Resolution by Unassisted Top3 Points to Template Switch Recombination Intermediates during DNA Replication*

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Background: The Sgs1/Top3/Rmi1 (STR) complex resolves DNA linkages formed during replication of methyl methanesulfonate (MMS)-damaged templates.

Results: Even without Sgs1 and Rmi1, Top3 can provide some resistance to MMS and resolve linkages that have recombination-dependent X-molecule (Rec-X) topology.

Conclusion: Rec-Xs form during replication of MMS-damaged DNA.

Significance: The findings provide novel insight into replication, STR function, and genome maintenance.

The evolutionarily conserved Sgs1/Top3/Rmi1 (STR) complex plays vital roles in DNA replication and repair. One crucial activity of the complex is dissolution of toxic X-shaped recombination intermediates that accumulate during replication of damaged DNA. However, despite several years of study the nature of these X-shaped molecules remains debated. Here we use genetic approaches and two-dimensional gel electrophoresis of genomic DNA to show that Top3, unassisted by Sgs1 and Rmi1, has modest capacities to provide resistance to MMS and to resolve recombination-dependent X-shaped molecules.

The X-shaped molecules have structural properties consistent with hemicatenane-related template switch recombination intermediates (Rec-Xs) but not Holliday junction (HJ) intermediates. Consistent with these findings, we demonstrate that purified Top3 can resolve a synthetic Rec-X but not a synthetic double HJ in vitro.

We also find that unassisted Top3 does not affect crossing over during double strand break repair, which is known to involve double HJ intermediates, confirming that unassisted Top3 activities are restricted to substrates that are distinct from HJs. These data help illuminate the nature of the X-shaped molecules that accumulate during replication of damaged DNA templates, and also clarify the roles played by Top3 and the STR complex as a whole during the resolution of replication-associated recombination intermediates.

The Saccharomyces cerevisiae STR complex, consisting of the DNA helicase Sgs1, the type 1A topoisomerase Top3, and the OB-fold containing protein Rmi1, functions in multiple facets of genome maintenance (1). Loss of function mutations in STR complex members cause sensitivity to a variety of DNA damaging agents, increased rates of chromosome loss, and chromosomal rearrangements (2–6). Similarly, mice and humans lacking homologs of STR complex members, such as the Werner and Bloom syndrome DNA helicases, have phenotypes ranging from strong cancer predisposition to early embryonic lethality, highlighting the evolutionarily conserved importance of the complex in maintaining genomic integrity (7, 8).

How the STR complex functions to maintain genomic stability is an active subject of investigation with evidence pointing to roles in DNA damage checkpoint responses, replication fork stability, exonucleolytic processing of DNA ends, and the resolution of homologous recombination (HR) intermediates (9). Of note, emerging in vitro and in vivo evidence supports roles for the STR complex in the resolution of at least two different types of HR-dependent linkages: Holliday junctions (HJ) formed, for example, during double strand break repair (DSBR), and so-called Rec-X structures (also sometimes called sister chromatid junctions) formed during template switch recombination arising from the perturbation of DNA replication (10–15). In one type of HR-based DSBR, both broken ends invade the target, and following repair synthesis, each extended end is ligated to yield a double Holliday junction (dHJ). The dHJ can be processed by a classical HJ resolvase, which can generate either crossover or non-crossover products. Alternatively, the STR complex can branch migrate the two HJs into one another to achieve dissolution without crossing over (16). In the case of template switch recombination, when a replicating polymerase encounters a block to DNA synthesis, it is thought to switch to using the newly replicated strand of the sister chromatid as a template, and then eventually return to the original template once the stall-inducing lesion has been bypassed. Alternatively, the switch could occur at a gap left behind the advancing repli-
and supercoils in several contexts, Top3 is most active on negatively twisted (i.e. unwound) DNA (23). To relieve negative twist, Top3 induces a single strand nick into the phosphodiester backbone by forming a covalent linkage between its catalytically essential tyrosine residue 356 and a phosphorus present in the DNA backbone. This reaction generates a transient gate through which the intact complementary strand of DNA can pass, thus relieving superhelical tension, and is followed by restoration of the phosphodiester backbone and release of Top3 (24–26). Unlike Top1 and Top2, which provide the majority of in vivo topological relaxation, Top3 appears to function mainly in disentangling intermediates associated with DNA replication and repair (24–27). Indeed, in support of such roles, top3Δ mutants display slowed chromosome replication, chromosome missegregation, inability to undergo meiosis, and a strong sensitivity to genotoxic stressors (5, 28, 29). Because Top3 interacts physically with Sgs1 and Rmi1, and mutations that disrupt these interactions compromise DNA repair activities, Top3 is generally thought to work only within the context of the STR complex (24, 30, 32–35). However, sgs1Δ top3Δ mutants are more sensitive to MMS than sgs1Δ mutants (34, 36, 37), suggesting that Top3 might have residual Sgs1-independent activities, but this possibility remains largely unexplored.

