A DNA nick at Ku-blocked double-strand break ends serves as an entry site for exonuclease 1 (Exo1) or Sgs1–Dna2 in long-range DNA end resection

Weibin Wang, James M. Daley, Youngho Kwon, Xiaoyu Xue, Danielle S. Krasner, Adam S. Miller, Kevin A. Nguyen, Elizabeth A. Williamson, Eun Yong Shim, Sang Eun Lee, Robert Hromas, and Patrick Sung

From the Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, Connecticut 06520 and Departments of Biochemistry and Structural Biology, Medicine, Radiation Oncology, and Molecular Medicine, University of Texas Health Science Center, San Antonio, Texas 78229

The repair of DNA double-strand breaks (DSBs) by homologous recombination (HR) is initiated by nucleolytic resection of the DNA break ends. The current model, being based primarily on genetic analyses in Saccharomyces cerevisiae and companion biochemical reconstitution studies, posits that end resection proceeds in two distinct stages. Specifically, the initiation of resection is mediated by the nuclease activity of the Mre11–Rad50–Xrs2 (MRX) complex in conjunction with its cofactor Sae2, and long-range resection is carried out by exonuclease 1 (Exo1) or the Sgs1–Top3–Rmi1–Dna2 ensemble. Using fully reconstituted systems, we show here that DNA with ends occluded by the DNA end-joining factor Ku70–Ku80 becomes a suitable substrate for long-range 5′–3′ resection when a nick is introduced at a locale proximal to one of the Ku-bound DNA ends. We also show that Sgs1 can unwind duplex DNA harboring a nick, in a manner dependent on a species-specific interaction with the ssDNA-binding factor replication protein A (RPA). These biochemical systems and results will be valuable for guiding future endeavors directed at delineating the mechanistic intricacy of DNA end resection in eukaryotes.

DNA double-strand breaks (DSBs) are induced by reactive metabolites and upon exposure to ionizing radiation or perturbations during DNA replication. If left unrepaird or repaired inappropriately, these lesions can cause chromosome aberrations and rearrangements, leading to senescence or apoptosis, cell transformation, and cancer. The repair of DNA double-strand breaks (DSBs) are induced by reactive metabolites and upon exposure to ionizing radiation or perturbations during DNA replication. If left unrepaird or repaired inappropriately, these lesions can cause chromosome aberrations and rearrangements, leading to senescence or apoptosis, cell transformation, and cancer.
and the tumor suppressor BRCA2 in complex with the DSS1 protein in humans (27–31).

Previous studies from our laboratory and by others have provided direct biochemical evidence that Ku shields DNA ends from exonucleolytic digestion but triggers endonucleolytic scission by MRX–Sae2 (21, 22, 32). However, whether the DNA nick introduced by MRX–Sae2 serves directly as a suitable entry site for Exo1 or Sgs1–Dna2 to initiate long-range resection remains to be determined. Here, using reconstituted systems with highly purified end resection factors and plasmid-length DNA substrates, we show that 1) Ku binding to DNA ends poses an obstacle to Exo1 or Sgs1–Dna2, but this inhibitory effect is relieved by the introduction of a DNA nick proximal to one of the Ku-bound DNA ends; 2) both Exo1 and Sgs1–Dna2 can carry out long-range DNA end resection from a DNA nick with a 5’ to 3’ polarity; and 3) Sgs1 helicase is able to unwind double-stranded DNA (dsDNA) harboring a nick or a gap, which depends on its species-specific interaction with RPA.

These findings support a model wherein a DNA nick created by MRX–Sae2 at Ku-blocked DSB ends can serve as the entry site for Exo1 or Sgs1–Dna2. These findings are likely relevant for understanding the mechanism of resection of other types of protein-blocked DSB ends, such as Spo11-conjugated ends at meiotic DSBs or arrested topoisomerase–DNA conjugates (33, 34).

Results

Relief of Ku-mediated end resection restriction by a DNA nick

MRX–Sae2 incises the 5’-terminated DNA strand endonucleolytically within the vicinity of Ku-occluded DNA ends (21, 22). To mimic the endonucleolytically cleaved DNA generated by MRX–Sae2, we inserted an Nt.BbvCI nicking endonuclease recognition sequence proximal to the DNA end. An incision site 59 nucleotides (nt) away from the 5’-terminated DNA end was introduced by Nt.BbvCI digestion (Fig. 1A). We first tested the nuclease activity of Exo1 on the 3’-32P-labeled nicked dsDNA. As shown in Fig. S1A, Exo1 was able to resect dsDNA or nicked dsDNA with a comparable efficiency when there was no Ku at the DNA ends. Likewise, in the absence of Ku, Sgs1–Dna2 was also able to resect dsDNA with or without a nick equally well (Fig. S1B).

