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CHARACTERIZATION OF THE DNA REPAIR ROLE OF THE *SACCHAROMYCES CEREVISIAE NSE1* GENE

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CHARACTERIZATION OF THE DNA REPAIR ROLE OF THE *SACCHAROMYCES CEREVISIAE NSE1* GENE

by

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To my family and to Felicia, my lovely wife

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CHARACTERIZATION OF THE DNA REPAIR ROLE OF THE *SACCHAROMYCES CEREVISIAE NSE1* GENE

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In eukaryotes, the structural maintenance of chromosomes (SMC) complexes play important roles in chromosome dynamics. The *Saccharomyces cerevisiae* Smc5-Smc6 complex is composed of eight essential subunits and is particularly required for a normal response to DNA damage. Hypomorphic mutations in these subunits render cells sensitive to DNA damaging agents. Of the non-SMC subunits, Nse1 and Mms21 are of particular interest, because Nse1 contains a RING finger motif characteristic of ubiquitin ligases and Mms21 is a SUMO ligase that sumoylates different subunits of the complex and also other DNA repair proteins.

In this study, we have isolated a mutation, *nse1-101*, which results in sensitivity to UV irradiation and MMS. We used this mutant for genetic and postreplication repair (PRR) experiments to examine the role of Nse1 in promoting the bypass of DNA lesions. From epistasis analyses, we inferred a role for Nse1 in the Rad52-dependent repair pathway. Our PRR experiments further support our genetic results, as the newly

synthesized DNA does not attain the normal size in UV irradiated *nse1-101* cells. On the basis of these and other studies we have provided evidence for the role of Nse1 in the PRR of UV-damaged DNA in a Rad5-independent but Rad52-dependent manner.

We also constructed mutations in the RING domains of Nse1 and Mms21 to investigate the role of their ligase functions in DNA repair. As was observed with *nse1-101*, epistasis analyses with the *nse1* and *mms21* RING mutants also suggest a role for their ligase activities in Rad52-dependent PRR. As this pathway has been suggested to promote PRR when the lesion is on the lagging strand, our studies have indicated a role for Nse1 and Mms21 in promoting PRR on the lagging strand together with the proteins of the Rad52 group. We propose that the Smc5-Smc6 complex functions in this pathway by holding the DNA duplexes in close proximity using the ring structure formed by the Smc5-Smc6 dimer. Further, the ubiquitin ligase and SUMO ligase functions of Nse1 and Mms21, respectively, can contribute to this process by mediating physical interactions between the Smc5-Smc6 complex and the Rad52 group of recombinational proteins.

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CHAPTER 1: INTRODUCTION

Our genetic material is continually exposed to a large variety of exogenous as well as endogenous agents, including ultraviolet (UV) light from the sun, reactive oxygen species resulting from aerobic respiration, chemical pollutants, among others. As a consequence, cells have developed repair systems to remove a wide spectrum of DNA lesions, many of which are highly conserved throughout evolution (23). The importance of DNA repair is illustrated by the fact that mutations in many genes involved in these processes lead to genetic disorders in humans (14, 23, 126).

Depending on the type of damage, different repair systems restore the integrity of the DNA. Direct reversal of damage is the simplest and most efficient of all repair mechanisms. In bacteria and yeast, for example, lesions caused by UV irradiation can be removed by the action of a single enzyme that requires light in a process called enzymatic photoreactivation (23). However, most repair processes rely on the excision and subsequent resynthesis of the damaged stretch of DNA. Base excision repair (BER) recognizes and repairs lesions that produce small modifications in the DNA molecule (e.g., oxidized and methylated bases). Nucleotide excision repair (NER), on the other hand, recognizes large, bulky lesions that cause distortions of the DNA helix, such as damage caused by UV irradiation and DNA cross-linking agents. These excision repair pathways are of little benefit when the type of damage incurred is double strand breaks (DSBs), which are caused mainly upon exposure to clastogenic agents like ionizing radiation. The two primary pathways of DSB repair in eukaryotes are homologous recombination (HR) and non-homologous end joining (NHEJ). Both pathways involve the coordinated action of several proteins with the final objective of rejoining the broken

ends of the DNA molecule via genetic exchange of homologous sequences or by binding the free ends and facilitating their rejoining, respectively.

DNA DAMAGE TOLERANCE

Although DNA damage can be removed by the concerted action of these repair processes, many lesions escape repair and present a block to transcriptional elongation by RNA polymerases and to replication by DNA polymerases. In the absence of a system to resolve a stalled replication fork, the resulting replication arrest could ultimately lead to cell death. For this reason, all living organisms have developed DNA damage tolerance mechanisms that allow the bypass of lesions and the completion of replication without actual removal of the damage.

The phenomenon of DNA damage tolerance was first observed in both prokaryotes and eukaryotes using NER-deficient cells irradiated with UV light (19, 105, 112). Two of the most common DNA lesions caused by UV light and which block the continuation of synthesis by the replicative polymerases are the *cis-syn* cyclobutane pyrimidine dimers (CPDs) and the (6-4) photoproducts (23). Although UV-induced DNA lesions are normally repaired by NER, under certain conditions, such as NER deficiency due to mutational inactivation, or the presence of lesions at the replication fork where NER cannot function, the lesions will persist into S phase and interfere with DNA replication. This is manifested by the appearance of DNA breaks in the newly synthesized DNA, resulting from the stalling of replication forks opposite the lesions. This can be observed through sedimentation in alkaline sucrose gradients of genomic DNA synthesized after UV irradiation. After an incubation period the small DNA fragments are converted into high molecular weight DNA, similar in size to that observed in unirradiated cells. This restoration process has been referred to as postreplication

repair (PRR) (112). In *Escherichia coli*, PRR requires RecA and the RecA-mediated SOS response and is accomplished by recombinational mechanisms (113).