An essential requirement for resolution of both dHJs and Rec-Xs is that DNA strands pass through transient DNA breaks, and we reasoned that Top3 may thus be the most critical component of the STR complex for resolution of these X-shaped intermediates. Although Sgs1 and Rmi1 stimulate Top3 catalysis, in principle, Top3 should have some intrinsic capacity to resolve entangled DNA strands. Here we present evidence that Top3 indeed plays a more critical role than Sgs1 or Rmi1 in the resistance to MMS-induced DNA damage, and that in the absence of Sgs1 and Rmi1, Top3 has some capacity to resolve recombination intermediates. We utilize this Top3-dependent rescue to characterize the properties of the recombination intermediates that accumulate during replication of damaged DNA templates. Using genetic and biochemical assays, we show that the intermediates resolved by Top3 during bypass of stall-inducing lesions caused by MMS have characteristics of Rec-X structures rather than HJs. These findings clarify how the conserved STR complex promotes genome stability, and provide support for the role of Rec-X structures in DNA replication.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains**—All yeast strains are derived from the BY4741/2 background. Experiments using α-factor were carried out in bar1Δ strains. The following strains were constructed: YAC174, sgs1Δ::HIS3; YAC173, sgs1Δ::HIS3, top3Δ::KanMX; YAC833, sgs1Δ::HIS3, rad52Δ::HygMX; YAC1177, sgs1Δ::HIS3, top3Δ::KanMX, rad18Δ::HsgMX; YAC1637, sgs1Δ::HIS3, top3Δ::CaLIra3; YAC1640, sgs1Δ::HIS3, top3Δ::CaLIra3, mph1Δ::KanMX, shu1Δ::NatMX; YAC1644, sgs1Δ::HIS3, mph1Δ::KanMX, shu1Δ::NatMX; YAC1646, rmi1Δ::KanMX; YAC1887, sgs1Δ::HIS3, rad18Δ::HygMX; YAC2005, sgs1Δ::HIS3, top3Δ::NatMX, Δrad52Δ::HsgMX; YAC2341, rmi1Δ::KanMX, top3Δ::NatMX; YBB26, sgs1Δ::KanMX, top3Δ::NatMX, bar1Δ::HygMX.
Plasmids—All plasmids were based on Gateway-compatible pAG CEN/ARS vectors (38). The constitutive GPD or NOP1 promoters, which drive high or moderate levels of transcription, respectively, were each tested but gave indistinguishable results for TOP3-mediated rescue (data not shown). Plasmids used and constructed were as follows: pAG143GD-ccdb; pAG415GD-ccdb; pAG416GD-ccdb; AC207, pDONR221-TOP2; AC246, pDONR221-TOP1; AC273, pAG416NOP1-ccdb; AC739, pDONR221-TOP3; AC1314, pAG416TOP3-MYC-ccdb (i.e., a TOP3 promoter-driven vector); AC1332, pDONR221-hTOP3αc; AC1337, pDONR221-top3-Y356.

