rNMPs.

Neither biased repair nor R-loops account for the NTS bias for 2-bp deletions

The data presented above suggest that, relative to the NTS, the TS of a highly transcribed gene is a very poor substrate for the rNMP-dependent mutagenesis initiated by Top1. One possible explanation for this bias is that cleavage of the NTS by Top1 is more efficient than cleavage of the TS. Alternatively, it is possible that the cleavage efficiency of the two strands is similar, but that incision of the NTS is much more likely to produce the 2-bp deletions detected by our reporter constructs. This could reflect either some feature of the NTS strand that promotes mutagenesis and/or some feature of the TS that limits mutagenesis.

There are two well-characterized asymmetries between the DNA strands during transcription: DNA damage on the TS is repaired more efficiently and the TS can stably hybridize to the transcript to form an R-loop (Figure 4). With respect to asymmetric repair, the nucleotide excision repair (NER) pathway is triggered when RNAPII stalls at a lesion on the TS, resulting in more efficient repair of lesions on the TS than on the NTS (35). Either a Top1 cleavage complex (Top1cc) or a Top1-generated nick on the TS would be expected to stall RNAPII. To examine whether transcription-coupled NER (TC-NER) limits mutagenesis when Top1 cleaves the TS, we deleted the *RAD14* gene, the product of which is essential for NER [reviewed in (36)]. If TC-NER were solely responsible for preventing mutagenesis initiated by Top1 cleavage of the TS, then we would predict that a similar rate of 2-bp deletions for all constructs in a *rad14*Δ background. That is, the relatively low deletion rates for the $(TG)_2$ constructs (Figure 2B) should be greatly elevated and should be similar to those observed with the

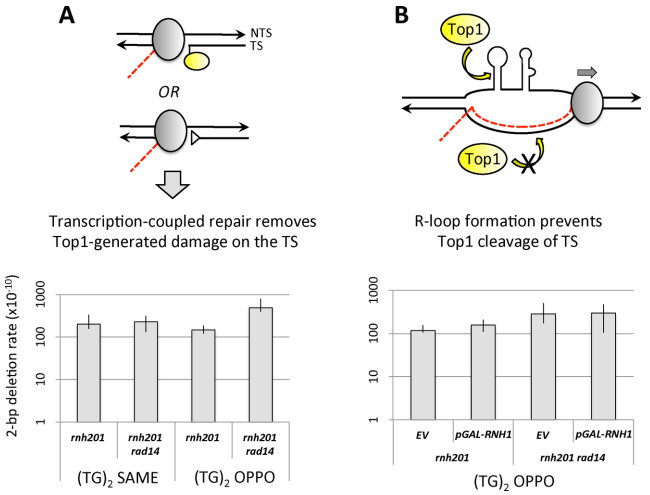


Figure 4. Neither TC-NER nor R-loop formation limits mutagenesis on the TS under high-transcription conditions. In the cartoons shown, the gray and yellow ovals represent RNAPII and Top1, respectively; DNA and RNA are black and dotted red lines, respectively. In panel (**A**), the triangle corresponds to the 2′,3′ cyclic phosphate that results from Top1 incision at an rNMP. EV, empty vector.

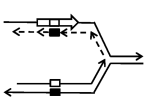
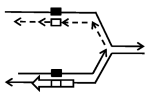
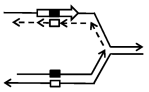
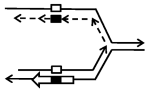
corresponding $(TG)_2$-inv constructs. There was no effect of Rad14 loss on 2-bp deletions in the $(TG)_2$ SAME construct, however, and only a modest, 3.3-fold increase in the rate of 2-bp deletions in $(TG)_2$ OPPO construct (Figure 4A and Supplementary Table S3). With regard to the latter, the rate of 2-bp deletions was still at least 30-fold less than with the corresponding $(TG)_2$-inv constructs, leading us to conclude that TC-NER is not responsible for the transcription-related asymmetry.

Within an R-loop, the TS is base-paired with the transcript and such RNA:DNA hybrids are not a substrate for Top1 (16). Although the single-stranded NTS within an R-loop would also not be a substrate for Top1 (37), it could potentially fold into secondary structures that can be cleaved by the enzyme (38). We examined whether R-loops limit mutagenesis initiated by Top1 cleavage of the TS by overproducing RNase H1, which degrades R-loops in yeast. Overproduction of RNase H1 did not significantly alter the rate of 2-bp deletions in the $(TG)_2$ OPPO construct in either the presence or absence of Rad14 (Figure 4B and Supplementary Table S4). Our data thus indicate that neither R-loops nor biased repair is responsible for the transcription-related strand bias in Top1-dependent mutagenesis.

