Requirement of Nse1, a Subunit of the Smc5-Smc6 Complex, for Rad52-Dependent Postreplication Repair of UV-Damaged DNA in *Saccharomyces cerevisiae*[∇]

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In *Saccharomyces cerevisiae*, postreplication repair (PRR) of UV-damaged DNA occurs by a Rad6-Rad18- and an Mms2-Ubc13-Rad5-dependent pathway or by a Rad52-dependent pathway. The Rad5 DNA helicase activity is specialized for promoting replication fork regression and template switching; previously, we suggested a role for the Rad5-dependent PRR pathway when the lesion is located on the leading strand and a role for the Rad52 pathway when the lesion is located on the lagging strand. In this study, we present evidence for the requirement of Nse1, a subunit of the Smc5-Smc6 complex, in Rad52-dependent PRR, and our genetic analyses suggest a role for the Nse1 and Mms21 E3 ligase activities associated with this complex in this repair mode. We discuss the possible ways by which the Smc5-Smc6 complex, including its associated ubiquitin ligase and SUMO ligase activities, might contribute to the Rad52-dependent nonrecombinational and recombinational modes of PRR.

Genetic studies of the yeast *Saccharomyces cerevisiae* have revealed a prominent role for the Rad6-Rad18 ubiquitin-conjugating enzyme (Ubc) complex (2, 3) in promoting replication through DNA lesions that block the progression of the replication fork. The Rad6-Rad18-dependent lesion bypass processes include translesion synthesis (TLS) by DNA polymerase η (Pol η) and Pol ζ and an Mms2-Ubc13-Rad5-dependent postreplication repair (PRR) pathway which repairs the discontinuities that form in the DNA synthesized from damaged templates (23, 34, 39, 44).

For UV-induced DNA lesions, Pol η contributes to error-free TLS by virtue of its proficient and accurate ability to synthesize DNA opposite from the cyclobutane pyrimidine dimers (CPDs) (23, 26, 48, 49). By contrast, Pol ζ contributes to mutagenic TLS by means of its proficient ability to extend from the nucleotides inserted opposite the CPD or the (6-4) photoproduct by another DNA polymerase (21, 25). The Mms2-Ubc13-Rad5-controlled PRR pathway is likely to involve a transient template-switching mechanism and a copy choice type of DNA synthesis wherein the newly synthesized daughter strand of the undamaged complementary strand is used as a template for bypassing the lesion in an error-free way (4).

The Rad6-Rad18 enzyme complex modulates lesion bypass through its role in PCNA ubiquitination. In DNA-damaged yeast cells, PCNA is first monoubiquitinated at its lysine 164 residue by the Rad6-Rad18 enzyme; subsequently, this lysine residue is polyubiquitinated via a lysine 63-linked polyubiquitin chain by the Mms2-Ubc13-Rad5 enzyme complex (16). In this complex, Mms2-Ubc13 carries out the assembly of the lysine 63-linked polyubiquitination chain (17), and Rad5 functions in

the ubiquitination reaction as an E3 ubiquitin ligase (16, 46). In addition to having a regulatory role as an E3 ligase in PCNA polyubiquitination, Rad5 is expected to act directly in the template-switching process via its DNA helicase activity (4, 9). Rad5, a member of the Swi2/Snf2 family of ATPases, has a DNA-dependent ATPase activity (22, 24), and we have recently shown that Rad5 has a DNA helicase activity that is uniquely suited for carrying out the replication fork regression reaction which is a prerequisite for template switching and the copy choice type of DNA synthesis (4).

Genes belonging to the *RAD52* epistasis group function independently of *RAD6* and *RAD18*, and in contrast to the very high UV sensitivity conferred by the *rad6* Δ and *rad18* Δ mutations, the deletion of *RAD52* or of any other gene of this group confers only a modest increase in UV sensitivity (for example, see reference 41). More recently, we have provided evidence for the involvement of *RAD52* group genes in promoting PRR in UV-damaged yeast cells. The efficiency of PRR is reduced in yeast strains harboring the *rad51* Δ , *rad52* Δ , or *rad54* Δ mutation, and an almost complete inhibition of PRR occurs in the absence of both the Rad5 and Rad52 proteins (10). Thus, PRR in yeast cells is modulated by two pathways: a Rad5-dependent pathway, which in addition requires the Ubc functions of the Rad6-Rad18 and Mms2-Ubc13 enzymes, and a Rad52-dependent pathway, which in addition requires Rad51, Rad54, and presumably the other proteins that function with this group of proteins.

Structural maintenance of chromosome (Smc) proteins play fundamental roles in chromosome organization and dynamics and also in DNA repair (20, 31). In eukaryotes, including in yeast, six different Smc proteins have been identified. Smc1 and Smc3 form the core of the cohesin complex which holds the sister chromatids together following replication, Smc2 and Smc4 form the core of the condensin complex involved in chromosome condensation, and the Smc5-Smc6 complex functions in DNA repair (20, 31). In the Smc heterodimers, each

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Smc subunit folds onto itself by antiparallel coiled-coil interactions, forming an elongated arm with an ATP-binding "head" domain at one end and a "hinge" domain at the other. The two Smc subunits associate with each other at their hinge domain, forming a V-shaped structure. The opening and closing of the head-head domains are dependent upon ATP hydrolysis and have been proposed to drive the interactions of the Smc complexes with the DNA (15).