Analysis of X-shaped Recombination Intermediates by Two-dimensional Gel Electrophoresis—Preparation of cell cultures, DNA, two-dimensional gel electrophoresis, and Southern blots for analyzing the ARS305 region has been previously described (39), and we employed the following modifications. Briefly, α-factor was added at a final concentration of 50 nM to logarithmically growing cultures for 4 h. The synchronized cells were collected by centrifugation and inoculated at $1.2 \times 10^7$ cells/ml into fresh YPAD containing a final concentration of 0.033% MMS. Samples were either harvested 1.5 h later or the cells were washed at this point and inoculated into fresh YPAD (without MMS) at $4 \times 10^6$ cells/ml and harvested 2 and 3 h later. All comparisons were made between samples grown, prepared, and run in parallel to remove inter-experimental variation. For two-dimensional gel electrophoresis of the ribosomal DNA (rDNA) and the region adjacent to the ARS305 region, DNA was digested with BglII, and run under identical conditions as for the ARS305 region. The rDNA probe was made using PCR amplification of genomic DNA with primers rDNA-1053 and rDNA-447 as previously described (40). The ARS305-region adjacent probe was made using PCR amplification of genomic DNA with primers 5'-GTAGGAACAAAGGTTTG-GCACGG-3' and 5'-CTTCCAGATAGCAGCATGGG-3'. Gels were quantified using ImageQuant analyses of PhosphorImager scans. p values for spike:arc ratios were calculated using one-tailed t tests. MBN sensitivity of X-shaped molecules was performed by digesting 1 μg of samples with BglII in New England Biolabs Buffer 2 followed by addition of 45 units of MBN (New England Biolabs) at 30 °C for 1 h. To confirm the specificity of MBN under these conditions, 0.5 fmol of a 5'-CCACCTTTTCTGCTGAGACCTACACGGTACGACCCACCAGGCAATGGCGCTGGACTAACGCTCGACACCC-3' oligo (which self-anneals to form a structure containing a ligatable nick in one of its hairpinned duplex arms), and the second step generated the full Rec-X and dHJ products; this approach allowed for higher yield and purity of the Rec-X substrate. To ensure consistency between substrates, their components were processed identically and in parallel. For the first step, the dHJ1 and Rec-X1 oligonucleotides (12.5 pmol each) were phosphorylated using T4 polynucleotide kinase and a molar excess of [γ-32P]ATP, followed by purification using Centri-Spin 20 columns (Princeton Separations) containing 10 mm Tris-HCl, pH 7.5, 0.1 mm EDTA. The oligos were then subjected separately to annealing and ligation conditions (to which only Rec-X1 responded, by forming a closed circle) as follows: 6.25 pmol of oligonucleotide was suspended in 35 μl containing 50 mm Tris-HCl, pH 7.5, 10 mm MgCl2, denatured at 99 °C for 1 min; and cooled rapidly to 4 °C. Samples were then ligated by addition of 1 mm ATP, 5 mm DTT, and 280 units of T4 DNA ligase (New England Biolabs) and incubated at 8 °C for 1 h, 10 °C for 1 h, 12 °C for 4 h, 16 °C for 12 h, and 20 °C for 12 h. The products were mixed 2:1 with formamide containing 0.1% bromphenol blue and xylene cyanol, and resolved via electrophoresis through an 8% polyacrylamide gel (19:1 acrylamide: bisacrylamide) under denaturing conditions (7.8 μ urea in 0.5X TBE). The gel was exposed to a PhosphorImager screen for ~15 min, and the image was printed out on a clear plastic sheet and placed over the gel to guide excision of the bands corresponding to the circularized Rec-X1 oligo and the linear dHJ1 oligo. The oligos were electroeluted from the gel slices using D-Tube Dialyzer Midi tubes (Novagen) with a 6–8-kDa cutoff at 6 V/cm for 1 h in 0.5X TBE, followed by dialysis overnight at 4 °C versus 1 liter of 10 mM Tris-Cl, pH 7.5, 0.1 mM EDTA. In the morning the dialysis buffer was exchanged with fresh buffer and allowed to equilibrate for an additional 2 h (this step was repeated twice). Samples were precipitated by adding 0.1 volume of NaOAc, pH 5.2, and 2.5 volumes of 100% ethanol, and washed with 70% ethanol.

For the second step, the pellets were resuspended in 20 μl of 10 mM Tris-Cl, pH 7.5, 0.1 mM EDTA containing 5'-phosphorylated dHJ2 (due to differences in substrate synthesis efficiency, dHJ2 was added at 100-fold molar excess to the Rec-X1; and at 15-fold molar excess to the dHJ1). Samples were annealed in buffer conditions as described above by denaturing at 95 °C for 3 min, shifted to 80 °C, and cooled to 5 °C at a rate of 0.125 °C/min. Ligation, electrophoresis, gel extraction, electroelution, dialysis, and precipitation were performed as above. The final products were resuspended in 10 mM Tris, pH 7.5, 0.1 mM EDTA at
0.5 fmol/μl. Substrate concentrations were estimated based on radioactive counts, measured using Image Quant analyses of PhosphorImager scans, compared with originally end-labeled oligos.

Confirmation of the Rec-X structure involved incubation of 0.5–1 fmol of substrate with restriction enzymes (New England Biolabs) and exonucleases in 50 mM KAc, 20 mM Tris acetate, 10 mM MgAc, 1 mM DTT, pH 7.9. Rsal (5 units), and Hhal (20 units) digests were carried out at 37 °C for 3 h. TaqI (20 units) digests were carried out at 47 °C for 3 h. Escherichia coli ExoI (2.5 units; U. S. Biochemical) and ExoIII (50 units; Promega) digests were carried out at 37 °C for 30 min. After digests, enzymes were heat inactivated at 80 °C for 20 min. Electrophoresis and image analysis were performed as above.

