Topoisomerase I Alone Is Sufficient to Produce Short DNA Deletions and Can Also Reverse Nicks at Ribonucleotide Sites*  

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Background: DNA deletions at short repeat sites containing ribonucleotides are Top1-dependent.  
Results: Top1 generates DNA deletions at short repeat sites in vitro and also reverses nicks at ribonucleotide sites.  
Conclusion: The impact of Top1 on the ribonucleotide sites is both negative and positive.  
Significance: We elucidate the detailed mechanism of Top1 interacting with ribonucleotide sites, the most frequently misincorporated non-canonical nucleotides.

Ribo nucleotide monophosphates (rNMPs) are among the most frequent form of DNA aberration, as high ratios of ribonucleotide triphosphate:deoxyribonucleotide triphosphate pools result in approximately two misincorporated rNMPs/kb of DNA. The main pathway for the removal of rNMPs is by RNase H2. However, in a RNase H2 knock-out yeast strain, a topoisomerase I (Top1)-dependent mutator effect develops with accumulation of short deletions within tandem repeats. Proposed models for these deletions implicated processing of Top1-generated nicks at rNMP sites and/or sequential Top1 binding, but experimental support has been lacking thus far. Here, we investigated the biochemical mechanism of the Top1-induced short deletions at the rNMP sites by generating nicked DNA substrates bearing 2',3'-cyclic phosphates at the nick sites, mimicking the Top1-induced nicks. We demonstrate that a second Top1 cleavage complex adjacent to the nick and subsequent faulty Top1 religation led to the short deletions. Moreover, when acting on the nicked DNA substrates containing 2',3'-cyclic phosphates, Top1 generated not only the short deletion, but also a full-length religated DNA product. A catalytically inactive Top1 mutant (Top1-Y723F) also induced the full-length products, indicating that Top1 binding independent of its enzymatic activity promotes the sealing of DNA backbones via nucleophilic attacks by the 5'-hydroxyl on the 2',3'-cyclic phosphate. The resealed DNA would allow renewed attempt for repair by the error-free RNase H2-dependent pathway in vivo. Our results provide direct evidence for the generation of short deletions by sequential Top1 cleavage events and for the promotion of nick religation at rNMP sites by Top1.

The chemical stability of deoxyribonucleotide monophosphates makes them the preferred nucleic acids for storing genetic information. DNA replicative polymerases are highly selective for the deoxyribonucleotides over ribonucleotides due to the steric hindrances present at their substrate-binding sites. However, the selectivity of the DNA polymerases for the correct sugar structures is not absolute, which could be further exacerbated in some cancers bearing mutations in the replicative polymerases (1–4). Combined with the vast excess of ribonucleotide triphosphate over deoxyribonucleotide triphosphate pools, ribonucleotides are frequently misincorporated into the genome during DNA synthesis (5–7). Based on recent estimates, ribonucleotide monophosphates (rNMPs)2 are the most frequently misincorporated non-canonical nucleotides during replication (for review, see Refs. 8 and 9).

The majority of the misincorporated rNMPs are removed by a repair pathway termed ribonucleotide excision repair (RER), which is initiated by RNase H2 nicking the DNA backbone on the 5'-side of the rNMPs (6, 10–12). In addition, nucleotide excision repair has been implicated as backup pathway for rNMP removal (13, 14). Genetic studies in yeast and mammalian cells show that blocking RER by inactivating RNase H2 leads to heightened cellular distress and genomic instability (10, 15).

The phenotypes exhibited by yeast strains harboring high levels of genomic rNMPs include DNA deletion at hot spots consisting of short repeats, increased rate of recombination, and replication stress (6, 10). Strikingly, inactivating DNA topoisomerase I (Top1) in these yeast strains reverses these phenotypes (10, 16–18), firmly implicating processing of rNMPs by Top1 in the observed cellular responses.

Top1 is an ubiquitous enzyme that relaxes DNA supercoiling induced by DNA replication, transcription, and chromatin remodeling (19–21). The catalytic mechanism of Top1 involves a reversible transesterification reaction, initiated by the nucleophilic attack by the active-site tyrosine (Tyr-723 for human Top1) on the scissile phosphate, generating a DNA nick and a covalent protein-DNA complex (termed the Top1 cleavage complex (Top1cc)). The Top1cc allows the nicked duplex to