A role for the Smc5-Smc6 complex in DNA repair has been suggested from the sensitivity conferred by mutations in the various subunits of this complex to DNA-damaging agents (7, 8, 18, 29, 32, 38, 47). The Smc5-Smc6 complex has been purified from *S. cerevisiae*, and it has six additional subunits: Nse1, Mms21 (Nse2), Nse3 (YDR288w), Nse4 (Ori2), Kre29 (Nse5), and YML023C (Nse6) (14). Smc5, Smc6, and the other six subunits of this complex are all essential for cell viability (14).

A role for the Smc5-Smc6 complex in the repair of double-strand (ds) breaks in DNA has been inferred from genetic analyses of hypomorphic mutations in the Smc6 subunit (*rad18*) of *Schizosaccharomyces pombe*. These mutations confer a defect in the repair of ds breaks resulting from ionizing radiation damage, and based upon epistasis analyses, a role for Smc6 in Rad52-dependent recombinational repair has been suggested (7, 29, 47). Hypomorphic mutations of the Nse1, Mms21, and Nse4 subunits have also been identified in *S. cerevisiae*, and they confer sensitivity to DNA-damaging agents (8, 18, 38, 50). Of these, the Nse1 and Mms21 proteins are of particular interest since they contain sequence motifs characteristic of the E3 ubiquitin ligases and E3 small-ubiquitin-related modifier (SUMO) ligases, respectively, and Mms21 has been shown to promote the sumoylation of different protein substrates (1, 50).

Since mutations in the Nse1 and Mms21 subunits confer sensitivity to UV, methyl methanesulfonate (MMS), and gamma rays (8, 38, 50), in this study we have sought to examine the role of these subunits in the repair of UV-damaged DNA. In particular, we identify a temperature-sensitive (*ts*) mutation of *NSE1* and show that it inactivates the *RAD52*-dependent PRR pathway at the restrictive temperature. We discuss the possible involvement of the Nse1 and Mms21 E3 ligases and of the whole Smc5-Smc6 complex in promoting the PRR of UV-damaged DNA via the Rad52-dependent pathway.

MATERIALS AND METHODS

Strains. All yeast strains used for epistasis analyses and for alkaline sucrose gradient sedimentation experiments are isogenic to the wild-type strain EMY74.7 (*MATa his3-Δ1 leu2-3,112 trp1Δ ura3-52*). The following strains (shown with their relevant genotypes) were used for epistasis experiments: YRP727 *nse1-101*, YRP787 *nse1 C274A*, YMMS21-46 *mms21 C200A H202A*, YR14-43 *rad14Δ*, YR14-185 *nse1-101 rad14Δ*, YR14-217 *nse1 C274A rad14Δ*, YR14-215 *mms21 C200A H202A rad14Δ*, YR18-148 *rad18Δ*, YR18-131 *nse1-101 rad18Δ*, YR18-152 *nse1 C274A rad18Δ*, YR18-150 *mms21 C200A H202A rad18Δ*, YR52-90 *rad52Δ*, YR52-117 *nse1-101 rad52Δ*, YR52-121 *nse1 C274A rad52Δ*, YR52-123 *mms21 C200A H202A rad52Δ*, YR5-51 *rad5Δ*, YR5-326 *nse1-101 rad5Δ*, YR5-390 *nse1 C274A rad5Δ*, YMMS2-6 *mms2Δ*, YMMS2-92 *nse1-101 mms2Δ*, YRP551 *ubc13Δ*, YRP772 *nse1-101 ubc13Δ*, YPCNA41 *pol30-119*, YPCNA101 *nse1-101 pol30-119*, YPCNA103 *nse1 C274A pol30-119*, YPCNA106 *mms21 C200A H202A pol30-119*, and YR52-51 *pol30-119 rad52Δ*. The following yeast strains, which were made [*rho*⁰] by growth in ethidium bromide to generate strains lacking mitochondrial DNA, were used for PRR experiments: YR1-65 *rad1Δ*, YR1-373 *nse1-101 rad1Δ*, YR1-118 *rad1Δ rad5Δ*, YR1-398 *nse1-101 rad1Δ rad5Δ*, YR1-231 *rad1Δ rad52Δ*, and YR1-394 *nse1-101 rad1Δ rad52Δ*.

To avoid confusion between low-molecular-size nuclear DNA synthesized

after UV irradiation and undamaged mitochondrial DNA, [*rho*⁰] strains are used for PRR experiments. Even though [*rho*⁰] strains are respiration deficient, in medium containing glucose as the carbon source, the growth rates of [*rho*⁰] and [*rho*⁺] cells are very similar and any differences in PRR rates can be ascribed to the inactivation of the gene being studied.