Top3 Decatenation Reactions—Top3 protein was prepared as described previously (44). Reactions shown in Fig. 8, A and B, were carried out using the indicated amounts of protein, and 50 pm DNA substrate in 40 mM Hepes, pH 7.0, 42% glycerol, 5 mM sodium acetate, 65 mM NaCl, 60 mM KCl, 10 μg/ml of BSA, 2 mM MgCl2, 0.2 mM EDTA, 0.2 mM DTT, 0.002% Nonidet P-40, 0.02 mM PMSF, and 1 mM spermidine, at the indicated temperatures, for 2 h. Reactions shown in Fig. 8, C and D and E and F, were carried out as above but in 7% glycerol buffer, 5 mM ATP, no or 20 mM KCl, and 4 or 6 mM MgCl2, respectively, at 37 °C. All reactions were stopped by the addition of 0.5% SDS and 0.05 mg/ml of proteinase K, incubated at 37 °C for 20 min, and electrophoresis and image analysis were performed as above. p values were calculated using two-tailed t tests.

Western Blot—Strains were grown to log phase and 108 cells were harvested by centrifugation and frozen for subsequent processing by mechanical disruption with glass beads in a 20% TCA solution. Samples were electrophoresed in 4–15% gradient acrylamide gels (Bio-Rad) and transferred to nitrocellulose TCA solution. Samples were electrophoresed in 4–15% gradient acrylamide gels (Bio-Rad) and transferred to nitrocellulose membranes for probing with mouse anti-MYC antibody (Abcam #AB32) at a 1:2000 dilution, followed by HRP/chemiluminescence detection.

ADE2 Crossover Assay—Similar to a previously described crossover assay (45), a URA3 marked ARS209-containing plasmid with a ~600-bp internal fragment of the ADE2 locus was linearized at the Hpal site within the ADE2 fragment. The resulting linear DNA was transformed into the desired yeast strains, cells were selected on SC-URA media and the percentage of red colonies was determined. Red colonies represent crossover repair events involving the formation of a dHJ that is resolved to disrupt the endogenous ADE2 locus. White colonies represent non-crossover events, e.g., repair events where the dHJs were convergently migrated and dissolved. p values were calculated using two-tailed t-tests.

RESULTS

Top3 Promotes DNA Damage Tolerance in sgs1Δ Mutants—As sgs1Δ top3Δ mutants are more MMS-sensitive than sgs1Δ mutants (34, 36, 37), we hypothesized that Top3 might promote DNA damage tolerance, in an Sgs1-independent fashion, through the resolution of toxic recombination intermediates. The alternative explanation, that Top3 impacts checkpoint responses or cell cycle kinetics in sgs1Δ mutant cells, has been ruled out previously (28). To first confirm that Top3 confers DNA damage tolerance in our genetic background, we compared the growth of MMS-treated sgs1Δ and sgs1Δ top3Δ mutants and found a 10-fold increase in DNA damage sensitivity when TOP3 was deleted from sgs1Δ cells (Fig. 2A). To provide further evidence for autonomous Top3 activity, we tested if overexpression of Top3 might further promote DNA damage tolerance in cells lacking Sgs1. A small (5–10-fold) but repeatable increase in MMS resistance was observed upon Top3 overexpression (Fig. 2B and data not shown). These data prompted us to further investigate the mechanism of Top3-dependent DNA damage resistance.

Rescue of sgs1Δ Mutants by Topoisomerase Activity Is Specific to Top3, and Can Be Conferred by Its Human Ortholog Top3α—To test if the identified role for Top3 in promoting DNA dam-
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MMS resistance provided by unassisted Top3 is evolutionarily conserved and can occur without RMI1. Spot assays comparing effects of MMS on the growth of: A, sgs1Δ top3Δ cells containing either control vector or human TOP3α expression plasmid; B, rmi1Δ or rmi1Δ top3Δ mutants; and C, rmi1Δ cells with vector or TOP3 overexpression plasmid and sgs1Δ strains containing a TOP3 overexpression plasmid together with an additional control vector or plasmid overexpressing RMI1.

FIGURE 3.

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FIGURE 4. Rescue by unassisted Top3 requires factors that enable the accumulation of unresolved recombination intermediates within sgs1Δ mutants. A, sgs1Δ cells lacking additional factors that render them unable to accumulate recombination intermediates, with or without endogenous TOP3, were spotted onto YPAD containing the indicated concentrations of MMS. B, identical to A except comparing the effects of TOP3 overexpression.

age resistance within sgs1Δ cells is specific to Top3 or might be a general property of topoisomerases, we compared the capacity of Top1, Top2, or Top3 overexpression to rescue the MMS sensitivity of sgs1Δ and sgs1Δ top3Δ mutants. Unlike Top3, overexpression of Top1 or Top2 provided no increased DNA damage resistance in either context (Fig. 2D). The catalytic activity of Top3 was required for rescue, because overexpression of the top3Δ-Y356F mutant, which is defective in the formation of the 5′ phosphotyrosine covalent bond between DNA and Top3, was unable to rescue DNA damage sensitivity in sgs1Δ or sgs1Δ top3Δ mutants, despite equal accumulation of wild-type and Y356F proteins (Fig. 2, E–G) (24, 36).