Postreplication repair (PRR) in eukaryotes

Eukaryotic cells share a similar DNA damage tolerance pathway. Studies in *S. cerevisiae* and in mammalian cells have shown that lesions caused by UV irradiation block progression of the replication fork, yielding smaller DNA fragments than those observed in unirradiated cells and that represent regions of single-stranded gaps (19, 72, 105). As described in *E. coli*, further incubation results in the conversion to larger DNA molecules, approaching to that observed in unirradiated controls. Furthermore, the size of the DNA fragments synthesized following UV irradiation corresponds to the average distance between UV lesions, which remain in the template strand even after the single-stranded gaps have been filled (105).

To study the genetic control of PRR in eukaryotes, different *S. cerevisiae* mutants were analyzed for their ability to resolve the single-stranded gaps created after UV irradiation (105). These mutants were selected based on their sensitivity to a variety of DNA damaging agents. Three of these mutants contain mutations in genes that belong to the *RAD6* epistasis group (*rad6-1*, *rad18-2*, and *rev3-1*), and one of them is a recombination-deficient mutant (*rad52-1*). The *rad6* mutant was not able to carry out PRR, while the *rad18* and *rad52* mutants were unable to fully restore to high molecular weight DNA, indicating that they were partially deficient for PRR. The *rev3-1* mutant, on the other hand, showed no deficiency in PRR. Together, these results indicated that PRR of UV-induced DNA damage in *S. cerevisiae* is dependent upon the *RAD6*, *RAD18*, and *RAD52* genes, and suggested that both recombinational and nonrecombinational mechanisms could play a role in PRR (105).

Rad6-Rad18-dependent lesion bypass pathways

Genetic studies in the yeast *S. cerevisiae* have played an important role in the identification of genes involved in the DNA damage response in eukaryotes and in the elucidation of their roles in this process. In *S. cerevisiae*, PRR is controlled by the *RAD6* and *RAD18* genes. Rad6, a ubiquitin-conjugating enzyme (E2), forms a stable complex with Rad18, a DNA binding protein that contains a C_3HC_4 RING motif at its N terminus (8, 9). Other genes that belong to the *RAD6* epistasis group were first implicated in this pathway by epistasis analysis and by their involvement in damage-induced mutagenesis. Genetic studies have indicated that the Rad6-Rad18 complex controls the bypass of UV-damaged DNA via at least three separate pathways: an error-free translesion synthesis (TLS) pathway dependent on the *RAD30* gene; a mutagenic TLS pathway dependent on the *REV1*, *REV3*, and *REV7* genes; and a third pathway that is also error-free and dependent on the *RAD5*, *MMS2*, and *UBC13* genes (Figure 1.1) (109, 128). It is important to note that most of the genes involved in this pathway, including *RAD6*, *RAD18*, *MMS2*, *UBC13*, *RAD5*, *POL30*, and the majority of the TLS polymerases (48, 87, 127, 139, 140), have been conserved from yeast to humans.

Translesion synthesis (TLS)

In eukaryotes, DNA polymerases that belong to the Y family, and the *REV3*-, *REV7*-encoded DNA polymerase ζ (Polζ), which is a member of the B family, promote replication through DNA lesions, *i.e.,* translesion synthesis (92, 107). In contrast to replicative DNA polymerases which synthesize DNA with great accuracy and are blocked by lesions that introduce distortions in the DNA, TLS polymerases synthesize opposite undamaged DNA with much higher error rates and are able to synthesize DNA past lesions that block replicative polymerases (107).