Isolation of *nse1* and *mms21* mutations. Random mutations of the *NSE1* gene were generated by hydroxylamine treatment of plasmid pPM1162, a *CEN LEU2 NSE1* plasmid. A 3-kb DNA fragment containing the *NSE1* gene was first amplified from yeast genomic DNA by PCR and cloned into a *CEN LEU2* vector. The wild-type *NSE1* gene was then isolated from yeast by gap repair, generating plasmid pPM1162. pPM1162 (3 μg) was incubated with hydroxylamine for 1 h at 65°C before its introduction into the yeast strain YRP723, an *nse1Δ::URA3* isogenic derivative of EMY74.7 which is viable because it carries the wild-type *NSE1* gene on a *CEN URA3* vector (pPM1168). Transformants were replica plated on medium containing 5-fluorouracil to select for loss of the *NSE1 CEN URA3* plasmid. Colonies which grew on 5-fluorouracil were screened for the inability to grow at 37°C and for sensitivity to UV light and to MMS. One isolate conferred a growth defect at 37°C, exhibited sensitivity to MMS and UV at 30°C, and was named the *nse1-101* mutant. The *nse1-101* mutant gene was sequenced and found to contain three amino acid changes: glycine 175 to glutamic acid (G175E), serine 207 to threonine (S207T), and glycine 332 to aspartic acid (G332D). The *nse1 C274A* mutant containing a mutation in one of the residues of the RING finger motif of Nse1 was generated by site-directed mutagenesis of plasmid pPM1162. The *mms21 C200A H202A* mutant (*mms21 RING*) was generated by site-directed mutagenesis using plasmid pMMS21-44, which carries DNA encoding the C-terminal region of Mms21 in pUC19. The *URA3* gene blaster fragment was then cloned downstream of the *mms21 RING* open reading frame, generating pMMS21-47, and this RING mutation was then integrated into the genome by direct replacement.

Assays for UV sensitivity. Cells were grown overnight at 30°C to mid-logarithmic phase in yeast extract-peptone-dextrose (YPD) medium, washed, sonicated to disperse cell clumps when necessary, and resuspended in sterile distilled water to a density of 2×10^8 cells per ml. A 200-μl aliquot of serial 10-fold dilutions was pipetted into a 96-well microtiter dish, followed by transfer to YPD plates. The plates were then UV irradiated at a dose rate of 0.1 or 1 J/m²/s and incubated at 30°C for 2 days before being photographed.

UV irradiation and sedimentation in alkaline sucrose gradients. Yeast strains were grown overnight at 30°C in synthetic complete medium lacking uracil but supplemented with uridine at 5 μg/ml. When the asynchronously growing cells reached a density of 0.5 to 1.0×10^7 cells per ml, they were UV irradiated at a dose rate of 0.1 J/m²/s in the same growth medium in 150- by 20-mm petri dishes, with constant stirring. All operations during and after UV irradiation were performed in yellow light to prevent photoreactivation of UV-induced CPDs. Cells were collected by filtration after UV irradiation and resuspended in fresh uridine medium at a density of 1×10^8 to 2×10^8 cells per ml. For pulse-labeling of DNA, 100 μCi of [⁶-³H]uracil (20 to 25 Ci/mmol, 1 mCi/ml; Moravsek Biochemicals and Radiochemicals, Brea, CA) was added to 1 ml of cells, followed by vigorous shaking for 15 min at 30°C. Cells were then washed, resuspended in high-uracil medium (synthetic complete medium containing uracil at 1.67 mg/ml), and incubated for an additional 30 min or 6 h at 30°C or at 37°C. Conversion of cells to spheroplasts, alkaline sucrose sedimentation, and the processing of samples were done as described previously (37, 45), except that alkaline sucrose gradients were centrifuged at 21,000 rpm for 15.5 h and acid precipitation of alkali-hydrolyzed samples was carried out with 1 N HCl containing 0.1 M sodium pyrophosphate.

RESULTS

Isolation of a *ts* mutation of *NSE1*. Since *NSE1* is an essential gene, we isolated a *ts* mutation of *NSE1*, *nse1-101*, which confers growth at 30°C but not at 37°C and which also confers sensitivity to DNA-damaging agents at the permissive temperature. The *nse1-101* mutant allele encodes three amino acid substitutions, G175E, S207T, and G332D (Fig. 1A). The *nse1-101* cells held at 37°C arrest in the cell cycle as large doublets, indicative of arrest at the G₂/M boundary, and even at 30°C, the mutant shows a preponderance of larger, multibudded cells compared to those of the wild-type strain (Fig. 1B). In addition to displaying *ts* growth at 37°C, the mutant cells display sensitivity to UV and MMS at 30°C (Fig. 1C).