The selective role for Top3 in the rescue of MMS sensitivity prompted us to ask if this role might be conserved and thus shared by the human Top3 ortholog TOP3α (46). Remarkably, expression of TOP3α, which might be expected to interact poorly with endogenous yeast proteins, provided robust rescue of sgs1Δ top3Δ DNA damage sensitivity (Fig. 3A). This finding supports the idea that Top3 can function independently to confer resistance to MMS, and raises the possibility that this function is conserved between yeast and human cells.

Top3 Promotes DNA Damage Tolerance Independent of Other STR Complex Members—In addition to its association with Sgs1, Top3 binds the OB-fold containing protein Rmi1 (2, 3). Because Rmi1 binds DNA and is reported to stimulate Top3 reaction kinetics in vitro, we asked if DNA damage tolerance provided by Top3 depends upon Rmi1 (11, 35, 44). No role for Rmi1 in Top3-mediated rescue was found as rmi1Δ top3Δ mutants were more sensitive to MMS than rmi1Δ controls, and Top3 overexpression in rmi1Δ mutants provided MMS resistance (Fig. 3, B and C). Furthermore, Rmi1 overexpression did not augment MMS resistance provided by Top3 overexpression in sgs1Δ cells (Fig. 3C). Altogether, our findings indicate that even without assistance by other STR complex members Top3 can provide some resistance to MMS-induced DNA damage.

Recombination Intermediate Resolution by Unassisted Top3 Provides DNA Damage Tolerance—We sought to determine whether the same DNA repair factors that enable the accumulation of HR intermediates during replication of MMS-treated sgs1Δ mutants are also required for the MMS resistance provided by unassisted Top3. Previous biochemical and biophysical analyses employing two-dimensional gel electrophoresis indicated that these intermediates are Rec-Xs (13, 18, 19). On two-dimensional gels, Rec-Xs run as a prominent near-vertical spike originating at the end of the replication arc (e.g. see Fig. 5A, below), as would be expected for joint linkages arising from template switch recombination between sister chromatids. Importantly, not all molecules running within the X-spike are HR-dependent, as low levels of X-shaped species are still observed in homologous recombination-deficient rad51Δ or rad52Δ strains, particularly near origins of replication (47). However, the elevated levels of Rec-Xs that accumulate when sgs1Δ mutants replicate through damaged DNA templates are entirely HR-dependent (13).

Several factors are necessary for Rec-X formation in sgs1Δ mutants, including the HR factor Rad52 and the post-replicative repair protein Rad18 (13, 18, 20). Furthermore, it was shown recently that cells defective in another protein complex critical for Rec-X resolution, the Smc5/6 complex, accumulate Rec-X intermediates in a fashion dependent on the DNA helicase Mph1 and the pro-recombination Shu protein complex (consisting of Shu1, Shu2, Psy3, and Csm2) (22, 48–50). Combined deletion of MPH1 and SHU1 completely suppresses Rec-X formation in smc5/6 mutants, and given the biochemical similarity between the Rec-X molecules in smc5/6 and sgs1Δ mutants, we predicted and confirmed similar suppression of X-shaped molecule formation in sgs1Δ top3Δ by combined deletion of MPH1 and SHU1 (data not shown). Therefore, MPH1 or SHU1, like RAD52 and RAD18, are required for accumulation of X-shaped structures in sgs1Δ top3Δ mutant cells.

If the Sgs1-independent functions of Top3 involve resolution of Rec-X intermediates, Top3 should not impact MMS resistance in backgrounds lacking Sgs1 and factors required for Rec-X formation. As predicted, sgs1Δ rad52Δ, sgs1Δ shu1Δ mph1Δ, and sgs1Δ rad18Δ mutants showed little to no effect of top3 deletion (Fig. 4A). Similarly, Top3 overexpression did not improve the MMS sensitivity of these mutants (Fig. 4B).