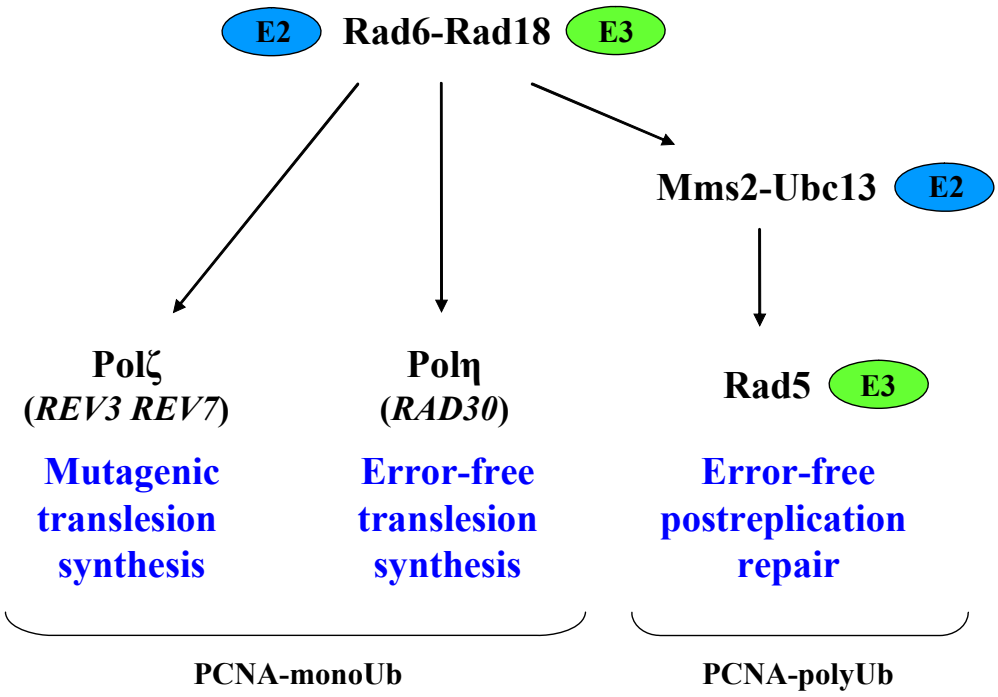

FIGURE 1.1. THE RAD6-RAD18-DEPENDENT BYPASS PATHWAY FOR THE REPLICATION OF UV-DAMAGED DNA**.** Upon exposure to UV light, PCNA becomes monoubiquitinated by Rad6-Rad18, which in turn activates TLS. Polη and Polζ carry out error-free and mutagenic replication past the UV lesions, respectively. Subsequently, PCNA can become polyubiquitinated by the action of Mms2-Ubc13 and Rad5, and this promotes error-free PRR by a copy choice type of DNA synthesis.

The *RAD30*-encoded DNA polymerase η (Polη) was first identified in yeast, and strains carrying a deletion of the *RAD30* gene conferred enhanced UV-induced mutagenesis (58, 82). Mutations of Polη in humans cause the cancer-prone syndrome, the variant form of xeroderma pigmentosum (XP-V) (56, 81). Cells from XP-V individuals are deficient in the replication of UV-induced DNA damage and they are hypermutable upon UV light exposure (17, 74, 142). Polη contributes to error-free TLS by virtue of its proficient and accurate ability to carry out error-free replication through CPDs (58, 61, 143, 144). Remarkably, Polη replicates through this UV lesion with the

same efficiency and accuracy with which it replicates through undamaged DNA (61, 143). The proficiency of Polη to efficiently bypass UV lesions derives from its unique structure. The X-ray crystal structure of yeast Polη modeled with a thymine-thymine CPD in the template DNA and an incoming dATP has provided clear evidence that its active site is open enough to accommodate both residues of the cyclobutane ring, compared to the tight active site present in replicative polymerases (135), thus enabling it to replicate past this normally blocking lesion.

The *REV3*- and *REV7*-encoded proteins form DNA polymerase ζ (Polζ), which promotes mutagenic bypass of DNA lesions induced by UV light and by other DNA damaging agents (91). Polζ contributes to error-prone TLS by its proficient ability to extend from nucleotides inserted opposite a thymine-thymine dimer or (6-4) photoproducts by another DNA polymerase (54, 60). This is consistent with the observation that mutations in the *REV3* and *REV7* genes in yeast cause a significant decrease in the incidence of mutagenesis induced by UV light or by other DNA damaging agents (67-69, 111). *REV1* encodes a highly specialized DNA polymerase that preferentially inserts a C residue opposite template G (36, 90). Like Polζ, Rev1 is also required for UV mutagenesis (66, 67, 69). However, its DNA polymerase activity seems to have no role in UV mutagenesis because C incorporation rarely occurs opposite UV lesions, and TLS through UV-induced damage and various other lesions does not depend upon its DNA synthetic activity (28, 29). Recent evidence suggests a role for Rev1 in the assembly of other Y-family polymerases at the replication fork to promote TLS (1-3).

The Mms2-Ubc13-Rad5-dependent PRR pathway

Besides TLS, the Rad6-Rad18-dependent pathway promotes replication through DNA lesions via a second error-free pathway that repairs the discontinuities that form in the DNA synthesized from damaged templates. This pathway is dependent upon the

RAD5, *MMS2*, and *UBC13* genes (Figure 1.1) (128). Rad5 is a member of the SWI/SNF family of ATPases that exhibits a DNA-dependent ATPase activity and that possesses a C_3HC_4 RING motif characteristic of ubiquitin ligase (E3) proteins (55, 59). In addition, it has been recently shown that yeast Rad5 is a structure-specific DNA helicase that is able to carry out replication fork regression (10). This pathway also requires Mms2 and the ubiquitin-conjugating enzyme Ubc13, which form a complex that promotes the assembly of polyubiquitin chains linked through lysine 63 of ubiquitin (47). It has been proposed that the Mms2-Ubc13-Rad5-controlled PRR pathway is likely to involve a copy choice type of DNA synthesis, in which the damage is bypassed by a template switching mechanism wherein the newly synthesized daughter strand of the undamaged complementary strand is used as the template, and this template switching process requires the replication fork regression activity of Rad5 (10).