DISCUSSION

Top1 relieves torsional stress associated with transcription and replication, and Top1-dependent deletions that reflect incision at rNMPs are associated with both processes. Although both Polδ and Polε insert rNMPs at a low level during replication, only those inserted by the Polε leading-strand DNA polymerase are mutagenic (24). A favored explanation for the replication-associated asymmetry is that Top1 is needed to remove supercoils that accumulate on the leading strand behind the replication fork; nicks that are naturally generated during lagging-strand synthesis can serve a similar role. For the $(TG)_2$ hotspot used here, syn-

Table 1. Summary of rNMP-dependent 2-bp deletions rates in the $(TG)_2$ hotspot

Construct	$(TG)_2$ Location		2-bp deletion rate relative to $(TG)_2$ SAME, low txn		High/low transcription
	Replication	Transcription	Low txn	High txn	
$(TG)_2$ SAME 	nascent lagging	TS	1.0X	57X	57X
$(TG)_2$ OPPO 	nascent leading	TS	15X	41X	2.7X
$(TG)_2$-inv SAME 	nascent leading	NTS	54X	15 000X	280X
$(TG)_2$-inv OPPO 	nascent lagging	NTS	0.8X	3700X	4600X

The open arrow corresponds to the *LYS2* coding sequence and indicates the direction of transcription. Filled and open boxes correspond to the $(TG)_2$ and $(CA)_2$ sequence, respectively.

thesis of the $(TG)_2$-containing strand by Polε is associated with rNMP-dependent, 2-bp deletions at a level that is orders of magnitude higher than when Polε synthesizes the complementary $(CA)_2$-containing strand (4). This behavior specifically identifies the $(TG)_2$-containing strand as the target of Top1-dependent mutagenesis *in vivo*.

Top1-dependent mutagenesis that occurs in the context of transcription can be rNMP dependent or independent (17), and the primary goal of the current study was to examine transcriptional effects on rNMP-dependent mutagenesis. The $(TG)_2$ hotspot was thus moved into a reporter that (i) efficiently detects 2-bp deletions, (ii) can be transcribed at either a low or high level and (iii) was inserted in both orientations relative to a strong replication origin (*ARS306*). A unique aspect of our system is that, in addition to inverting the entire reporter relative to a replication origin (SAME and OPPO constructs), we inverted only the hotspot-containing fragment within the reporter [$(TG)_2$ and $(TG)_2$-inv constructs]. Each type of inversion switches the $(TG)_2$ sequence between the leading and lagging strands of replication, but the hotspot-only inversion additionally switches $(TG)_2$ between the TS and NTS of the reporter (see Figure 1). Data obtained using these constructs are summarized in Table 1 and discussed below.

Under low-transcription conditions, we recapitulated and extended results obtained when the $(TG)_2$ hotspot was at its natural position within *URA3* (4). The effect of replication was evident with both the $(TG)_2$ and $(TG)_2$-inv constructs, confirming that deletions occur when the $(TG)_2$-containing strand is synthesized by the Polε leading-strand polymerase. Given the high sensitivity of our assay for 2-bp deletion formation, we were able to more accurately quantitate the magnitude of the replication effect. With the $(TG)_2$ construct, the direction of replication had a 15-fold effect; with the $(TG)_2$-inv construct, the effect was ∼70-fold.

Unexpectedly, the replication-associated bias for the $(TG)_2$ hotspot was abolished when the reporter was highly transcribed. In contrast to low-transcription conditions, the rate of 2-bp deletions correlated with whether the $(TG)_2$ sequence was on the TS or the NTS of the highly transcribed reporter. When the $(TG)_2$ sequence was on the NTS, deletion rates were several orders of magnitude higher than when it was on the TS (Table 1). Replacing a leading-strand bias under low-transcription conditions with an NTS bias under high-transcription resulted in a highly variable effect of transcription on mutagenesis. As summarized in the final column of Table 1, the stimulatory effect of transcription on 2-bp deletions was only 3-fold for the $(TG)_2$ OPPO construct, but almost 5000-fold for $(TG)_2$-inv OPPO. The observation that mutagenesis is driven by the location of the $(TG)_2$ sequence on the TS versus NTS of the reporter indicates that the mutagenic rNMP can be inserted by the lagging- as well as the leading-strand DNA polymerase. We confirmed this for the $(TG)_2$-inv OPPO construct, where altering the rNTP permissiveness of Polδ, but not that of Polε, affected the deletion rate. This provides the first example of rNMP-dependent mutagenesis driven by rNMPs inserted by Polδ rather than by Polε.