To test directly for effects of Top3 on replication intermediates, we employed two-dimensional gel electrophoresis fol-
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FIGURE 5. Top3 overexpression reduces the level of X-shaped molecules within sgs1Δ top3Δ cells. A, representative two-dimensional gel electrophoresis Southern blots examining replication intermediates that accumulate in sgs1Δ top3Δ cells containing either vector or TOP3 overexpression plasmid during exposure to MMS and following release into MMS-free medium. An rDNA fragment containing the replication fork block, a fragment adjacent to an ARS305-containing fragment, as well as the ARS305-containing fragment, are shown on the left, middle, and right sets of panels, respectively. MMS indicates cells grown for 1.5 h in 0.033% MMS, and 2 h, 3 h, and 4 h indicate cells 2, 3, and 4 h after release from MMS. Right, schematic showing the location of DNA structures visualized by two-dimensional gel electrophoresis, including the Y-spot containing non-replicating duplex, the Y-arc containing single replication forks, and the X-spike containing two duplexes interlinked at different positions along their lengths. B, quantification of the ratio of X-shaped molecules to those running within the replication arc (n = 3 biological replicates for the rDNA fragment and ARS305 adjacent fragment, and error bars represent the S.E.; *, p = 0.034; **, p = 0.009).

followed by Southern blotting to visualize events at different genomic regions. As is standard (13, 51–53), higher levels of MMS (0.01–0.033%) were used in two-dimensional gel electrophoresis assays to enable visualization of enhanced X-structure levels under conditions where cells are exposed to MMS for only a single S-phase and where replication must be perturbed in any given genomic fragment that is visualized; importantly, we confirmed that Top3 overexpression improves growth even when cells are exposed to these higher levels of MMS (Fig. 2C).

Logarithmically growing cells were synchronized with α-factor, and released into media containing MMS. Samples were taken at 1.5 h after treatment, and also at 2 and 3 h after release from MMS into fresh YPAD. In agreement with our MMS-resistance analyses, Top3 overexpression within sgs1Δ top3Δ mutants decreased X-shaped intermediates during recovery from MMS treatment (Fig. 5). Whereas there was a trend toward suppression by Top3 overexpression of X-shaped molecule accumulation in a genomic fragment containing ARS305, this suppression did not achieve statistical significance in our assays (Fig. 5 and data not shown). We reasoned that the background of origin-dependent X-structures may have obscured a difference in the MMS-dependent X-structures. To address this, we examined genomic fragments replicated by forks emanating from origins outside the fragments, including an rDNA region containing the replication fork block and a region adjacent and telomeric to the fragment containing ARS305. Significant and reproducible suppression of X-shaped molecules by Top3 overexpression was observed within both of these fragments (Fig. 5). Overall, our findings indicate that Top3 is capable of providing MMS resistance and promoting the resolution of recombination intermediates independently of other STR members.

Unassisted Top3 Does Not Resolve HJ Intermediates Efficiently—Recently, HJ-cleaving enzymes (RusA, Gen1, Mus81/Mms4) were shown to diminish levels of MMS-induced X-shaped molecules within STR complex mutants, leading to the suggestion that the X-structures may be HJs rather than Rec-Xs (see “Discussion”) (52, 53). Because Top3 confers resistance to DNA damage and decreases the level of X-shaped molecules that accumulate in STR mutant cells, we used Top3 manipulation to further dissect the nature of the X-shaped molecules. The X-shaped molecules that accumulate during DSBR are known to be HJs (10), and we therefore tested whether Top3, independent of Sgs1, could affect the outcomes of DSBR. We employed a previously characterized ADE2 recombination repair assay (45) in which the rate of crossover and non-crossover events are reflected by the frequencies of red and white colonies, respectively. As demonstrated previously, sgs1Δ mutants show a statistically significant increase in the ratio of crossover events as compared with wild-type strains (Fig. 6A) (12). However, in contrast to its effects on MMS resistance and replication-associated X-shaped molecules, and consistent with earlier findings, the deletion of TOP3 from sgs1Δ mutants causes no additional change in crossover events (12, 54). Similarly, overexpression of Top3 in the sgs1Δ top3Δ background
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FIGURE 6. Unassisted Top3 promotes Rec-X resolution but not HJ resolution. A, wild-type, sgs1Δ, and sgs1Δ top3Δ cells were transformed with the ADE2 cassette, and the ratios of crossover-containing to total (crossover + non-crossover) colonies are plotted on the y axis. Data are from three independent experiments and error bars represent the S.E. * p < 0.05; NS, not significant. B, identical to A except testing the effects of Top3 overexpression. C and D, schematic: resolved products are indicated by the new spot in the right panel. Southern blot of genomic DNA extracted from sgs1Δ top3Δ cells containing either vector or Top3 overexpression plasmid, showing resolution of X-shaped molecules into branch migrated products (arrows) at the ARS305 adjacent fragment (C) and rDNA fragment (D). Samples were incubated at 65 °C in branch migration buffer (MBB) ± Mg2+ between the first and second dimensions of electrophoresis. Percentages of branch migration products are indicated in the top right corner of panels. E, MBB treatment of genomic DNA extracted from sgs1Δ top3Δ cells containing either vector or Top3 overexpression plasmid, showing alteration in rDNA migration, consistent with Rec-X structures as indicated in the schematic. F, 8% native polyacrylamide gel showing MBB digestion products of a 5'-32P-end labeled 50-base long oligonucleotide alone (ssDNA) or annealed into a synthetic HJ substrate that was added into genomic DNA MBB digests under the same conditions as in E. The percentages of substrates digested are shown on the right. Note that the samples for C and E are from cells grown for 1.5 h in 0.033% MMS, prior to Top3-induced differences in X-structure resolution, whereas samples for D are from cells 2 h after release from MMS.