Regulation of Rad6-Rad18-dependent pathways

The Rad6-Rad18 complex modulates lesion bypass through its role in PCNA ubiquitination. Ubiquitin (Ub) is an essential protein of 76 amino acids that is conserved from yeast to humans and best known for its function in the targeting of proteins for proteasome-mediated degradation via lysine 48 polyubiquitin chains. In addition to its role in proteasomal degradation, protein ubiquitination has many other possible consequences (98-101). Attachment of ubiquitin to a target protein usually requires the coordinated action of at least three different enzymes. First, ubiquitin is activated by a ubiquitin-activating enzyme (E1) through an ATP-dependent reaction to form an E1-Ub thioester linkage. Then, the activated ubiquitin is transferred to the active-site cysteine of a ubiquitin-conjugating enzyme (E2), which finally attaches the ubiquitin moiety to an internal lysine residue of a substrate protein through an isopeptide bond. This final reaction is usually aided by a ubiquitin protein ligase (E3). The most remarkable feature

of the ubiquitin conjugation pathway is the diversity of its substrates and the specificity for each of them. It is now clear that the recognition of substrates for ubiquitination is governed by the presence of primary sequence or structural motifs in the substrate that are recognized by cognate E3s (65). Thus, E3 ligases are the main determinants of specificity in ubiquitination. Of all the known families of E3s, really interesting new gene (RING) finger proteins are particularly interesting. The RING domain consists of a short motif rich in cysteine and histidine residues, which coordinate two zinc ions, and that is believed to increase the probability of reaction by bringing together the substrate lysine and the E2-Ub intermediate (11, 100, 148).

The *S. cerevisiae POL30* gene encodes the proliferating cell nuclear antigen (PCNA), the eukaryotic sliding clamp required for processive DNA synthesis. PCNA is essential for viability, and conditional lethal *pol30* mutations confer defects in DNA replication (5, 7). In addition to its role in DNA replication, PCNA is required for various DNA repair processes, including NER and BER (62). More recent studies have shown that PCNA plays a critical role in the regulation of different lesion bypass processes and provides the central scaffold to which the TLS polymerases bind in yeast and humans (33-35, 38).

Genetic studies in yeast have indicated that PCNA is involved in the Rad6-Rad18-dependent bypass of UV-damaged DNA (129). Upon treatment of yeast cells with DNA damaging agents, PCNA becomes monoubiquitinated at its lysine 164 residue in a Rad6-Rad18-dependent manner; subsequently, this lysine residue is polyubiquitinated via a lysine 63-linked ubiquitin chain in an Mms2-Ubc13-Rad5-dependent manner (46). These chains, unlike lysine 48-linked chains, do not promote proteasomal degradation. Rad5 and Rad18 are two RING finger proteins that play a critical role in this process. It has been demonstrated that Rad5 interacts with Rad18, and that this association brings the

Mms2-Ubc13 complex into contact with Rad6-Rad18 (138). Thus, interaction between these two RING finger proteins promotes the formation of a heteromeric complex in which the two ubiquitin-conjugating activities of Rad6-Rad18 and Mms2-Ubc13 can be closely coordinated.

Epistasis analyses of the yeast *pol30-119* mutant in which the lysine 164 residue of PCNA has been changed to arginine (K164R) have suggested that this mutation inactivates all three Rad6-Rad18-dependent lesion bypass processes, as it impairs Polζ-dependent UV mutagenesis, Polη-dependent TLS, and PRR of discontinuities that form in the newly-synthesized DNA across from UV lesions (37, 46). Thus, whereas PCNA monoubiquitination appears to activate Polη- and Polζ-dependent TLS, polyubiquitination of PCNA is required for Rad5-dependent PRR (Figure 1.1). In addition to ubiquitin, PCNA is also modified by the small ubiquitin-like modifier (SUMO) at the lysine 164 residue and also at lysine 127; lysine 164, however, is the primary site of this modification (46). SUMO is a protein with about 18 % sequence identity to ubiquitin that also shares a similar activation and conjugation process, and it has been suggested to mediate protein-protein interactions or to function as a ubiquitin antagonist (46, 100, 137).

Rad52-dependent lesion bypass

Although various genetic observations strongly suggest that the *pol30-119* mutation impairs all three Rad6-Rad18-dependent lesion bypass processes, this mutation actually confers a much lower degree of UV sensitivity than mutants carrying deletions in the *RAD6* (*rad6*Δ) or *RAD18* (*rad18*Δ) genes (37). In addition, double mutants containing *rad6*Δ or *rad18*Δ and the *pol30-119* mutation are not as UV sensitive as the single *rad6*Δ or *rad18*Δ mutants, implying that the *pol30-119* mutation suppresses the

repair defectiveness of the deletion mutants by activating or promoting repair by a different pathway (37).

In addition to Rad6-Rad18-dependent PRR of UV-damaged DNA, a role for the Rad52-dependent recombinational pathway has also been indicated (105). Genes belonging to the *RAD52* epistasis group function independently of *RAD6* and *RAD18*, and the deletion of *RAD52* or of any other member of this group confers only a slight increase in UV sensitivity (120). It has been recently shown that strains carrying the *rad51*Δ, *rad52*Δ, or *rad54*Δ mutations are partially deficient in PRR of UV-induced DNA damage, and that an almost complete inhibition of PRR occurs in the absence of both Rad5 and Rad52 (26). Thus, PRR of UV-damaged DNA in yeast is modulated by two separate pathways: a Rad5-dependent pathway, which in addition requires the ubiquitin-conjugating functions of the Rad6-Rad18 and Mms2-Ubc13 complexes, and a Rad52-dependent pathway that in addition requires Rad51, Rad54, and presumably the other proteins that function with this group of recombinational proteins (26).