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2The abbreviations used are: rNMP, ribonucleotide monophosphate; RER, ribonucleotide excision repair; Top1cc, Top1 cleavage complex(es); CIP, calf intestinal alkaline phosphatase; nt, nucleotide(s).
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Protein Expression and Purification—Human recombinant Top1 and Top1-Y723F were purified using a baculovirus expression system from Sf9 insect cells as described previously (26). Human recombinant tyrosyl-DNA phosphodiesterase 1 (TDP1) was overexpressed and purified from *Escherichia coli* as described previously (27). Overexpression and purification of N-terminally His<sub>10</sub>-tagged *E. coli* RNA 3′-terminal phosphate cyclase (RtcA) were carried out essentially as described previously with minor modifications (28). Briefly, pET16b-RtcA (2–339) was transformed into BL21(DE3) competent cells and grown in LB medium containing 100 µg/ml ampicillin to *A<sub>600</sub> = 0.6–0.8 before the culture was chilled and grown for another 16 h in the presence of 0.1 mM isopropyl β-D-1-thiogalactopyranoside and 2% (v/v) ethanol at 17°C. All subsequent procedures were performed at 4 °C. The cells were harvested, and the pellet was resuspended in buffer A (50 mM Tris–HCl (pH 7.4), 250 mM NaCl, 2 mM 2-mercaptoethanol, and 10% sucrose supplemented with Complete EDTA-free protease inhibitor mixture tablets (Roche Applied Science). Lysozyme was added to the concentration of 0.2 mg/ml, and the mixture was allowed to gently mix at 4 °C for 1 h before sonication, followed by clarification by ultracentrifugation at 30,000 × g for 45 min. The soluble lysate was loaded on a HiTrap HP column (GE Healthcare) precharged with nickel ions and pre-equilibrated with buffer A. The column was washed first with buffer A and then with wash buffer (buffer A with the addition of 25 mM imidazole) before elution with a gradient of 0–500 mM imidazole, with the protein peak eluting at 340 mM imidazole. The protein peak fractions (>95% pure assayed by SDS-PAGE) were buffer-exchanged into storage buffer (10 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, and 10 mM ATP) and concentrated using centrifugal filters (Millipore). The protein sample was brought to a final concentration of 50% (v/v) glycerol and stored at −80 °C.

**Generation of 2′,3′-CyclicPhosphate Ends**—The 12-mer oligonucleotide bearing a 3′-terminal ribonucleotide and a 3′-phosphate with sequence 5′-AGCGTTGAAGArU-P (final concentration of 4 µM) was incubated with RtcA (final concentration of 0.1 µg/µl) in buffer containing 50 mM Tris–HCl (pH 7.4), 10 mM MgCl<sub>2</sub>, 200 µM ATP, and 2 mM DTT at 37 °C for 30 min. Calf intestinal alkaline phosphatase (CIP; 1 unit/µl) was added to the samples where indicated, and the reaction mixtures were further incubated at 37 °C for 30 min before the addition of 1 volume of gel loading buffer (96% (v/v) formamide, 10 mM EDTA, 1% (w/v) xylene cyanol, and 1% (w/v) bromphenol blue) to terminate the reaction. The resulting products were analyzed on a 20% denaturing polyacrylamide gel and stained with SYBR Gold (Life Technologies, Inc.).

**DNA Substrates**—Custom-synthesized oligonucleotides (Midland Certified Reagent Co. and Integrated DNA Technologies, Inc.) were either 3′- or 5′-labeled prior to annealing with the unlabeled complementary strand at a 1:1 ratio. For nicked DNA substrates, RtcA- or mock-treated oligonucleotides with different 3′-ends were passed through mini Quick Spin Oligo columns (Roche Applied Science) before 5′-labeling with [γ-<sup>32</sup>P]ATP (PerkinElmer Life Sciences) by T4 polynucleotide kinase (3′-phosphatase minus, New England Biolabs). After passing through the mini Quick Spin Oligo columns to remove residual [γ-<sup>32</sup>P]ATP, the 5′-radiolabeled oligonucleotides were annealed to unlabeled complementary strands 5′-ATGTGGC-AAAACCTTTGTCACAAGGGT-T-CTCACGCT at a 1:1:1 ratio.

**In Vitro Top1 Cleavage Assays, Alkaline and RNase H2 Treatments**—The *in vitro* Top1 cleavage assays were carried out as described previously (29). The reaction mixture contained 50–200 nM labeled double-stranded or nicked DNA construct and 70 or 140 nM human recombinant Top1. The reaction mixture also contained 10 µM camptothecin or a 2-fold serial dilution of human recombinant TDP1 (1–0.125 µM) where indicated. The reaction was stopped by the addition of 0.5% SDS (final concentration) after incubation for 1 h at 25 °C. For the alkaline treatments, nicked DNA constructs containing 2′,3′-cyclic phosphate ends were allowed to react with 70 or 140 nM Top1 for 1 h at 25 °C before the reactions were stopped by the addition of 0.5% SDS (final concentration). The samples were then split in two equal parts, and one part of the reaction mixture was further treated with 0.3 M KOH (final concentration) at 55 °C for 1 h. Alternatively, RNase HII (New England Biolabs) at 0.05 units/µl (final concentration) was added to the reaction mixture and further incubated at 25 °C for 15 min. All reactions were stopped by the addition of 1 volume of gel loading buffer and analyzed on 20% sequencing gels.