We next tested the effect of Ku on Exo1- or Sgs1–Dna2-mediated long-range DNA end resection. On dsDNA without a nick, digestion by Exo1 or Sgs1–Dna2 was attenuated when we preincubated the DNA substrate with Ku, indicating that Ku restricts the access of Exo1 or Sgs1–Dna2 to DNA ends (Fig. 1, B and C). Importantly, the inhibitory effect of Ku on Exo1- or Sgs1–Dna2-mediated resection was significantly lessened upon introducing the site-specific nick into the DNA substrate (Fig. 1, B and C). Altogether, these results indicate that Ku protects DNA ends from nucleolytic digestion by Exo1 or Sgs1–Dna2 and also provide evidence that Exo1 and Sgs1–Dna2 can initiate DNA resection from a DNA nick. The latter premise is further validated in studies described below. Our previous findings indicate that MRX–Sae2 creates nicks 40 or 60 nt away from Ku-blocked DNA ends (21). To evaluate the effect of the distance between nick and DNA end on resection activity, DNA substrates with nicks in different positions (41, 59, or 82 nt away from DNA ends) were utilized. As shown in Fig. S1C, Exo1 processed these nicks equally well when DNA ends were blocked by Ku. Likewise, no obvious difference was seen with Sgs1–Dna2 on these DNA substrates (Fig. S1D).

DNA nick processing activity of Exo1 and Sgs1–Dna2

Based on the above observations (Fig. 1), we surmised that a DNA nick can serve as an entry site for Exo1- or Sgs1–Dna2-mediated resection. To eliminate the possibility of strand resection by Exo1 or Sgs1–Dna2 initiating from a free end, we constructed a circular dsDNA substrate that harbors a unique nick at which the 3’ terminus was labeled with 32P (Fig. 2A). As shown in Fig. S2A, Exo1 alone was able to digest the circular dsDNA.
nicked dsDNA substrate. The cleavage pattern of Exo1 on this DNA substrate was similar to that seen with bacteriophage T7 exonuclease, which has a strict 5′ → 3′ polarity (Fig. S2B) and is known to catalyze 5′ nucleotide removal at a DNA nick (35, 36).

We further tested Exo1 alone or in combination with RPA and/or MRX on the nick-containing DNA substrate. As shown in Fig. 2B, the digestion of the circular nicked dsDNA by Exo1 was enhanced by RPA or MRX. The stimulatory effect of RPA stems from its ability to sequester ssDNA to prevent the formation of nonproductive Exo1–ssDNA complexes (37), and MRX likely acts by recruiting Exo1 to the DNA substrate (38).

Dna2, Sgs1, and RPA constitute the minimal set of proteins capable of long-range DNA end resection (25, 26), and the combination of these proteins was sufficient to digest the nicked substrate (Fig. 2C). As expected, the final reaction product was not recognized by the radiolabeled probe P2500R that is complementary to the nicked DNA strand in the substrate (Fig. 3, D and F). Although Dna2 alone was able to digest circular ssDNA (39), this nuclease activity was undetectable when RPA was also present (Fig. 4, A and B). Taken together, these results support a model wherein Exo1 or Sgs1–Dna2–RPA specifically resects DNA in the 5′ → 3′ direction from the nick, generating a ssDNA circle that is protected by RPA from digestion.

Figure 2. Nuclease activity of Exo1 and Dna2–Sgs1–RPA on circular dsDNA with a nick. A, reaction schematic. The asterisk denotes the 32P label in the substrate. B, the circular nick-containing substrate (1 nM) was incubated with Exo1 (0.25 nM) in the presence of RPA (800 nM) and/or MRX (16 nM) for the indicated times. The results from three independent experiments were graphed with the error bars representing S.D. C, the activity of Dna2 (8 nM) was tested with combinations of Sgs1 (8 nM), RPA (800 nM), TR (8 nM), and MRX (16 nM) as indicated. The results were graphed as in B. See also Fig. S2.