Whereas ubiquitin conjugation of PCNA at lysine 164 activates the different Rad6-Rad18-dependent bypass processes, SUMO modification of this same residue appears to inactivate Rad52-dependent recombinational bypass, which explains the suppression observed in *pol30-119* mutants. SUMO conjugation of PCNA might be a mechanism by which the Rad52 recombinational pathway is kept in check to avoid the risk of chromosomal rearrangements (37). A similar role has been observed for the Sgs1 and Srs2 helicases (27, 71). Indeed, physical interactions between sumoylated PCNA and the Srs2 helicase result in the recruitment of Srs2 to replication forks (94, 97). Srs2 blocks recombinational repair by disrupting the formation of Rad51 nucleoprotein filaments necessary for recombinational repair (94, 97). Thus, these findings suggest a

mechanism by which ubiquitin and SUMO cooperatively control the choice of pathway for DNA lesion bypass.

STRUCTURAL MAINTENANCE OF CHROMOSOMES (SMC)

Chromosomal organization is a dynamic process that facilitates gene expression, DNA repair and recombination, sister chromatid cohesion, and chromosome condensation. Within the past fifteen years, genetic and biochemical approaches have identified a superfamily of proteins, the structural maintenance of chromosomes (SMC) proteins, as key components in all of these processes (51, 79, 89). These proteins constitute a highly conserved and ubiquitous family, found in all eukaryotes for which enough sequence data are available and in most of the currently sequenced prokaryotes (15). SMC proteins are large polypeptides (1,000 – 1,400 amino acid residues) that share a characteristic design and configuration of protein domains that provide the molecular basis for their various functions in chromosome dynamics (44). The primary structure of SMC proteins consists of five distinct domains. Two canonical nucleotide binding motifs, known as the Walker A and Walker B motifs, are located in the highly conserved N-terminal and C-terminal domains, respectively. The central domain is composed of a moderately conserved hinge sequence that is flanked by two long coiled-coil motifs. A number of studies have established that an SMC monomer folds back on itself through antiparallel coiled-coil interactions, creating an ATP-binding head domain at one end and a hinge domain at the other (Figure 1.2) (32, 41, 84, 114). Two monomers can then associate with each other at the hinge domain to form a V-shaped dimer, and it has been proposed that this V-shaped structure acts like a hook to trap DNA strands (41, 84). The conformation of SMC dimers is highly flexible, and a wide range of structures including open-V, closed-V, and ring-like molecules have been observed (84). The hinge domain

plays an essential role in modulating the mechanochemical cycle of SMC proteins (42). An initial interaction of the hinge domain with DNA leads to opening of the arms by triggering ATP hydrolysis at the head domains, which are located ~ 50 nm away from the hinge. This conformational change allows the inner surface of the hinge domain to stably interact with DNA in an ATP-independent manner and primes subsequent ATP-driven manipulations of DNA (42).

In eukaryotes, at least six members of the SMC protein family have been identified, and each of them has a specific partner with which it forms a heterodimer (15). Eukaryotic SMC heterodimers can be classified into three distinct groups: Smc1-Smc3, Smc2-Smc4, and Smc5-Smc6. These heterodimers further associate with different non-SMC subunits to assemble fully functional SMC holocomplexes. The *S. cerevisiae* cohesin (Smc1-Smc3) complex consists of the SMC heterodimer and at least two non-SMC subunits (Scc1/Mcd1 and Scc3), and participates actively in sister chromatid cohesion and meiotic chromosome pairing and segregation (32, 43). It is thought that cohesin forms a ring around the sister chromatids with Scc1 and Scc3 holding the head domains, and that Scc1 is then cleaved by a protease so that the ring can open during anaphase, thus enabling the sister chromatids to separate (31, 136). A role for cohesin in holding sister chromatids for DSB repair has also been proposed (73). The condensin (Smc2-Smc4) holocomplex contains three non-SMC subunits (Ycs4, Ycs5/Ycg1, and Brn1), and is mainly involved in chromosome condensation and global gene regulation (43). Condensin might induce DNA compaction by dynamically and reversibly introducing and stabilizing loops along the DNA (89, 122). Phylogenetic analyses have revealed that eukaryotes possess two additional members of the SMC family, the Smc5 and Smc6 proteins. The Smc5-Smc6 heterodimer forms the third SMC complex together with additional non-SMC subunits, and is likely to play a role in higher-order

chromosome organization that is essential for a coordinated response to DNA damage and for normal genomic integrity.

The Smc5-Smc6 complex

Smc5 and Smc6 were initially identified by the analysis of radiation-sensitive mutants in *Schizosaccharomyces pombe* (22, 75). Studies in fission and budding yeast then reported the existence of a high molecular mass complex containing Smc5, Smc6, and six other non-SMC proteins (Nse1, Nse2/Mms21, Nse3, Nse4/Qri2, Nse5, and Nse6) (Figure 1.2) (24, 39, 40, 83, 86, 95, 96, 121). In the budding yeast *S. cerevisiae* all of these proteins are essential for cell viability and DNA repair. Nse1 (non-SMC element 1) was the first non-SMC subunit of the Smc5-Smc6 complex to be identified, and *nse1* mutant cells were found to be sensitive to DNA damaging agents and presented aberrant morphologies (24, 83). Nse2 (Mms21 in *S. cerevisiae*) is an E3 SUMO ligase that modifies target proteins involved in a variety of cellular processes and is required for the response to DNA damage (6, 12, 83, 103, 104, 147). Nse3 is an essential nuclear protein related to the mammalian MAGE protein family required for normal mitotic chromosome segregation and cellular resistance to different genotoxic agents (95, 125). Nse4 (Qri2 in *S. cerevisiae*) resembles a kleisin subunit and may bridge the heads of the Smc5-Smc6 dimer (86, 93). Nse5 and Nse6 also associate with the head domains of the dimer, potentially forming a second bridge important for the function of this complex (96). The identification of these non-SMC subunits and subsequent protein interaction studies has allowed the development of a model for the Smc5-Smc6 complex (88, 121).