**Results**

**Top1 Binding at rNMP Sites Generates DNA Nicks with 2′,3′-Cyclic Phosphate Ends**—To study the mechanism of Top1-dependent DNA deletions at short repeat sites on a high rNMP background, we focused on the (AT)<sub>2</sub> hot spot from the *CANI* gene, which exhibits a strong Top1-dependent deletion signature (16–18). We carried out Top1 cleavage assays with DNA constructs containing the (AT)<sub>2</sub> hot spot (Fig. 1A) in the absence and presence of camptothecin, a highly selective and potent Top1 poison (20, 30). The constructs contained dT, dU, or rU at the first dT position within the (AT)<sub>2</sub> hot spot (the
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X-position in Fig. 1A; the designation of the Top1 sites on the (AT)₂ hot spot is adopted from our previous study (16), indicating the position of the phosphotyrosyl linkage in the Top1cc.

With DNA constructs 3'-labeled on the transcribed strand, the Top1 site (site f) was greatly enhanced when rU was present at the X-position independently of camptothecin (Fig. 1B, lane 9). By contrast, the Top1 site (site f) was barely visible with either dT or dU at the X-position (Fig. 1B, lanes 3 and 6), although the addition of camptothecin enhanced Top1 cleavage at site f (lanes 4 and 7). This indicated that although the Top1cc at site f was susceptible to camptothecin trapping, the 2'-hydroxyl at the X-position was capable of enhancing site f cleavage independently of camptothecin. Furthermore, the addition of camptothecin promoted other Top1 sites on the same construct, competing with site f under our experimental conditions.

S₀₂ attack on the scissile phosphate of a Top1cc by the 2'-hydroxyl at ribonucleotide sites results in a nick in the DNA backbone, accompanied by the release of Top1 (see Fig. 5, inset) (16, 24). We confirmed that the Top1-induced product with rU at the X-position was an enzyme-free DNA nicked at site f by performing Top1 cleavage assay with substrates radiolabeled at the 5'-end of the transcribed strand (Fig. 1C). The appearance of the Top1-induced band corresponding to a 12-nucleotide (nt) piece of DNA bearing 2',3'-cyclic phosphates (termed 12CP) indicates that Top1 was not covalently linked to the product (Fig. 1C). Accordingly, the product resulting from Top1 interaction with rU at the X-position was a nicked DNA with the 2',3'-cyclic phosphate at the nick site (upper panel in Fig. 2A, with the 2',3'-cyclic phosphate indicated by the inverted red triangle) (24, 31).

We reasoned that subsequent Top1 binding to the nicked DNA substrate likely led to formation of additional Top1cc (25, 32), which did not enter the gel due to the covalent linkage of Top1 to the 3'-end of the DNA. The appearance of the band at the bottom of the well in the presence of Top1 confirmed this (Fig. 1D, lane 2, Top1cc band). We took advantage of the fact that TDP1 directly hydrolyzes such complexes (33, 34) to map the position of the phosphotyrosyl linkage on the substrate. Although TDP1 does not resolve intact Top1cc efficiently (35), the amount of Top1cc decreased with increasing TDP1 (Fig. 1D, lanes 2-6, Top1cc band). Most importantly, TDP1 generated two products in a dose-dependent manner, corresponding to the Top1cc immediately upstream from the ribonucleotide-induced nick, at sites h and i (Fig. 1, A and D), with the Top1cc at site i being the dominant product.

Generation of Substrates Containing 2',3'-Cyclic Phosphate—To investigate how a nicked DNA with 2',3'-cyclic phosphates leads to short deletions (Fig. 2A), we sought to generate DNA substrates mimicking Top1-induced nicked DNA at rNMP sites. Although Top1 readily generated 2',3'-cyclic phosphates, such products required gel purification, and the 2',3'-cyclic phosphates were not sufficiently stable through the lengthy purification steps. To circumvent such complex and

**FIGURE 1.** **Top1 induces DNA nicks at rNMP sites and generation of 2',3'-cyclic phosphates.** A, sequence of the DNA construct containing the (AT)₂ short repeat site from the CANT reporter gene is shown. The (AT)₂ short repeat site is marked by the gray box. The top strand is the transcribed strand (TS), and the bottom strand is the non-transcribed strand (NTS). The transcribed strand was radiolabeled at the 3'-end (for B) or at the 5'-end (for C and D). Five Top1 sites in the transcribed strand are marked e–i, indicating the positions of the phosphotyrosyl linkage in the Top1cc. B, representative Top1 cleavage assay with the construct sequence (3'-labeled on the transcribed strand) shown in A resolved on a denaturing sequencing gel. The nucleotide at the X-position is dT, dU, or rU. Top1-induced cleavage products are labeled e, f, and g (annotated with their respective length), corresponding to the positions shown in A. CPT, camptothecin. C, representative Top1 cleavage assay with the construct sequence (5'-labeled on the transcribed strand) shown in A containing rU at the X-position. The reactions contained a 2-fold serial dilution of human recombinant TDP1 (1–0.125 μM). Top1cc bands correspond to the bottom of the gel wells. Top1 sites f, h, and i are annotated with their respective length. TDP1-generated products bear 3'-phosphate ends as indicated.
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![Diagram of Top1 reaction](image)