FIG. 1. Isolation of the *nse1-101* mutant of *S. cerevisiae*. (A) Sequence alignment of the C termini of Nse1 proteins from various organisms. Multiple-sequence alignment was performed with CLUSTAL W version 1.82. Sequences aligned are *S. cerevisiae* Nse1 (ScNse1; 336 amino acids; GenBank accession no. NP_013107), *S. pombe* Nse1 (SpNse1; 232 amino acids; NP_588097), *Homo sapiens* Nse1 (HsNse1; 256 amino acids; NP_659547), and *Mus musculus* Nse1 (MmNse1; 280 amino acids; NP_080606). Shaded residues correspond to the C₄H₉ RING zinc finger-like motif. Residues with an asterisk (G175E, S207T, and G332D) are mutated in the *nse1-101* mutant. Cysteine 274 (●) was mutated to alanine (C274A) in the *nse1-101* mutant. (B) Photomicrograph of wild-type *NSE1* and *nse1-101* mutant cells grown at 30°C. (C) Sensitivity of the *nse1-101* mutant to DNA-damaging agents. (1) Growth on a YPD plate at 30°C for 2 days. (2) Growth deficiency on a YPD plate at the nonpermissive temperature (37°C). (3) Cells were spotted on a YPD plate, UV irradiated at 80 J/m², and incubated at 30°C for 2 days. (4) Cells were spotted on a YPD plate containing 0.035% MMS and incubated at 30°C for 2 days.

Epistasis analyses of *nse1-101* for UV sensitivity. To determine the epistasis relationship of *nse1-101* with genes that function in different repair pathways for UV damage, we combined the *nse1-101* mutation with the *rad14Δ*, *rad18Δ*, or *rad52Δ* mutation, defective in nucleotide excision repair, in the promotion of replication through DNA lesions, or in ds break repair and recombination, respectively. As shown in Fig. 2, both the *nse1-101 rad14Δ* and *nse1-101 rad18Δ* double mutant strains exhibit higher degrees of UV sensitivity than the corresponding *rad14Δ* and *rad18Δ* strains, whereas the UV sensitivity of the *nse1-101 rad52Δ* strain is more similar to that of the *rad52Δ* strain. These observations suggested that Nse1 functions in the repair of UV-damaged DNA in conjunction with the Rad52-dependent repair pathway. To further verify that Nse1 promotes repair in a Rad6-Rad18-independent manner, we examined the UV sensitivity conferred by the *nse1-101* mutation in combination with the *rad5Δ*, *mms2Δ*, or *ubc13Δ* mutation, which inactivates the Rad6-Rad18-dependent PRR pathway controlled by the Rad5-Mms2-Ubc13 complex (Fig. 2). The greater UV sensitivity of the *nse1-101 rad5Δ*, *nse1-101 mms2Δ*, and *nse1-101 ubc13Δ* double mutants than that of the corresponding *rad5Δ*, *mms2Δ*, and *ubc13Δ* single mutants adds further support for a role for Nse1 in a pathway that acts independently of the Rad6-Rad18 and Rad5-Mms2-Ubc13 enzyme complexes.

Impaired PRR in the *nse1-101* mutant. To determine if the *nse1-101* mutation has an adverse effect on the PRR of UV-damaged DNA, we examined the size of newly synthesized DNA in *rad1Δ nse1-101* mutant cells following UV irradiation. Because of the nucleotide excision repair defect, UV lesions persist in the *rad1Δ* strain, and replication through such lesions

requires the various lesion bypass processes. When *rad1Δ* cells are UV irradiated at 3.5 J/m² at 30°C and the size of the newly synthesized DNA is examined by pulse-labeling of DNA with [³H]uracil for 15 min and then chased for 30 min at 30°C, the DNA sediments toward the top of the alkaline sucrose gradient, indicative of the presence of discontinuities in the newly synthesized DNA. Further incubation of *rad1Δ* cells for 6 h at 30°C following the 15-min pulse after UV irradiation restores normal-sized DNA (Fig. 3A). When *rad1Δ nse1-101* cells are UV irradiated at 30°C and then given a 15-min pulse and a 6-h incubation at 30°C, normal-sized DNA is not reconstituted; instead, the size attained is intermediate, between that of the low-molecular-weight DNA that is formed in UV-irradiated cells following the 15-min pulse and 30-min chase period and that of the normal-sized DNA that is seen in unirradiated cells pulse-labeled for 15 min and then given a 6-h incubation period at 30°C (Fig. 3B). Commensurate with the *ts* phenotype, the *rad1Δ nse1-101* mutant displays a higher degree of the PRR defect at 37°C than at 30°C (Fig. 3B). In the unirradiated *rad1Δ nse1-101* mutant, however, normal-sized DNA is reconstituted in cells incubated at 37°C (Fig. 3B). These experiments were repeated a minimum of three times, and similar results were obtained. Since we have found no evidence for a defect in DNA replication in unirradiated *rad1Δ nse1-101* cells at the restrictive temperature and normal-sized DNA is formed in unirradiated *rad1Δ nse1-101* cells pulse-labeled for 15 min and then incubated for 6 h at 37°C (Fig. 3B), the persistence of low-molecular-weight DNA in UV-irradiated mutant cells held at 37°C must result from the PRR defect.