Like cohesin and condensin, Smc5 and Smc6 form a V-shaped heterodimer with a hinge domain at one end and two head domains at the other, separated by two long coiled-coil motifs (Figure 1.2). Interestingly, different subcomplexes have been identified in fission yeast (121). One subcomplex is composed of the Smc5-Smc6 dimer

and Nse2/Mms21, and a second one consists of Nse1, Nse3, and Nse4, which binds to Smc5 and Smc6 via interaction of Nse4 and the head domains of the heterodimer. A second dimer formed by Nse5 and Nse6 also binds to Smc5 and Smc6 and seems to form a second bridge in addition to Nse4 (Figure 1.2) (88, 96, 121).

Genetic evidence has placed the DNA repair function of the Smc5-Smc6 complex in the homologous recombination repair pathway (18, 22, 24, 39, 75, 83, 86, 141). Based on the mechanochemical activities of cohesin and condensin, it has been proposed that the Smc5-Smc6 complex can hold together sister chromatids or broken ends in the vicinity of DSBs to facilitate repair by homologous recombination. However, the fact that this complex is essential for viability whereas the Rad52-dependent recombinational pathway is not, might imply a separate essential function other than in DNA repair (145). Subsequent studies have found a role for this complex in other processes, including rescue of collapsed replication forks, resolution of recombination-like structures, telomere maintenance, and ribosomal DNA replication and stability (76, 104, 130-134, 147).

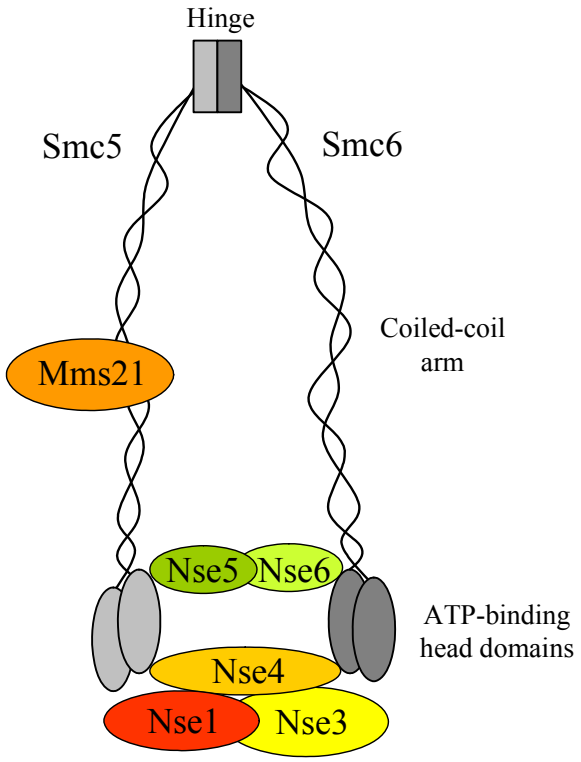

FIGURE 1.2. PROPOSED ARCHITECTURE OF THE YEAST SMC5-SMC6 COMPLEX. Smc5 and Smc6 form a V-shaped dimer with a hinge region at one end and two ATP-binding heads at the other, separated by long coiled-coil motifs. Mms21 is a SUMO ligase that interacts with Smc5. Nse1, Nse3, and Nse4 form a subcomplex that binds to Smc5-Smc6 via the interaction of Nse4 with the head domains. In addition, Nse5 and Nse6 form a second dimer that also binds to these domains.

The non-SMC element 1 (Nse1)

As mentioned above, Nse1 was the first non-SMC component of the Smc5-Smc6 complex to be identified, first in *S. cerevisiae*, and then in *S. pombe* (24, 83). *NSE1* is an essential gene conserved in eukaryotes from yeast to humans. In *S. cerevisiae* it encodes a nuclear protein of 336 amino acids with a predicted molecular weight of 38 kDa. Analysis of its amino acid sequence reveals a conserved C_4HC_3 motif located near the C terminal cysteine and histidine-rich region, suggesting that it might function as a RING finger-like motif (116, 121). Recently, a considerable number of RING finger proteins