**FIGURE 2. Generation of 2',3'-cyclic phosphates.** A, scheme depicting the generation of the 2-nt deletion from nicked DNA substrates containing 2',3'-cyclic phosphate ends (shown as an inverted red triangle). TS, transcribed strand; NTS, non-transcribed strand. B, biochemical scheme of the RtcA reaction. RtcA generates a 2',3'-cyclic phosphate end (12CP) from ssDNA with the 3'-ribonucleotide containing a 3'-phosphate group (12rUP). The resulting 2',3'-cyclic phosphate (12CP) is not susceptible to digestion by CIP, whereas the 3'-phosphate group on the 3'-ribonucleotide is. C, CIP digestion verified the completion of 2',3'-cyclic phosphate generation. The ssDNA with the 3'-ribonucleotide (12rU) was not susceptible to CIP. The same DNA construct with an additional 3'-phosphate group (12rUP) was dephosphorylated by CIP, generating 12rU, as demonstrated by the decreased migration mobility on the sequencing gel. However, the product generated by RtcA from 12rUP was completely resistant to CIP treatment, as shown by the unaltered migration mobility, indicating that conversion of 12rU to 12CP by RtcA was complete. To produce the DNA constructs mimicking Top1-induced DNA nicks (as shown in A), we used RtcA to generate a 12-nt piece of DNA bearing 2',3'-cyclic phosphates (12CP), followed by annealing with the complementary DNA strands.

inefficient purification steps, we took advantage of the fact that RtcA efficiently converts a ssDNA with a 3'-rU and 3'-phosphate group (termed 12rUP) into a 2',3'-cyclic phosphate (Fig. 2B) (36). Because 2',3'-cyclic phosphates are resistant to dephosphorylation by CIP (24), CIP digestion confirmed complete conversion of the substrates by RtcA into ssDNA bearing 2',3'-cyclic phosphates (12CP) under our experimental conditions (Fig. 2C). The resulting 12CP was then 5'-radiolabeled before annealing to the unlabeled complementary strands to produce the nicked DNA constructs mimicking nicked DNA generated by Top1 at ribonucleotide sites (upper panel in Fig. 2A), with the 2',3'-cyclic phosphate indicated by the inverted red triangle.

**Sequential Binding of Top1 Induces the 2-nt Deletion at the (AT)_2 Repeat Site**—To examine whether Top1 alone is able to generate the 2-nt deletion by acting on the substrates containing 2',3'-cyclic phosphates (Fig. 2A), we allowed Top1 to react with substrates bearing different 3'-ends at the nicked site (Fig. 3A, Nicked-12X). Using these nicked DNA substrates allowed us to focus on the subsequent events induced by Top1 after the initial nicking at the nRNMP sites. As shown in Fig. 3A, the top strand (transcribed) of the (AT)_2 hot spot construct was split into a 5'-radiolabeled 12-nt piece bearing different 3'-ends and an 18-nt piece bearing a 5'-hydroxyl. Therefore, any product longer than 12 nt represented a religation product.

The addition of Top1 to all nicked DNA constructs generated Top1cc (Fig. 3B, Top1cc band, corresponding to the bottom of the gel wells) (21, 37). These Top1cc constructs initiate complexes generated by Top1 binding to the 5'-side of the existing nick (32). In addition, we observed a Top1-induced 28-nt product with all substrates tested. Because direct sealing of the DNA nicks would generate a 30-nt (12 + 18 nt) product, the 28-nt product represents a faulty religation product with a 2-nt deletion. We hypothesized that the second Top1cc 2 nt upstream from the nicked site (site h in Fig. 1D) was responsible for the observed 2-nt loss. Even though the Top1cc formed at site i was more abundant than that at site h, the Top1cc at site i appeared inefficient in religating across the gap, as few if any 27-nt products were generated (Fig. 3B). Top1 induced varying amounts of the 28-nt products in the different substrates, which were the most abundant when the 3'-end was a deoxyribose without phosphate (termed Nicked-12dT) (Fig. 3B, lanes 2 and 3). The difference in local structures of the various 3'-ends at the DNA nicks most likely modulated the occurrence of the Top1cc at site h.

**Top1 Binding at DNA Nicks with 2',3'-Cyclic Phosphates Promotes Religation**—In the case of the construct bearing 2',3'-cyclic phosphate at the nicked site (termed Nicked-12CP), Top1 induced, in addition to the 2-nt deletion (28-nt) product, a prominent 30-nt product (Fig. 3B, lanes 14 and 15). The same construct also generated a small amount of 30-nt products in the absence of Top1 (Fig. 3B, lane 13), indicating that 2',3'-cyclic phosphates are susceptible to direct nucleophilic attack by the 5'-hydroxyl at the DNA nicks (38, 39). However, the presence of Top1 substantially enhanced the formation of the 30-nt product.