Involvement of Nse1 in Rad52-dependent PRR. Since Rad5 and Rad52 function in alternate PRR pathways for UV dam-

FIG. 2. Epistasis analysis of the *nse1-101* mutation. The *nse1-101* mutation enhances the UV sensitivity of the *rad14Δ*, *rad18Δ*, *rad5Δ*, *mms2Δ*, and *ubc13Δ* mutants but not of the *rad52Δ* mutant. The UV sensitivity of the *pol30-119* mutant, defective in lysine 164 ubiquitination and the sumoylation of PCNA, is enhanced in the presence of *nse1-101*. YPD plates containing 10-fold dilutions of exponentially growing yeast cells were UV irradiated at the indicated doses and incubated in the dark for 2 days at 30°C.

age (10), we examined whether, as suggested by the epistasis of the *nse1-101* mutation with the *rad52Δ* mutation, Nse1 functions together with the Rad52 group of proteins in PRR. As shown in Fig. 4C, compared to that in the *rad1Δ nse1-101* (Fig. 3B) and *rad1Δ rad5Δ* (Fig. 4A) cells, the PRR defect is greatly enhanced in *rad1Δ rad5Δ nse1-101* cells that are UV irradiated, pulse-labeled for 15 min, and then incubated for 6 h at 37°C. In fact, there is no evidence of any residual PRR in the mutant cells, indicating that Nse1 functions in PRR in a Rad5-independent manner. To verify that Nse1 promotes PRR through the Rad52 pathway, we examined PRR in *rad1Δ rad52Δ nse1-101* mutant cells (Fig. 4D) and found that the PRR defect in these mutant cells was no greater than that seen in the *rad1Δ nse1-101* mutant (Fig. 3B) or the *rad1Δ rad52Δ* mutant (Fig. 4B). From these observations, we conclude a role for Nse1 in the Rad52-dependent PRR of UV-damaged DNA. Since normal-size DNA was reconstituted in unirradiated *rad1Δ rad5Δ nse1-101* cells and in *rad1Δ rad52Δ nse1-101* cells which were pulse-labeled for 15 min and then incubated for 6 h at 37°C

(see Fig. 4C and D), the persistence of small-molecular-size DNA in these mutants when they are UV irradiated and held at 37°C reflects the PRR defect.

Interestingly, in the UV-irradiated *rad1Δ rad52Δ nse1-101* cells held at 37°C, the newly synthesized DNA shows more of a shift to a larger molecular size than is seen in the *rad1Δ nse1-101* cells (compare Fig. 3B with Fig. 4D). This suggests that in the absence of Nse1 function, the DNA structures that are generated from the action of Rad52 and other proteins, such as Rad51 and Rad54, are more prone to nucleolytic attack and degradation. We elaborate upon the implications of this observation for the role of the Smc5-Smc6 complex in Rad52-dependent PRR in Discussion.

A mutation in the E3 ubiquitin ligase motif of Nse1 affects DNA repair. Since the *nse1-101* mutation does not involve any alteration in the C₄HC₃ E3 ubiquitin ligase sequence motif, we changed the cysteine 274 present in this motif to alanine and examined the effect of the *nse1* C274A mutation on growth and sensitivity to DNA-damaging agents. The *nse1* C274A mutant

FIG. 3. Requirement of the *NSE1* gene for PRR of UV-damaged DNA. Sedimentation in alkaline sucrose gradients of nuclear DNA from cells incubated for different periods following UV irradiation. The *rad1Δ* (A) and *rad1Δ nse1-101* (B) strains were UV irradiated at 3.5 J/m² and then pulse-labeled with [³H]uracil for 15 min, followed by a 30-min chase (Δ), a 6-h chase at 30°C (●), or a 6-h chase at 37°C (■) in high-uracil medium. DNA synthesized in unirradiated cells was pulse-labeled with [³H]uracil for 15 min, which was followed by a 6-h chase (○) at 30°C for the *rad1Δ* strain and at 37°C for the *rad1Δ nse1-101* strain; in unirradiated *rad1Δ nse1-101* cells that were treated similarly but kept for 6 h at 30°C, normal-size DNA was reconstituted in a manner similar to that seen in cells held at 37°C. The data for the *rad1Δ* strain in panel A shown here for comparison are taken from reference 9.

exhibits a *ts* phenotype at 37°C (Fig. 5B); cells stop division as large, multibudded cells, indicative of a G₂/M checkpoint at 37°C, and even at 30°C, very large and multibudded clusters of cells are seen (Fig. 5A). Also, the mutant displays sensitivity to UV and MMS (Fig. 5B). Next, we examined the UV sensitivity conferred by the *nse1* C274A mutation in combination with the mutations in genes affecting different DNA repair pathways. As shown in Fig. 5C, the UV sensitivity resulting from the *rad14Δ*, *rad18Δ*, and *rad52Δ* mutations is enhanced when they are combined with the *nse1* C274A mutation, whereas the *nse1* C274A *rad52Δ* double mutant displays nearly the same degree of UV sensitivity as the *rad52Δ* mutant. These epistasis relationships suggest a role for the Nse1 E3 ubiquitin ligase function also in the Rad52-dependent PRR pathway. Because of the poor growth characteristics of the *nse1* C274A mutation, we have been unable to directly examine its effect on PRR.