have been characterized as E3 factors for ubiquitin conjugation of target proteins, and these E3s are usually ubiquitinated as well by their cognate E2 ubiquitin conjugating enzymes (52, 78). Although the classic RING domain contains a C_3HC_4 motif, recently many proteins containing the variant C_4HC_3 RING domain, as in Nse1, have been shown to act as E3 ubiquitin ligases (20, 80). Therefore, Nse1 may be involved in mediating the ubiquitin modification of unknown substrates by bringing the E2 enzyme closer to its protein substrate. The importance of the C_4HC_3 motif is also supported by the observation that substitution of the first two cysteine residues with serine leads to growth defects in *S. cerevisiae* (24). *nse1* mutants exhibit a high degree of sensitivity to a variety of DNA damaging agents and they display abnormal cellular morphologies, highlighting the importance of Nse1 in maintaining genomic stability. In addition, studies in fission yeast have shown that *nse1* and *nse2* mutants are epistatic to Rhp51 (Rad51 in *S. cerevisiae*) in response to ionizing irradiation, suggesting that the Smc5-Smc6 complex may facilitate the repair of DSBs, either by tethering the broken ends near the repair template or by modifying local DNA structure (83). Nevertheless, little is known about the molecular mechanisms of how Nse1 participates in the Smc5-Smc6 complex during DNA repair and cell proliferation.

The non-SMC element 2 (Nse2/Mms21)

S. cerevisiae MMS21 encodes an essential protein of 267 amino acids with a predicted molecular weight of 30 kDa. The *MMS21* gene was first identified over thirty years ago in a screen for genes affecting resistance to the alkylating agent methyl methanesulfonate (MMS) (106). The *mms21-1* mutation confers sensitivity to UV light, MMS and gamma irradiation, poor growth, a morphology of large multibudded cells, a large increase in the rate of spontaneous mutations, and a hyper-recombinational phenotype (85, 108). The *mms21-1* mutation contains a nonsense codon at nucleotide

position 590 (R.E. Johnson, L. Prakash, and S. Prakash, unpublished observations) resulting in deletion of the amino acids downstream of residue 196. Interestingly, Mms21 carries a C-terminal motif that resembles the SP-RING domain that is exclusively found in E3 SUMO ligases (6, 110, 147). Further biochemical and genetic characterization have demonstrated that Mms21 is indeed a SUMO ligase that belongs to the essential Smc5-Smc6 complex in yeast and humans, and that mutations in the RING domain result in loss of its ligase function and render cells sensitive to DNA damaging agents (6, 103, 147). Mms21 sumoylates a variety of target proteins, including members of the Smc5-Smc6 complex, and proteins involved in DNA repair (6, 103, 147). It has been proposed that the SUMO ligase function of Mms21 is required for several processes, including DNA repair, nucleolar integrity, telomere maintenance, and resolution of pathological recombinogenic structures that arise during replication (6, 12, 102-104, 147). Moreover, the Smc5-Smc6 complex is thought to facilitate proper sumoylation by localizing Mms21 and its SUMO ligase activity to specific chromosomal regions (147).

DOCTORAL DISSERTATION WORK

The budding yeast *S. cerevisiae* has been extensively used as a biological model for the genetic and biochemical characterization of mutagenesis and the DNA damage response. My doctoral research has focused on the genetic characterization of the *S. cerevisiae NSE1* gene and its role in DNA repair. In addition, we have carried out studies to better elucidate the role of the ligase activities of Nse1 and Mms21 in postreplication repair of UV-induced DNA damage. The information we obtain from these studies will allow us to better understand the molecular role of Nse1, Mms21, and the entire Smc5-Smc6 complex in DNA repair and mutagenesis.

CHAPTER 2: MATERIALS AND METHODS

GENERATION OF *S. CEREVISIAE* STRAINS

All yeast strains used for epistasis and mutagenesis analyses, and for alkaline sucrose gradient sedimentation experiments are isogenic to the wild type yeast strain EMY74.7, *MAT*a *his3*-Δ*1 leu2-3*,*112 trp1*Δ *ura3-52*, and are listed in Tables 2.1 and 2.2.

Generation of gene deletions in *S. cerevisiae* strains

Deletions of different genes were made by using the one-step gene disruption method to replace all or most of their open reading frames (ORFs) with the *URA3*, *HIS3*, or *TRP1* genes as selectable markers. Briefly, cells grown to logarithmic phase at 30 ºC in liquid yeast extract-peptone-dextrose (YPD) medium were washed once in sterile water, resuspended in 1 ml 100 mM lithium acetate, pH 7.5, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5 (LiAc/TE), and incubated for 1 hour at 30 ºC, resulting in competent cells. For integrative transformation, 200 µl of competent cells, 7 µl of 10 mg/ml sonicated herring sperm DNA, and 5 µg of digested deletion generating plasmid DNA were mixed and incubated for 30 minutes at 30 ºC, followed by addition of 1 ml LiAc/TE containing 40 % PEG_{4000}. After a 30-minute incubation at 30 ºC, the cells were heat shocked at 42 ºC for 15 minutes, washed in sterile water and plated on synthetic complete (SC) media lacking either uracil, histidine, or tryptophan, depending on the selectable marker used. Yeast colonies were grown for three to five days at 30 ºC and screened for integration by standard polymerase chain reaction (PCR) of genomic DNA. To remove the *URA3* gene from the integrated "gene blaster", strains were plated on SC plates containing 1 mg/ml 5-fluorouracil (FOA) and incubated at 30 ºC for three to five days. PCR was performed on genomic DNA of each strain to confirm loss of the *URA3* gene. Multiple deletions

were generated by sequential gene deletion using the same method. The various deletion generating plasmids used in these studies are given in the Appendix.