The finding that rNMPs inserted by Polδ initiate mutagenesis under high-transcription conditions is consistent with (i) high levels of transcription creating a new, Polδ-dependent rNMP insertion site that does not exist under low-transcription conditions, (ii) high levels of transcription increasing the amount of rNMPs incorporated by Polδ, and/or (iii) high levels of transcription recruiting Top1 to DNA replicated by either DNA polymerase. Unscheduled DNA synthesis that occurs outside of S phase, when the rNTP:dNTP ratio is elevated, is most likely catalyzed by Polδ. This does not appear to be the primary source of rNMPs inserted by Polδ, however, as we were unable to detect a change in rNMP levels in our *lys2* reporter under high-transcription conditions (data not shown). We also think it is unlikely that high transcription modifies the sites of rNMP insertion because the positions of hotspots in a forward mutation assay are similar under low- and high-transcription conditions (11,17). We suggest that Polδ inserts the same amount of rNMPs irrespective of transcription level, but that Top1 efficiently incises at rNMPs incorporated by Polδ only under high transcription conditions. Increased recruitment of Top1 to highly transcribed genes is a well-established phenomenon and may partially reflect interaction with the phosphorylated C-terminal domain of RNAPII (13).

The most striking aspect of the data reported here is that under high-transcription conditions, Top1-dependent deletions occur much more frequently when its target site is on the NTS than when on the TS of the reporter. It should be noted that this bias is not limited to the $(TG)_2$ hotspot, and has been observed for at least one other hotspot following transplantation into the *lys2*-based reversion assay (data not shown). Transcription establishes two well-characterized asymmetries between the two DNA strands:

repair occurs more efficiently on the TS and the TS can be extensively paired with the transcript as part of an R-loop. The former might eliminate premutagenic lesions specifically on the TS while the latter would preclude cleavage of the TS by Top1. Neither elimination of an essential NER protein (*rad14*Δ mutant) nor overexpression of RNase HI, however, stimulated deletions when the $(TG)_2$ target site was on the TS.

The basis of the distinctive NTS bias for hotspot activity under high-transcription conditions remains an enigma. One possibility is that Top1 may have a strong preference for cleaving the NTS relative to TS of a highly active gene. How this might occur is unclear, but one intriguing possibility is that the bias might be related to the interaction of Top1 with elongating RNAPII (13). Two activities have been implicated in limiting rNMP-dependent mutagenesis, and either might limit mutagenesis in a strand-specific manner. First, processing of a trapped Top1 cleavage complex by Tdp1/Tpp1 promotes error-free removal of rNMPs and precludes deletion formation *in vitro* (18), but whether a similar mechanism operates *in vivo* is not known. Second, the Srs2 helicase cooperates with Exo1 to prevent rNMP-dependent mutagenesis by removing the 5′-OH generated by the initial Top1 cleavage (39). An additional possibility is that a Top1-dependent deletion intermediate arising on TS is more efficiently converted into a double-strand break (DSB) during replication. Such a DSB would be repaired via homologous recombination with the sister chromatid, which is a genetically silent event. Finally, the sequential-cleavage model of Top1-dependent mutagenesis requires that the strand covalently bound by Top1 realign with respect to the complementary strand. This realignment converts a 2-nt gap into a nick with a 5′-OH positioned correctly for efficient Top1 ligation. If the Top1cc is attached to the NTS, RNAPII might 'push' the Top1cc *toward* the 5′-OH to drive realignment and ligation. When the Top1cc is on the TS, however, RNAPII would push Top1 *away* from 5′-OH and thereby preclude the final ligation reaction. Regardless of the underlying molecular mechanism, the results presented here further extend the range of transcriptional effects on genetic stability, and underscore the complexity of factors that together produce a highly dynamic mutation landscape across the eukaryotic genome.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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