We reasoned that the binding of the Top1 enzyme to Nicked-12CP at site f potentially promoted a favorable alignment of the 2',3'-cyclic phosphate and the 5'-hydroxyl for nucleophilic attack (see Fig. 5, inset). Therefore, we tested the ability of the catalytically inactive Top1-Y723F mutant enzyme (20, 40) to
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Figure 3. Induction of both short deletions and religation by Top1 at rNMP misincorporation sites. A, nicked DNA constructs (Nicked-12X) containing the (AT)2 short repeat site from the CAN1 reporter gene (see upper panel in Fig. 2A). The length of each of the three fragments constituting the nicked DNA substrate is indicated. The 5′-end of the 12-mer was radiolabeled, and each construct contained a different 3′-end (A). The structures of the different 3′-ends are illustrated on the right. TS, transcribed strand. B, Top1-induced religation of nicked DNA substrates bearing the indicated 3′-ends (Nicked-12X). The nicked DNA constructs shown in A were reacted with two different concentrations of Top1 (70 or 140 nM) until reaching equilibrium and then resolved on denaturing sequencing gels. A representative gel is shown. C, same as in B except that reaction mixtures contained the catalytically inactive Top1 mutant Top1-Y723F.

Discussion

Genomic misincorporation of rNMPs occurs in many domains of living organisms, from bacteria to yeast to mammalian cells (5, 6, 15, 41). Cells have developed specific RNase H2-dependent pathways for the removal of rNMPs, termed RER. In general, the misincorporated rNMPs are first recognized by RNase H2, which signals the beginning of the repair process by nicking the DNA on the 5′-side of the rNMP. These nicks serve as the entry point for a host of repair enzymes to simultaneously remove the pieces of rNMP-containing DNA and synthesize rNMP-free DNA. In E. coli, removal of the rNMP-containing piece and synthesis of the new piece are carried out by the 5′ to 3′ exonuclease activity and the polymerase activity of DNA polymerase I (41). In eukaryotes, replicative polymerases carry out strand displacement synthesis in the presence of helicases, and the displaced DNA flap-containing rNMPs are excised by structural nucleases, such as FEN1 (flap structure-specific endonuclease 1) (12). DNA ligase then restores the continuity of the DNA backbone by sealing the final nicks. In addition, cells likely possess redundant repair pathways, as nucleotide excision repair has also been implicated in the removal of rNMPs (13, 14).
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This poses an important question: what are the consequences of unremoved rNMPs? Despite the labile nature of rNMPs, experiments carried out in yeast strains defective in RNase H2 and encoding a modified DNA polymerase prone to misincorporate rNMPs show that high levels of genomic rNMPs are tolerated. However, these yeast strains develop DNA deletions at short repeat sites and experience increased recombination rates (16, 17). Additionally, the large number of genomic rNMPs in RNase H2-null mouse embryonic fibroblasts leads to a strong p53-dependent DNA damage response and significantly increases micronucleus occurrence and chromosomal rearrangements (15). In humans, mutations in RNase H2 give rise to the neuroinflammatory disease Aicardi-Goutières syndrome. Importantly, in yeast strains with high levels of genomic rNMPs, additional deletion of the TOP1 gene essentially eliminates all of the aforementioned phenotypes while the levels of genomic rNMPs remain high (10, 16–18). These results clearly establish that the cellular distress and genomic instability in RNase H2-defective yeast are due to the processing of rNMPs by Top1. Whether this effect of Top1 extends to mammalian cells with defective RNase H2 and whether it has any potential connection to Aicardi-Goutières syndrome require further investigations.

Biochemical studies have demonstrated that Top1 nicks DNA at rNMP sites with a RNase H2-like ribonuclease activity, but with the opposite polarity from RNase H2 (16, 42). The scissile phosphate of the Top1cc at a rNMP site is susceptible to nucleophilic attack by the 2′-hydroxyl on the nearby sugar moiety, forming a 2′,3′-cyclic phosphate and releasing Top1 (illustrated in Fig. 5, inset). In RNase H2-defective cells, the high levels of rNMPs combined with the ubiquitous presence of Top1 (43–44), likely result in a large number of DNA nicks. Furthermore, the 2′,3′-cyclic phosphates on the Top1-induced DNA nicks are “dirty” ends, as they are suitable substrates neither for ligation nor for polymerase extension.

The events following the formation of Top1-induced nicks that eventually result in DNA deletions at short repeat sites have been the subject of much speculation (8, 9, 18, 25). Here, we provide experimental evidence for a sequential model (25). First, Top1 forms a cleavage complex at the rNMP site (Fig. 5a), which leads to the 2′,3′-cyclic phosphate ends (Fig. 5b). Second, a Top1cc forms 2 bases 5′ to the nick (Fig. 5d), leading to the release of the short DNA fragment with the 2′,3′-cyclic phosphates (Fig. 5e). The facile release of the dinucleotide is consistent with prior biochemical studies (32, 45), attributable to the lack of extensive Top1 interaction with its DNA substrates on the 3′-side of the scissile phosphates (46). Efficient religation across gaps is also consistent with previous reports (32, 45, 47). Thus, the sequential cleavage of Top1, as depicted in Fig. 5, results in short DNA deletions at repeat sequences. We note that while this manuscript was under review, a separate report by Sparks and Burgers (31) appeared, which corroborates our present findings remarkably well.