Effect of a mutation in the E3 SUMO ligase function of Mms21. To determine whether the Mms21 SUMO ligase activity also contributes to Rad52-dependent PRR, we constructed a mutation in the E3 motif by changing the cysteine 200 and histidine 202 residues to alanines (Fig. 6A). The resulting mutation, *mms21* RING, confers a growth defect at 37°C, and cells stop division with a terminal, large, multibudded morphology even at 30°C (Fig. 6B and C). At 30°C, the mutant cells exhibit sensitivity to UV and MMS (Fig. 6C). Similar to what occurs with the *nse1* mutations, the *mms21* RING mutation increases the UV sensitivity of the *rad14Δ* and *rad18Δ* mutants, whereas with increasing UV fluence, the UV sensitivity of the *mms21* RING *rad52Δ* double mutant increases to nearly the same extent as that of the *rad52Δ* mutant (Fig. 6D). The epistasis of the *rad52Δ* and *mms21* RING mutations suggests an involvement of the Mms21 SUMO ligase function in Rad52-dependent PRR. However, with the *mms21*

RING mutation also, we have been unable to directly examine its effects on PRR because of the severe growth impairment.

Requirement of Nse1 and Mms21 functions for Rad52-dependent repair in the absence of PCNA sumoylation. Whereas ubiquitin attachment at the lysine 164 residue of PCNA activates the Rad6-Rad18-dependent lesion bypass processes, SUMO attachment at this PCNA residue keeps the Rad52-mediated recombinational pathway in check by promoting the binding of the Srs2 DNA helicase to PCNA (35, 36). In the absence of SUMO modification, as occurs in the *pol30* K164R mutation, the Rad52-dependent recombinational pathway becomes activated; as a consequence, the sensitivity to DNA-damaging agents conferred by the *pol30* K164R mutation is greatly enhanced in the absence of Rad52 (13).

To examine whether Nse1 and Mms21 also contribute to the Rad52-dependent recombinational repair which becomes activated in the absence of PCNA sumoylation at lysine 164, we combined the *nse1* and *mms21* mutations with the *pol30-119* mutation, which encodes the K164R alteration in PCNA. The UV sensitivity of the *pol30-119* mutant was greatly increased in the presence of the *nse1-101* (Fig. 2), *nse1* C274A (Fig. 5), and *mms21* RING (Fig. 6) mutations, indicating a role for the Nse1 and Mms21 E3 ligase activities in Rad52-dependent recombinational repair.

The *pol30-119 rad52Δ* mutant is extremely UV sensitive, presumably because in the absence of any functional Rad6-Rad18-dependent pathways, lesion bypass becomes highly dependent upon the Rad52-dependent recombinational process. To examine if the Nse1 and Mms21 E3 ligase activities were required to the same extent as Rad52 activity, we compared the UV sensitivity of the *pol30-119 nse1* C274A and *pol30-119 mms21* RING double mutants with the UV sensitivity of the *pol30-119 rad52Δ* mutant. Our observation that the *pol30-119*

FIG. 4. Involvement of *NSE1* in the *RAD52*-dependent PRR pathway of UV-damaged DNA. Sedimentation in alkaline sucrose gradients of nuclear DNA from cells incubated for different periods following UV irradiation. The *rad1Δ rad5Δ* (A), *rad1Δ rad52Δ* (B), *rad1Δ rad52Δ nse1-101* (C), and *rad1Δ rad52Δ nse1-101* (D) strains were UV irradiated at 3.5 J/m² and then pulse-labeled with [³H]uracil for 15 min, followed by a 30-min chase (Δ), a 6-h chase at 30°C (●), or a 6-h chase at 37°C (■) in high-uracil medium. DNA synthesized in unirradiated cells was pulse-labeled with [³H]uracil for 15 min, which was followed by a 6-h chase at 30°C (○) for the *rad1Δ rad5Δ* and *rad1Δ rad52Δ* strains and at 37°C for the *rad1Δ rad52Δ nse1-101* and *rad1Δ rad52Δ nse1-101* strains. The data for the *rad1Δ rad5Δ* strain in panel A and for the *rad1Δ rad52Δ* strain in panel B, which are shown here for comparison, are taken from references 9 and 10, respectively.

nse1 C274A and *pol30-119 mms21* RING mutants are not as UV sensitive as the *pol30-119 rad52Δ* mutant (Fig. 5C and 6D) suggests that the Nse1 and Mms21 E3 ligase functions affect the proficiency of Rad52-dependent recombinational repair but that they are not absolutely required.

DISCUSSION

Hypomorphic mutations in the various subunits of the Smc5-Smc6 complex confer sensitivity to a variety of DNA-damaging agents, including UV, MMS, and gamma rays. From the epistasis of *S. pombe* *Smc6* mutations with the mutations in the *RAD52* group of genes, a role for this complex in the Rad52-mediated recombinational repair pathway was inferred, and

the observation that ds breaks induced by gamma rays were not repaired in these mutants added further support to this suggestion (7, 29, 47). However, even though the requirement of Smc6, and presumably of the other subunits of this complex, in the recombinational repair of ds breaks provides a satisfactory explanation for the underlying basis of the gamma-ray sensitivity conferred by mutations in the Smc5-Smc6 group of genes, it fails to explain the UV and MMS sensitivities of mutations in this group of genes, since base damage is the predominant lesion formed by these DNA-damaging agents.