Characteristics of Exo1- and Sgs1–Dna2-mediated resection from a DNA nick

We used Southern blotting to determine the directionality of Exo1- or Sgs1–Dna2-mediated resection that is initiated from a DNA nick in plasmid DNA. Two radiolabeled probes were used: P500 and P2500 that are complementary to the circular DNA strand near and further away from the 5′ terminus of the DNA nick (Fig. 3A). A time-course analysis showed that, at the early time points, nuclease products generated by Exo1 were detectable by P500 but not by P2500 (Fig. 3B), indicating that it carries out 5′ → 3′ resection from the nick. A 5′ → 3′ polarity of resection was also seen with Sgs1–Dna2 (Fig. 3C). The final reaction product, detectable by hybridization with either P500 or P2500 (Fig. 3, E and F), has the same size as that generated by T7 exonuclease (Fig. 3, E and F). As expected, the final reaction product was not recognized by the radiolabeled probe P2500R that is complementary to the nicked DNA strand in the substrate (Fig. 3, D and F).

Sgs1 unwinds circular dsDNA with a nick or gap

Sgs1 provides the helicase activity to unwind long stretches of duplex DNA during long-range end resection (25, 26). We therefore asked whether Sgs1 could release the strand that harbors the nick from the circular DNA substrate (Fig. 5A). Sgs1 could completely unwind a significant fraction of the nicked circular substrate when RPA was present (Fig. 5B). In contrast, the Mph1 helicase showed no activity on this substrate (Fig. S3A).
Consistent with published results (40, 41), affinity pulldown showed a robust physical interaction between Sgs1 and yeast RPA (Fig. S3C). In contrast, only a weak interaction of Sgs1 and human RPA (hRPA) was detected under the same reaction conditions (Fig. S3C). Importantly, the unwinding of the nicked DNA substrate by Sgs1 relies on its species-specific interaction with RPA as a much reduced level of fully unwound product occurred upon the substitution of yeast RPA with human RPA or with either of the human single-strand DNA-binding protein complexes SOSS1 and SOSS2 (Fig. 5C and Fig. S3B). The failure to stimulate Sgs1 is not due to protein inactivity as hRPA, SOSS1, and SOSS2 are fully capable of binding ssDNA (Fig. S3D). Although Escherichia coli SSB also supported DNA unwinding by Sgs1, its stimulatory effect was less than that of yeast RPA (Fig. 5C). Sgs1-mediated unwinding of the nicked substrate was also stimulated by TR and MRX individually (Fig. S3, E and F), and an additive effect was seen upon combining the latter two factors (Fig. 5D). We also verified that the stimulatory effect of TR and MRX on Sgs1-mediated unwinding is reliant on RPA (Fig. 5D).

A recent study from our laboratory has provided direct evidence that MRX–Sae2 utilizes its 3’ to 5’ exonuclease activity to create a DNA gap from an incision site (21). We therefore tested Sgs1 for the ability to unwind a 3-kb circular dsDNA containing a 46-nt DNA gap. As shown in Fig. 5E and Fig. S3G, Sgs1 was able to unwind the gapped substrate in the presence of RPA. Compared with the nicked DNA, the gapped substrate was unwound more efficiently by Sgs1 (Fig. 5E and Fig. S3G).