did not alter the frequency of crossover events (Fig. 6B). These findings are consistent with the idea that Top3 cannot by itself resolve HJ intermediates, and support the idea that the X-shaped intermediates that are resolved by such unassisted Top3 during replication are likely not HJ intermediates.

The X-shaped Molecules That Accumulate without Top3 Are Rec-X Structures—Previous analyses of the MMS-induced recombination intermediates that accumulate within sgs1Δ mutants revealed that they are Rec-X structures which, unlike HJs, can branch migrate unhindered by magnesium and are susceptible to cleavage by MBN (13). Although we expected a similar identity for the X-structures in sgs1Δ top3Δ, it was conceivable that they were instead HJs or regressed forks (i.e., “chicken foot” structures). We examined samples from cells grown for 1.5 h in 0.033% MMS, prior to Top3-induced differences in X-structure resolution. As predicted, the MMS-induced X-shaped molecules in sgs1Δ top3Δ mutants showed an equivalent capacity to be branch migrated to resolution in the absence of Mg2+ (Fig. 6, C and D). Furthermore, they demonstrated sensitivity to cleavage by MBN as well as a migration pattern consistent with the digested products of Rec-X structures (Fig. 6E). MBN specificity under these conditions was confirmed by demonstrating efficient cleavage of trace quantities of 5'-32P-end labeled ssDNA but not HJ substrates added into genomic DNA digestion reactions that were otherwise identical to those above (Fig. 6F).

Unassisted Top3 Decatenates Rec-X but Not dHJ Substrates in Vitro—To confirm that unassisted Top3 can directly and selectively resolve Rec-Xs, we generated synthetic Rec-X and dHJ substrates, and tested their resolution by purified Top3 in vitro. The dHJ substrate has been described previously (15, 42, 43) and was assembled from two 80-mer, each forming intrastand hairpinned duplex arms and two interstrand plectonemically coiled duplexes, each ~1.4 helical turns in length (Fig. 7A). The Rec-X was derived from the dHJ 80-mers, with one strand modified such that the two strands formed only one of the duplexes, and had unpaired “outside” strands (Fig. 7, A and B; see “Experimental Procedures”). The Rec-X structure was confirmed using site-specific endonucleases together with exonucleases (Fig. 7C). Each substrate contained a single 32P-labeled strand and a total of two interlinks between strands.

Top3 was incubated with each substrate and strand decatination was assessed using denaturing PAGE to separate the substrates from products (Fig. 8). A range of temperatures was examined, because Top3 topoisomerase activity is enhanced at elevated temperatures (25, 44, 55). Consistent with previous studies demonstrating that the dHJ substrate is resistant to Top3 alone but can be completely dissolved by Top3 combined with Sgs1 (11), Top3 was unable to decatenate the dHJ substrate even at high temperatures. However, Top3 displayed robust decatenation activity on the Rec-X substrate (up to 76%), in a temperature-dependent fashion (Fig. 8, A and B). Increased temperature alone did not result in decatenation of the Rec-X substrate, as evidenced by the no protein control, which was also treated at the highest temperature (47 °C). We suspect that the increased temperature could allow the Rec-X to adopt partial ssDNA character, making it a better substrate for Top3. However, we note that this does not appear to explain the specificity of Top3 for the Rec-X substrate because elevated temperatures should affect dHJ substrate similarly. It is also clear that the Rec-X retains significant duplex character at elevated temperatures because it is cleaved efficiently by the TaqI endonuclease at 47 °C (Fig. 7). Decatenation of the Rec-X was dependent on the catalytic activity of Top3, as the catalytically inactive Top3-Y356F was incapable of resolving the structure (Fig. 8, C and D). As expected, incubation with the entire STR complex showed robust decatenation of both the dHJ and the Rec-X (Fig.
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FIGURE 7. Characterization of synthetic Rec-X structure. A, schematic representations of the Rec-X and dHJ substrates, showing where the two oligonucleotides are topologically interlinked. For simplicity, the helical turns are not drawn in subsequent figures. B, Rec-X map showing the sequences of the RecX1 and dHJ2 oligos that compose it, regions of double-stranded DNA, and RsaI, HhaI, and TaqI cut sites. The 32P-labeled phosphate is indicated by an asterisk. C, 8% polyacrylamide denaturing gel confirming the structure of Rec-X.