Over 30 years ago, we identified a mutation in the *MMS21* gene (*mms21-1*) which confers sensitivity to UV, MMS, and gamma rays (38). Also, in the absence of any treatment with DNA-damaging agents, the mutant cells exhibited a number of

FIG. 5. Epistasis analysis of the *nse1* C274A mutation. (A) Photomicrograph of *nse1* C274A mutant cells showing aberrant morphology compared with that of wild-type cells at 30°C. (B) Sensitivity of the *nse1* C274A mutant to DNA-damaging agents. (1) Growth on a YPD plate at 30°C for 2 days. (2) Growth deficiency on a YPD plate at the nonpermissive temperature (37°C). (3) Cells were spotted on a YPD plate, UV irradiated at 80 J/m², and incubated at 30°C for 2 days. (4) Cells were spotted on a YPD plate containing 0.035% MMS and incubated at 30°C for 2 days. (C) The *nse1* C274A mutation enhances the UV sensitivity of the *rad14Δ*, *rad18Δ*, *rad52Δ*, and *pol30-119* mutants but has no significant effect on the UV sensitivity of the *rad5Δ* mutant. YPD plates containing 10-fold dilutions of exponentially growing yeast cells were UV irradiated at the indicated doses and incubated in the dark for 2 days at 30°C.

other defects, including poor growth, a preponderance of large multibudded cells, a large increase in the rate of spontaneous mutations, and a hyper-recombinational phenotype (33, 40). Subsequently, we found that this mutation harbors the change of the TCA serine codon at nucleotide position 590 to the TGA nonsense codon (R. E. Johnson, L. Prakash, and S. Prakash, unpublished observations). Because this mutation results in the deletion of Mms21 beyond amino acid residue 196, it lacks the E3 SUMO ligase function (Fig. 6A). The *mms21-1* mutation also confers a *ts* phenotype at 37°C. However, because of the mutant's poor growth and other associated phenotypes, we were unable to use this mutation to analyze the role of Mms21 in the repair of UV damage.

Here we identify a *ts* mutation of *NSE1* (*nse1-101*), with which we have been able to determine the role of Nse1 in promoting resistance to UV damage. The *nse1-101* mutation confers sensitivity to UV as well as to MMS at the permissive temperature (30°C), and from epistasis analyses, we inferred a role for Nse1 in Rad52-dependent lesion bypass. Consistent with this, we found that whereas at 30°C the proficiency of PRR is impaired to only a modest degree by the *nse1-101* mutation, a much higher degree of impairment in PRR occurs at 37°C. We also provide evidence that Nse1 functions in PRR in a Rad5-independent but Rad52-dependent manner. Interestingly, our observation that UV-irradiated *rad1Δ nse1-101* cells held at 37°C accumulate a much higher proportion of

FIG. 6. Epistasis analysis of the *mms21* RING mutation. (A) Sequence alignment of the C-terminal SP-RING domain of Mms21 proteins from various organisms. Multiple-sequence alignment was performed with CLUSTAL W version 1.83. Sequences aligned are *S. cerevisiae* Mms21 (ScMms21; 267 amino acids; GenBank accession no. NP010896), *S. pombe* Mms21 (SpMms21; 250 amino acids; Q4PIR3), *Homo sapiens* Mms21 (HsMms21; 247 amino acids; NP775956), and *Mus musculus* Mms21 (MmMms21; 247 amino acids; NP081022). Shaded residues correspond to the zinc-binding residues of the SP-RING domain. Residues marked with an asterisk (C200A and H202A) are mutated in the *mms21* RING mutant. (B) Photomicrograph of *mms21* RING mutant cells showing aberrant morphology compared to that of wild-type cells at 30°C. (C) Sensitivity of the *mms21* RING mutant to DNA-damaging agents. (1) Growth on a YPD plate at 30°C for 2 days. (2) Growth deficiency on a YPD plate at the nonpermissive temperature (37°C). (3) Cells were spotted on a YPD plate, UV irradiated at 80 J/m², and incubated at 30°C for 2 days. (4) Cells were spotted on a YPD plate containing 0.035% MMS and incubated at 30°C for 2 days. (D) The *mms21* RING mutations enhance the UV sensitivity of the *rad14Δ*, *rad18Δ*, and *pol30-119* mutants but have no significant effect on the UV sensitivity of the *rad52Δ* mutant. YPD plates containing 10-fold dilutions of exponentially growing yeast cells were UV irradiated at the indicated doses and incubated in the dark for 2 days at 30°C.

low-molecular-weight DNA than the *rad14 nse1-101 rad52Δ* cells implies a role for Nse1 (and presumably for the entire Smc5-Smc6 complex) in stabilizing the repair intermediates that would be generated from the action of Rad52 group proteins and in protecting them from nucleolytic degradation. Since the *nse1-101* mutation harbors no change in the C₄HC₃ E3 ubiquitin ligase motif, we constructed a mutation in this motif to examine whether the putative Nse1 ubiquitin ligase contributes to the Rad52 PRR pathway. Although the Nse1 ubiquitin ligase activity is not essential for cell viability, at