**Discussion**

By virtue of its abundance and high affinity for DNA ends, Ku is among the first protein factors that arrive at DSBs within the cellular setting where it not only promotes break repair by NHEJ (15, 16) but also restricts access of the end resection machinery in the G1 phase (42, 43). Recent studies have provided direct biochemical evidence that Ku shields DNA ends from exonucleolytic digestion but facilitates endonucleolytic scission by MRX–Sae2 (21, 22). The current model posits that the DNA nick introduced by MRX–Sae2 can be enlarged into a DNA gap via the 3’–5’ exonucleolytic action of this nuclease ensemble (21). However, whether DNA gap creation represents an obligatory step in long-range resection by Exo1 or the Sgs1–Dna2 complex or whether the DNA nick introduced by Mre11 is utilized as an entry site for either of the long-range resection nuclease entities is not known. This question has been addressed in our current study. Using reconstituted reactions with either Ku-bound linear dsDNA with a unique nick or nicked circular dsDNA, we have shown that resection by either
Exo1 or Sgs1–Dna2 occurs efficiently and with the 5′ to 3′ polarity observed with linear dsDNA harboring free ends (25, 26). We have also shown that DNA unwinding by Sgs1 initiated from either a DNA nick or a DNA gap is enhanced by the TR subcomplex and by MRX. In the case of the circular substrate, the covalently closed ssDNA is refractory to the endonucleolytic action of Dna2 when RPA is present, which is consistent with the observation that internal sites of RPA-bound 3′-tailed ssDNA are resistant to Sgs1–Dna2. Fig. 6 presents models depicting how a DNA nick introduced by MRX–Sae2 helps overcome the inhibitory effect of Ku on the initiation of long-range 5′ strand resection by Exo1 or Sgs1–Dna2. Herein, MRX generates the substrate for the recruitment of Exo1 or Sgs1–Dna2. This resembles the situation in mammalian DNA replication fork repair wherein the endonuclease EEPD1 nicks the stalled fork and loads Exo1 onto that structure (44).

The experimental systems described herein should be valuable for delineating mechanistic attributes of the eukaryotic resection machineries. For instance, it would be feasible to determine how DNA gap size may affect the recruitment and retention of Exo1 and the Sgs1–TR–Dna2 ensemble.

**Experimental procedures**

**DNA substrates**

The pBS-Nt.BbvCI plasmid was constructed by inserting the recognition sequence (CCTCAGC) for the DNA-nicking enzyme Nt.BbvCI into pBluescript II SK (−). To prepare the 3-kb linear dsDNA substrate, pBS-Nt.BbvCI plasmid DNA was digested with the restriction enzyme EcoRV (New England Biolabs). The resulting DNA was precipitated by adding isopropanol, washed with 70% (v/v) ethanol, and then dissolved in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). Then the DNA was 3′-labeled with [α-32P]dCTP (PerkinElmer Life Sciences) and terminal transferase (Roche Applied Science). The unincorporated radioactive nucleotide was removed using the QIAquick gel extraction kit (Qiagen). The radiolabeled DNA was treated with Nt.BbvCI (New England Biolabs) to introduce a DNA nick 59 nt away from the DNA end and then purified using the QIAquick gel extraction column. To prepare the linear DNA substrate with a DNA nick 41 or 82 nt away from the DNA end, pBS-Nt.BbvCI plasmid DNA was digested with Smal or Sall (New England Biolabs) followed by the procedures as described above.

The circular dsDNA substrate with a nick was prepared by digesting pBS-Nt.BbvCI plasmid DNA with Nt.BbvCI followed by DNA precipitation with isopropanol and purified as above. Circular nicked dsDNA was either 3′-labeled with 32P as described above or 5′-labeled with [γ-32P]ATP (PerkinElmer Life Sciences) and T4 polynucleotide kinase (New England Biolabs). Before 5′-end labeling, circular nicked dsDNA was dephosphorylated with shrimp alkaline phosphatase (New England Biolabs).

The pG46 plasmid was constructed by inserting five tandem repeats of Nt.BbvCI recognition sequences into pBluescript II SK (−). To prepare the circular gapped dsDNA substrate containing a 46-nt gap, pG46 plasmid DNA was digested by Nt.BbvCI followed by purification with a QIAquick gel extraction column at 80–85 °C. Circular gapped dsDNA was 3′-labeled with 32P as described above.

**Expression and purification of SOSS1 and SOSS2 complexes**

The coding sequences for hSSB1 with an N-terminal His6 tag, INTS3 with a C-terminal FLAG tag, and hSSBIP1 with a C-terminal Strep tag were amplified by PCR. The PCR products were inserted into the MacroBac 11A plasmid using ligation-independent cloning (45). To generate 11A-SOSS1 plasmid, the biobrick subcloning strategy was used to combine expression cassettes from 11A-hSSB1, 11A-INTS3, and 11A-hSSBIP1. Bacmid and baculovirus were prepared in DH10Bac and Trichoplusia ni High Five cells, respectively, following the manufacturer’s protocol (Bac-to-Bac baculovirus expression system, Invitrogen). For SOSS1 expression, Trichoplusia ni High Five cells (1 × 106 cells/ml) were infected with high-titer P3 baculovirus. Cells were harvested after 46 h and stored at −80 °C. All the subsequent steps were carried out at 0–4 °C.