Given the relative locations of the two Top1 binding events at the (AT)2 hot spot, the DNA deletions are accompanied by loss of rNMPs (Fig. 5, d–f). This may account for the fact that Top1 has been reported to function as a backup pathway to RER, as deletion of the TOP1 gene leads to higher accumulation of rNMPs in RNase H2-defective yeast strains (48). Moreover, the model of sequential Top1 binding is consistent with a recent report implicating Srs2 and Exo1 in a backup pathway to RER (17). Klein and co-workers (17) showed that Srs2 and Exo1 removed a small DNA fragment on the 5′-hydroxyl side of the Top1-induced nicks in vitro, creating a gap. From the biochemical point of view, Top1 and Srs2 compete for the same substrate: the nicks generated by the initial Top1 binding event. Genetic inactivation of SRS2 leads to higher rates of short DNA deletions (17), a finding that supports our interpretation because Top1 would have more opportunities to process the nicks in the absence of Srs2.

Notably, we also found that Top1 efficiently promoted resealing of the DNA nicks generated by the initial Top1cc at the rNMP sites (Fig. 3, B and C, and Fig. 5), consistent with previous reports (38, 39). The rNMPs are highly labile, as they are prone to spontaneous hydrolysis, forming nicks that are identical to the ones generated by Top1 at rNMP sites.

FIGURE 4. Top1-induced short deletion in rNMP-containing DNA, accompanied by excision of the rNMPs. A, scheme showing the 28-nt product accompanied by loss of rNMPs, which would be resistant to breakage by alkaline treatment. B, alternative scheme showing the 28-nt product retaining rNMPs, which would be sensitive to breakage by alkaline treatment, generating 2′,3′-cyclic phosphates. C, full-length religated product containing the rNMP, which is predicted to be cleaved by alkaline treatment. D, Top1-induced religation products with or without alkaline treatment. A representative gel is shown. Nicked DNA constructs containing 2′,3′-cyclic phosphate at the X-position were allowed to react with two different concentrations of Top1 (70 (1X) or 140 (2X) nm) until reaching equilibrium, and the samples were then split in two. One part of the reaction mixture was further treated with 0.3 M KOH (final concentration) at 55 °C for 1 h. Products were then resolved on a denaturing sequencing gel. Band intensities were normalized to that of the untreated 30-nt product containing 70 nM human recombinant Top1, arbitrary units.

(A) 5′-32p 28 KOH 5′-32p 28
(B) 5′-32p rU KOH 5′-32p nick
(C) 5′-32p 30 KOH 5′-32p 30
(D) Nicked-12CP
Top1 KOH
(rU)
Top1cc
(E) Band Intensity (A.U.)

| Band Intensity (A.U.) | Control KOH RNaseH2 |
|-----------------------|---------------------|
| 0.0                   | 1.2                 |
| 0.6                   | 1.0                 |
| 1.2                   | 1.0                 |
| 1.8                   | 1.2                 |

1 2 3 4 5 6 7 8
Marker 12rUP
Marker 30 28

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whereas the short deletion (Fig. 5) appears as the minor product (Fig. 3B). Direct resealing of this type of nick would allow renewed attempt for repair by the RNase H2-dependent pathway. Therefore, the consequences of Top1 can be viewed as both positive and negative, in a state of equilibrium (Fig. 5).

Finally, it is important to consider the potential impact of Top1 under normal conditions. Although RNase H2-dependent RER is the predominant repair pathway, how rapidly the Top1 under normal conditions. Although RNase H2-dependent RER is the predominant repair pathway, how rapidly the consequences of Top1 can be viewed as both positive and negative, in a state of equilibrium (Fig. 5).

Grid could have significant consequences.

Figure 5. Scheme for the processing of rNMP-containing substrates leading to Top1-induced deletions at short repeats or Top1-induced religation of the DNA nicks for subsequent RNase H2-dependent repair. a, rNMPs embedded in the genomic DNA, which can generate products (b or c). b, DNA breaks with 2',3' cyclic phosphates can be generated either by Top1 binding to the rNMPs or by spontaneous breakage. c, RNase H2-mediated DNA repair removes rNMPs from the genomic DNA. d, a second Top1cc adjacent to the nick enables the dissociation of the short DNA fragment, resulting in excision of the ribonucleotide. e, religation across the gap by Top1. f, Top1 dissociates, leaving a 2-nt deletion. g, alternatively, Top1 binding at the 2',3'-cyclic phosphates can reseal the DNA nicks, allowing RNase H2-mediated DNA repair to remove ribonucleotides to generate (c). Inset, chemical scheme of the key intermediates.

misincorporated rNMP undergoes spontaneous hydrolysis before RNase H2 locates it, the resulting DNA nicks (with 2',3'-cyclic phosphates) are no longer substrates for RNase H2 and therefore are potentially excluded from the error-free RNase H2-dependent repair. We note that when Top1 interacts with DNA nicks containing 2',3'-cyclic phosphates, it generates the resealed full-length construct (Fig. 5g) as the dominant product, whereas the short deletion (Fig. 5f) appears as the minor product (Fig. 3B). Direct resealing of this type of nick would allow renewed attempt for repair by the RNase H2-dependent pathway. Therefore, the consequences of Top1 can be viewed as both positive and negative, in a state of equilibrium (Fig. 5).