DISCUSSION

Here we show that the type 1A topoisomerase Top3 can confer significant resistance to MMS-induced DNA damage in the absence of its STR complex partners, Sgs1 and Rmi1. This function of Top3 requires its catalytic activity and also involves HR pathways, as strains defective in the accumulation of X-shaped recombination intermediates (sgs1Δ rad52Δ, sgs1Δ shut1Δ mph1Δ, or sgs1Δ rad18Δ mutants) failed to show altered DNA damage sensitivity upon TOP3 deletion or overexpression. Consistent with these findings, Top3 overexpression diminished X-structures visualized by two-dimensional gel electrophoresis and Southern blotting, indicating that Top3 promotes resolution of X-structures.

Although we have no reason to believe that Top3 functions apart from the STR complex under natural conditions, we took advantage of its unassisted activity to investigate the MMS-induced X-shaped replication intermediates, whose structure has been a matter of debate (56). In particular, recent investigations of factors (e.g. HJ resolvases) capable of resolving recombination intermediates that accumulate within STR mutants have concluded that the X-shaped intermediates previously characterized as Rec-Xs instead represent unresolved HJs (52, 53). However, to our knowledge, in all cases where X-structures induced by replication in the presence of MMS have been examined at a biochemical level, they have been found to have features most consistent with Rec-X species rather than HJs (13, 18, 20). These features include branch...
meflin unimpeded by Mg$^{2+}$, susceptibility of the outside single-stranded regions to cleavage by MBN, and resistance to cleavage by nucleases that cleave HJs, including T4 endonuclease VII and RuvC. On the other hand, it is clear that X-shaped intermediates formed during DSBR such as during meiotic crossover events are HJs (57). During DSBR, the STR complex promotes the branch migration of double HJs and ultimately their mutual removal by a dissolution mechanism. Consistent with a role for unassisted Top3 in the resolution of Rec-X intermediates but not HJs, we found that Top3 overexpression did not impact crossover frequencies in a DSBR assay, and that sgs1Δ top3Δ mutants had crossover levels no greater than sgs1Δ mutants. These observations are in agreement with previous studies (12, 54). We also note that although conversion of a stalled replication fork to a DSB is presumably followed by formation of a HJ during HR-dependent resumption of replication, MMS does not actually cause significant levels of DSBs, consistent with MMS-induced X-structures not being HJs (58). How then, can one reconcile the apparently conflicting viewpoints concerning the nature of replication-related X-structures? We suggest two non-mutually exclusive possibilities. First, the HJ resolvases identified as assisting in the resolution of X-shaped molecules within STR complex mutants (Mus81/Mms4, RusA, and Gen1(1–527)) may process Rec-X substrates,
although perhaps inefficiently. These nucleases clearly process HJs, but when carefully tested in vitro, each of these nucleases also binds and cleaves additional substrates (59–64). Because the discovery of Rec-X intermediates is still rather new, none of these identified HJ resolvases have been tested to see if they possess in vitro activity against Rec-X molecules. Indeed, to our knowledge, our in vitro studies with Top3 represent the first construction of a synthetic Rec-X substrate. We speculate that because Rec-X molecules have properties in common with HJs and replication forks, both of which Mus81/Mms4, RusA, and Gen1 all bind and act upon, it would not be surprising if each of these enzymes can process Rec-X molecules. Mus81/Mms4 in particular is a good candidate Rec-X resolvase as it cleaves HJs very inefficiently in vitro (65), but nonetheless has been demonstrated to be the enzyme responsible for resolution of MMS-induced X-spikes in the absence of the STR complex (31, 53). The second possibility is that Rec-Xs might transiently assume a more HJ-like character, potentially allowing processing by HJ resolvases. This conversion of a Rec-X into a HJ might take place by base-pairing the outside strands at a Rec-X junction (via paranemic coiling) to generate a HJ (Fig. 9). The very slow rate at which X-spikes are removed by overexpressed HJ resolvases is consistent with both of these models (52, 53). Ultimately, to settle this issue it will be important to test the activity of HJ resolvases on model Rec-X structures and also to perform more detailed structural studies on the X-shaped molecules that accumulate during replication of damaged templates.

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