The cell pellet (~16 g from 1 liter of culture) was suspended in 50 ml of 10 mM buffer (25 mM Tris-HCl, 10% glycerol, 0.5 mM EDTA) with 300 mM KCl and protease inhibitors. After sonication for 1 min, the cell lysate was clarified by ultracentrifugation at 16,000 g from 1 liter of culture. In the case of 11A-SOSS1 expression, the cell pellet (~16.9 g from 1 liter of culture) was suspended in 20 ml of buffer (10 mM Tris-HCl, 10% glycerol, 0.5 mM EDTA) with 300 mM KCl and protease inhibitors. After sonication for 1 min, the cell lysate was clarified by ultracentrifugation at 30,000 g from 1 liter of culture. The clarified lysate was treated with DNase I (New England Biolabs) before and after the purification steps, respectively.
DNA nick processing by Exo1 and Sgs1–Dna2

100,000 \times g for 1 h. The supernatant was incubated with 4 ml of nickel-nitrilotriacetic acid-agarose (Qiagen) for 1.5 h with constant mixing followed by washing the resin with 100 ml of T buffer containing 300 mM KCl and 20 mM imidazole. Bound proteins were eluted with 8 ml of T buffer containing 300 mM KCl and 200 mM imidazole for 10 min with gentle agitation. The protein eluate was mixed gently with 2 ml of anti-FLAG M2 affinity gel (Sigma) for 2 h. After washing the resin with 100 ml

Figure 5. Sgs1-mediated unwinding of circular dsDNA substrates with a nick or gap. A, reaction schematic involving the use of a nick-containing circular DNA substrate. The asterisk denotes the $^{32}$P label in the substrate. B, Sgs1 (1, 2, 4, 8, 16, and 32 nM) was tested on the nick-containing DNA substrate (1 nM) in the presence of RPA (800 nM). The results from three independent experiments were graphed with the error bars representing S.D. HD, heat denaturation. C, yeast RPA, hRPA, human SSO1 and SSO2, and E. coli SSB (800 nM each) were tested for their effect on unwinding of the nick-containing substrate by Sgs1 (32 nM) as in B. The results were graphed as in B. D, TR (4 nM) and MRX (8 nM) were tested, alone or in combination, for their effect on unwinding of the nick-containing substrate by Sgs1 (32 nM) and RPA (800 nM) as in B. The results were graphed as in B. E, the nick-containing or gapped circular dsDNA (0.5 nM each) was incubated with Sgs1 (8, 16, and 32 nM) and RPA (400 nM). The asterisk denotes the $^{32}$P label in the substrate. See also Fig. S3.

Figure 6. Exo1- or Dna2-mediated 5'-strand resection from an entry site created by MRX–Sae2. Ku, while protecting the DNA end from exonucleolytic digestion by Exo1, promotes 5'-strand endonucleolytic cleavage by MRX–Sae2. The resulting nick can serve as an entry site for Exo1 (A) or Sgs1–TR–Dna2–RPA (B) to carry out long-range DNA end resection in the 5’–3’ direction.
of T buffer containing 150 mM KCl, SOSS1 was eluted with 6 ml of T buffer containing 100 mM KCl and 250 ng/μl FLAG peptide for 20 min. The protein eluate was fractionated in a 6-ml SP Sepharose column (GE Healthcare) with a 72-ml gradient of 50–350 mM KCl in T buffer. The SOSS1 peak fractions were pooled, concentrated in an Amicon Ultra 30K microcentrator, and subjected to gel filtration in a 24-ml Superdex 200 column (GE Healthcare) in T buffer containing 30 mg KCl. The SOSS1 peak fractions were concentrated to ~7 mg/ml and stored in small aliquots at ~80 °C. The yield of highly purified SOSS1 was ~1.5 mg.

The 11A-SOSS2 plasmid was generated by combining the expression cassettes of 11A-hSSB2, 11A-INTS3, and 11A-hSSBP1. We followed the procedure developed for SOSS1 to express and purify the SOSS2 complex. The yield of highly purified SOSS2 was ~1.5 mg.