30°C, the *nse1* C274A mutation confers a higher degree of growth defect than the *nse1-101* mutation and the *nse1* C274A cells attain a much larger size than the *nse1-101* cells. Our observations that the UV sensitivity conferred by the *rad18Δ* and *rad52Δ* mutations is enhanced when they are combined with the *nse1* C274A mutation and that the UV sensitivity conferred by the *rad52Δ* mutation is not significantly affected in combination with the *nse1* C274A mutation suggest that the Nse1 ubiquitin ligase also contributes to the Rad52 PRR pathway. We have purified the Nse1 protein, and as was expected from

FIG. 7. Rad6-Rad18-dependent and Smc5-Smc6/Nse1-Mms21-dependent pathways for the replication of UV-damaged DNA in yeast. (A) Rad6-Rad18-dependent replication through UV lesions can occur either by TLS mediated by Pol η or Pol ζ or by Rad5-mediated fork regression and template switching. The Smc5-Smc6 complex functions in the Rad52-dependent PRR pathway, which is proposed to act in a nonrecombinational manner. (B) In the K164R PCNA mutant, because of the absence of PCNA sumoylation, the Rad52 recombinational pathway becomes activated and the Smc5-Smc6 complex acts in this pathway also (for more details, see Discussion).

our genetic analyses, we found no evidence for the physical or functional interaction of the Rad6-Rad18 enzyme complex with the Nse1 protein (S. R. Santa Maria, L. Prakash, and S. Prakash, unpublished observations). The Ubc with which Nse1 collaborates as a ubiquitin ligase and the protein substrates that are ubiquitinated by this enzyme complex remain to be identified.

In addition to the possible involvement of the putative Nse1 ubiquitin ligase in Rad52-dependent PRR, our epistasis analyses suggest the involvement of the Mms21 SUMO ligase activity in this PRR pathway. *S. cerevisiae* Mms21 has been shown to sumoylate Smc5 (50), and Nse2, the *S. pombe* counterpart of Mms21, has been shown to sumoylate Smc6 in vivo in a DNA damage-dependent manner (1). It is possible that the Mms21 (Nse2)-mediated sumoylation of Smc5-Smc6 and perhaps of the other subunits of this complex modulates the physical interactions of the Rad52-group proteins with the various subunits of this complex.

In yeast cells, replication through UV lesions can occur by a Rad6-Rad18- and Mms2-Ubc13-Rad5-dependent pathway or by a Rad52-dependent pathway (Fig. 7A). Since Rad5 has a DNA helicase activity specialized for replication fork regression, previously we suggested a role for Rad5 in promoting lesion bypass when the lesion is located on the leading-strand template (4) (Fig. 7A). Such a requirement for Rad5 in leading-strand lesion bypass then relegates the Rad52-dependent PRR pathway to function when the lesion is located on the lagging-strand template (10). Further, since recombination is normally kept in check in wild-type yeast cells by processes such as Siz1-mediated PCNA sumoylation (13, 35, 36) and by a host of protein factors, such as the Srs2 and Sgs1 proteins (11, 28), we have suggested that the Rad52-mediated PRR pathway

operates in a nonrecombinational manner, employing mechanisms such as those depicted in synthesis-dependent strand-annealing models (27, 42) (Fig. 7A).

The requirement of Nse1 for Rad52-dependent PRR and our suggestion that the Nse1 ubiquitin ligase and Mms21 SUMO ligase activities contribute to this pathway raise the strong possibility that the whole Smc5-Smc6 complex, including its associated ubiquitin ligase activities, function in promoting the Rad52-dependent repair of gaps that would occur in the lagging strand opposite DNA lesions. Since we expect little inhibition of replication fork progression when the lesion is on the lagging-strand template (5, 6, 30, 43), we assume that the replication fork continues its advance and that the gap that is left behind opposite the lesion site is then filled in by the Rad52 PRR pathway in a nonrecombinational manner. A likely role for the Smc5-Smc6 complex in this repair mode would be to hold the sister duplexes in close proximity, which it could achieve by encircling both DNA duplexes in the ring-like structure that Smc heteroduplexes are able to form (12, 15, 19). In addition, the Smc5-Smc6 complex could contribute via its role in mediating physical interactions with the Rad52-group proteins and via the protein modifications mediated by the Nse1 and Mms21 E3 ligases.

Our observations that the UV sensitivity conferred by the *pol30-119* mutation is enhanced in the presence of mutations in the Nse1 and Mms21 E3 ligase motifs implicate a role for the Nse1 and Mms21 E3 ligase activities in promoting Rad52-dependent recombinational lesion bypass. Since in *pol30-119* all the Rad6-Rad18-dependent lesion bypass pathways are inactivated because of the absence of lysine 164 ubiquitination of PCNA and the Rad52-dependent recombinational pathway becomes activated because of the absence of lysine 164 sumoylation of PCNA, presumably all the lesion bypass becomes entirely dependent upon the Rad52-dependent recombinational pathway in *pol30-119* (Fig. 7B). The Smc5-Smc6 complex, along with its associated E3 ligase activities, could function in the Rad52-dependent recombinational repair pathway (Fig. 7B) in a manner similar to that suggested above for its role in Rad52-dependent nonrecombinational repair.

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