Finally, it is important to consider the potential impact of Top1 under normal conditions. Although RNase H2-dependent RER is the predominant repair pathway, how rapidly the rNMPs are removed after misincorporation by replicative polymerases is not known. Of the estimated >10^6 rNMPs embedded in the genome per cycle of replication in mammalian cells (15, 49), what fraction is yet to be repaired by RNase H2-dependent RER before Top1-induced nicks can occur? Could Top1-induced DNA nicks pose greater threats in particular types of tissues? A recent key report highlights the potential damage of genomic rNMPs by showing that human ligases are prone to stall when they encounter RNase H2-generated nicks (50). The abortive ligation generates 5'-adenylation adducts, which require processing by aprataxin. Elimination of the aprataxin homolog in yeast leads to impaired growth and hypersensitivity to hydroxyurea in a RNase H2-dependent fashion (50). Mutations in human aprataxin lead to the late-onset neurological disease ataxia with oculomotor apraxia type 1 (AOA1).

Genomes of non-replicative cells, such as neurons, potentially accumulate high levels of rNMPs because DNA repair polymerases repeatedly misincorporate rNMPs, particularly with the low deoxyribonucleotide triphosphate:ribonucleotide triphosphate ratio in these cells. It is possible that physiological manifestation of AOA1 arises only on the background of high genomic rNMPs. The results from this study confirm that interaction of Top1 with embedded rNMPs on a similar background could have significant consequences.

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References

1. Meng, B., Hoang, L. N., McIntyre, J. B., Duggan, M. A., Nelson, G. S., Lee, C. H., and Köbel, M. (2014) POLE exonuclease domain mutation predicts long progression-free survival in grade 3 endometrioid carcinoma of the endometrium. Gynecol. Oncol. 134, 15–19.

2. Palles, C., Cazier, J. B., Howarth, K. M., Domingo, E., Jones, A. M., Broderick, P., Kemp, Z., Spain, S. L., Guarino Almeida, E., Salguero, I., Sherborne, A., Chubb, D., Carvajal-Carmona, L. G., Ma, Y., Kaur, K., Dobbins, S., Barclay, E., Gorman, M., Martin, L., Kovac, M. B., Humphray, S., The CORGI Consortium, The WGS500 Consortium, Lucassen, A., Holmes, C. C., Bentley, D., Donnelly, P., Taylor, J., Petridis, C., Roylance, R., Sawyer, E. J., Kerr, D. J., Clark, S., Grimes, J., Kearsey, S. E., Thomas, H. J., McVean, G., Houlston, R. S., and Tomlinson, I. (2013) Germ line mutations affecting the proofreading domains of POLE and POLD1 predispose to colorectal adenomas and carcinomas. Nat. Genet. 4(25), 136–144

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3. Abaan, O. D., Polley, E. C., Davis, S. R., Zhu, Y. J., Bilke, S., Walker, R. L., Pineda, M., Gindin, Y., Jiang, Y., Reinhold, W. C., Holbeck, S. L., Simon, R. M., Doroshov, J. H., Pommier, Y., and Meltzer, P. S. (2013) The exomes of the NCI-60 panel: a genomic resource for cancer biology and systems pharmacology. Cancer Res. 73, 4372–4382

4. Sousa, F. G., Matuo, R., Tang, S. W., Rajapakse, V. N., Luna, A., Sander, C., Varma, S., Simon, P. H., Doroshov, J. H., Reinhold, W. C., and Pommier, Y. (2015) Alterations of DNA repair genes in the NCI-60 cell lines and their predictive value for anticancer drug activity. DNA Repair 28, 107–115

5. Nick McElhinny, S. A., Watts, B. E., Kumar, D., Watt, D. L., Lundström, E. B., Burgers, P. M., Johansson, E., Chabes, A., and Kunkel, T. A. (2010) Abundant ribonucleotide incorporation into DNA by yeast replicative polymerases. Proc. Natl. Acad. Sci. U.S.A. 107, 4949–4954

6. Nick McElhinny, S. A., Kumar, D., Clark, A. B., Watt, D. L., Watts, B. E., Lundström, E. B., Johansson, E., Chabes, A., and Kunkel, T. A. (2010) Genome instability due to ribonucleotide incorporation into DNA. Nat. Chem. Biol. 6, 774–782

7. Clausen, A. R., Lujan, S. A., Burkholder, A. B., Orebaugh, C. D., Williams, J. S., Clausen, M. F., Male, E. P., Mieczkowski, P. A., Fago, D. C., Smith, D. J., and Kunkel, T. A. (2015) Tracking replication enzyme-in vivo by genome-wide mapping of ribonucleotide incorporation. Nat. Struct. Mol. Biol. 22, 185–191

8. Williams, I. S., and Kunkel, T. A. (2014) Ribonucleotides in DNA: origins, repair, and consequences. DNA Repair 19, 27–37

9. Potenski, C. J., and Klein, H. L. (2014) How the misincorporation of ribonucleotides into genomic DNA can be both harmful and helpful to cells. Nucleic Acids Res. 42, 10226–10234