Other proteins

Yeast Top3 and Rmi1 proteins were expressed in E. coli, purified to near homogeneity, and used for the assembly of the Top3–Rmi1 complex as described previously (25). Exo1 and Ku were expressed in insect and yeast cells, respectively, and purified to near homogeneity according to our published procedures (46, 47). Dna2 was purified from yeast cells overexpressing the protein as described previously (48). Sgs1 and Mph1 were expressed and purified from insect cells as reported before (25, 49). The yeast and human RPA complexes were purified as described previously (50, 51). E. coli SSB was purchased from Promega, and T7 exonuclease was purchased from New England Biolabs. Mre11, Rad50, and Xrs2 were expressed and purified from yeast, and the MRX complex was assembled according to our published procedures (52–54).

Nuclease reactions and Southern blotting hybridization

Nuclease reactions were performed with 1 mM DNA substrate and the indicated concentration of Exo1 or Dna2–Sgs1–RPA in 12 μl of R buffer (25 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 1 mM DTT, 100 μg/ml BSA, 2 mM ATP, 20 mM creatine phosphate, and 30 μg/ml creatine kinase) containing 100 mM KCl (final concentration). Where indicated, Ku was added prior to Exo1 or Dna2–Sgs1–RPA to occupy the DNA ends. After a 30-min incubation at 30 °C, the reactions were terminated by treatment with SDS (0.5%, w/v) and proteinase K (1 mg/ml) at 37 °C for 20 min. After adding 4 μl of 4× loading buffer (20 mM Tris-HCl, pH 7.5, 40% (v/v) glycerol, 2 mM EDTA, and 0.2% (w/v) orange G), the reaction mixtures were resolved in a 1% (w/v) agarose gel in TAE buffer (40 mM Tris acetate and 1 mM EDTA, pH 8.2). Gels were dried onto positively charged nylon membrane (GE Healthcare) and subjected to phosphorimaging analysis. Gel images were quantified using Quantity One software (Bio-Rad) based on the loss of the DNA substrate radioactivity. Nuclease reactions with circular ssDNA 5′-X174 were analyzed by agarose gel electrophoresis and ethidium bromide staining. Gel images were captured using the G:BOX gel imaging system (Syngene) and quantified using TotalLab software based on the disappearance of the substrate band.

Southern blotting hybridization with radiolabeled oligonucleotide probes was performed to analyze the resection polarity of Exo1 or Dna2 on circular nicked dsDNA. First, a standard nuclease reaction was carried out as described above. Upon deproteinization with SDS and proteinase K, 3′-radiolabeled probe P500, P2500, or P2500R was added. The reaction mixtures were incubated in a thermocycler (being held at 70 °C for 5 min and then cooled to 20 °C over 125 min) and further analyzed as described above.

Helicase assay

Helicase assays were carried out in 12 μl of R buffer containing 100 mM KCl. The radiolabeled DNA substrate (1 nM) was incubated with the indicated concentrations of Sgs1, TR, and/or MRX either with or without RPA (800 nM). After a 30-min incubation at 30 °C, reaction mixtures were deproteinized and analyzed as described above.

Electrophoretic mobility shift assay

The indicated concentrations of hRPA, SOSS1, and SOSS2 were incubated with 20 nM 90-nt ssDNA in 12 μl of buffer (25 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 1 mM DTT, and 100 μg/ml BSA) containing 100 mM KCl (final concentration). After a 10-min incubation at 37 °C, the reaction mixtures were resolved in a 4% (w/v) native polyacrylamide gel in TAE buffer on ice. Gels were dried onto filter paper and subjected to phosphorimaging analysis.

Affinity pulldown assay

To test for interaction between Sgs1 and yeast or human RPA, the protein pairs (350 ng of each) were incubated on ice in 30 μl of T buffer containing 50 mM KC1 and 20 mM imidazole for 1 h. Then 15 μl of nickel-nitrioltriacetic acid–agarose (Qia-gen) was added to the reaction mixture to capture the protein complex. After periodic gentle mixing over 1 h, the supernatant was removed, and the resin was washed three times with 200 μl of the same buffer. Bound proteins were eluted from the resin in 40 μl of 1× SDS-PAGE loading buffer (30 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 1% (w/v) SDS, and 0.01% (w/v) bromphenol blue). The supernatant, wash, and eluate fractions were subjected to Western blot analysis with antibodies as indicated.


References


DNA nick processing by Exo1 and Sgs1–Dna2

DNA nick processing by Exo1 and Sgs1–Dna2


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