10. Lazzaro, F., Novarina, D., Amara, F., Watt, D. L., Stone, J. E., Costanzo, V., Burgers, P. M., Kunkel, T. A., Plevani, P., and Muzzi-Falconi, M. (2012) RNase H and postreplication repair protect cells from ribonucleotides incorporated in DNA. Mol. Cell 45, 99–110

11. Shen, Y., Koh, K. D., Weiss, B., and Storic, F. (2012) Mispaired rNMPs in DNA are mutagenic and are targets of mismatch repair and RNases H. Nat. Struct. Mol. Biol. 19, 98–104

12. Sparks, J. L., Chan, H., Cerritelli, S. M., Kunkel, T. A., Johansson, E., Crouch, R. J., and Burgers, P. M. (2012) RNase H2-initiated ribonucleotide excision repair. Mol. Cell 47, 980–986

13. Kim, N., Cho, J. E., Li, Y. C., and Jinks-Robertson, S. (2013) RNA:DNA hybrids initiate quasi-palindrome-associated mutations in highly transcribed yeast DNA. PLoS Genet. 9, e1003924

14. Vaisman, A., McDonald, J. P., Huston, D., Kuran, W., Liu, L., Van Houten, B., and Woodgate, R. (2013) Removal of misincorporated ribonucleotides from prokaryotic genomes: an unexpected role for nucleotide excision repair. PLoS Genet. 9, e1003878

15. Reijns, M. A., Rabe, B., Rigby, R. E., Mill, P., Astell, K. R., Leitte, L. A., Boyle, S., Leitch, A., Keighren, M., Kilanowski, F., Devenney, P. S., Sexton, D., Grimes, G., Holt, I. J., Hill, R. E., Taylor, M. S., Lawson, K. A., Dorin, J. R., and Jackson, A. P. (2012) Enzymatic removal of ribonucleotides from DNA is essential for mammalian genome integrity and development. Cell 149, 1008–1022

16. Kim, N., Huang, S. N., Williams, J. S., Li, Y. C., Clark, A. B., Cho, J. E., Kunkel, T. A., Pommier, Y., and Jinks-Robertson, S. (2011) Mutagenic processing of ribonucleotides in DNA by yeast topoisomerase I. Science 332, 1561–1564

17. Potenski, C. J., Niu, H., Sung, P., and Klein, H. L. (2014) Avoidance of ribonucleotide-induced mutations by RNase H2 and Srs2-Exo1 mechanisms. Nature 511, 251–254

18. Takahashi, T., Burguier-Slezak, G., Van der Kemp, P. A., and Boiteux, S. (2011) Topoisomerase I provokes the formation of short deletions in repeated sequences upon high transcription in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U.S.A. 108, 692–697

19. Wang, J. C. (2002) Cellular roles of DNA topoisomerases: a molecular perspective. Nat. Rev. Mol. Cell Biol. 3, 430–440

20. Pommier, Y., Leo, E., Zhang, H., and Marchand, C. (2010) DNA topoisomerases and their poisoning by anticancer and antibacterial drugs. Chem. Biol. 17, 421–433
Top1 Causes Deletions and Religates DNA Nicks at Ribonucleotides

(2000) Conversion of topoisomerase I cleavage complexes on the leading strand of ribosomal DNA into 5’-phosphorylated DNA double-strand breaks by replication runoff. Mol. Cell. Biol. 20, 3977–3987

44. Pommier, Y., Poddevin, B., Gupta, M., and Jenkins, J. (1994) DNA topoisomerases I and II cleavage sites in the type 1 human immunodeficiency virus (HIV-1) DNA promoter region. Biochem. Biophys. Res. Commun. 205, 1601–1609

45. Shuman, S. (1992) DNA strand transfer reactions catalyzed by vaccinia topoisomerase I. J. Biol. Chem. 267, 8620–8627

46. Redinbo, M. R., Stewart, L., Kuhn, P., Champoux, J. J., and Hol, W. G. (1998) Crystal structures of human topoisomerase I in covalent and non-covalent complexes with DNA. Science 279, 1504–1513

47. Henningfeld, K. A., and Hecht, S. (1995) A model for topoisomerase I-mediated insertions and deletions with duplex DNA substrates containing branches, nicks, and gaps. Biochemistry 34, 6120–6129

48. Williams, J. S., Smith, D. J., Marjavaara, L., Lujan, S. A., Chabes, A., and Kunkel, T. A. (2013) Topoisomerase 1-mediated removal of ribonucleotides from nascent leading-strand DNA. Mol. Cell 49, 1010–1015

49. Clausen, A. R., Zhang, S., Burgers, P. M., Lee, M. Y., and Kunkel, T. A. (2013) Ribonucleotide incorporation, proofreading and bypass by human DNA polymerase delta. DNA Repair 12, 121–127

50. Tumbale, P., Williams, J. S., Schellenberg, M. J., Kunkel, T. A., and Williams, R. S. (2014) Aprataxin resolves adenylated RNA-DNA junctions to maintain genome integrity. Nature 506, 111–115