Isolation of the wild type *NSE1* gene and construction of the *nse1*Δ strain

The wild type *NSE1* gene was isolated by the gap-repair method using plasmid pPM1161, a *CEN LEU2* plasmid containing 988 bp upstream and 969 bp downstream of the *NSE1* ORF. These upstream and downstream DNA segments had been obtained by PCR amplification. pPM1161 DNA cut with HpaI and MscI was transformed into EMY74.7, generating the plasmid pPM1162 containing the wild type *NSE1*. Plasmid pPM1162 was transformed into EMY74.7, yielding the yeast strain YRP709 which contains both the genomic *NSE1* gene as well as the *NSE1* gene carried on the *CEN LEU2* plasmid. The genomic *NSE1* gene in YRP709 was deleted by transforming this strain with the *NSE1* deletion generating plasmid pPM1166 that had been digested with Asp718 and SphI. pPM1166 is a derivative of plasmid pPM1162 in which an internal deletion of 816 bp of the *NSE1* gene ORF has been replaced by a DNA fragment carrying the *S. cerevisiae URA3* gene flanked on either side by 1.1 kb direct repeats of the *Salmonella typhimurium hisG* gene (4). The 3.8 kb *URA3*-containing DNA fragment is referred to as the "gene blaster" because following targeted gene disruption by integrative transformation to Ura+, the high recombination frequency of 10^{-4} that occurs between the *hisG* repeats in vegetatively growing cells results in eviction of the *URA3* gene and the transformants become ura-, thus allowing for repeated targeted gene deletion by transformation to Ura+ followed by growth on FOA media to generate ura- cells. The resulting strain, YRP721 = EMY74.7 *nse1*Δ::*URA3*+, is viable because it carries the wild type *NSE1* gene in the *CEN LEU2* plasmid pPM1162. Growth of YRP721 on FOA resulted in YRP722, which is the ura- derivative of YRP721. Strain YRP722 was transformed to Ura+ by transformation with pPM1168 which carries the wild type *NSE1*

gene in the *CEN URA3* plasmid pTB363 (YCp lac33 *URA3*) (30). Transformants were screened for colonies which retained the *NSE1 CEN URA3* plasmid but had lost the *NSE1 CEN LEU2* plasmid. The resulting strain, EMY74.7 *nse1*Δ carrying the *NSE1* gene on the *CEN URA3* plasmid was designated YRP723 and was used for further studies.

Isolation of the *nse1-101* mutant

To introduce random mutations in *NSE1*, pPM1162 (3 μg) was incubated with hydroxylamine hydrochloride for 2 hours at 65 °C before its introduction into the yeast strain YRP723, the *nse1*Δ::*URA3* isogenic derivative of EMY74.7 which is viable because it carries the wild type *NSE1* gene on a *CEN URA3* plasmid. Ura+ transformants were replica plated on medium containing FOA to select for loss of the *NSE1 CEN URA3* plasmid. Colonies which grew on FOA were screened for sensitivity to UV light and to the alkylating agent methyl methanesulfonate (MMS). Two isolates exhibited sensitivity to MMS and UV at 30 °C, and were designated as *nse1-101* and *nse1-102* and the carrying strains named YRP727 and YRP728, respectively. Since in addition to its other phenotypes, the *nse1-101* mutant exhibited a growth defect at 37 °C, it was used in our subsequent genetic studies. 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). Deletion mutations of various DNA repair genes were generated in the *nse1-101* mutant strain YRP727 by the gene replacement method.

Generation of the *nse1* and *mms21* RING mutants

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 pPM1182, a derivative of pPM1162, using oligonucleotides LP1102 (5'-GAACACTTGCCAAAATGC

CCACAAGCTGGCAATTCAAGG-3') and LP1103 (5'-CCTTGAATTGCCAGCTTGTGGG CATTTTGGCAAGTGTTC-3'). The resulting plasmid, pPM1251, was then introduced into yeast strain YRP723, and transformants were replica plated on FOA plates to select for loss of the *NSE1 CEN URA3* plasmid. The *mms21* C200A H202A mutant (*mms21* RING) was generated by site-directed mutagenesis of plasmid pMMS21-44 using oligonucleotides LP1096 (5'-CCATTGATATCAAGAAAAGCAAATGCAGTCTTCGATAG AGACGGC-3') and LP1097 (5'-GCCGTCTCTATCGAAGACTGCATTTGCTTTTCTTGAT ATCAATGG-3'). pMMS21-44 carries DNA encoding the C-terminal region of Mms21 in pUC19. The *URA3* gene was then cloned downstream of the *mms21* RING open reading frame, generating plasmid pMMS21-47, and this RING mutation was then integrated into the genome by the gene replacement method described above.

UV SENSITIVITY ASSAYS (EPISTASIS EXPERIMENTS)

Wild type and mutant strains were grown overnight at 30 °C to mid-logarithmic phase in liquid YPD medium. Cells were washed in 1 ml sterile water, sonicated to disperse cell clumps when necessary, and resuspended in sterile water to a density of 2 x 10^8 cells per ml. 200-µl aliquots of serial 10-fold dilutions were pipetted into a 96-well microtiter sterile dish, followed by transfer to YPD plates. The plates were then UV irradiated at a dose rate of 0.1 or 1 J/m^2/s, depending on the total fluence desired for the given strain, and incubated at 30 °C for 2 days before being photographed. UV irradiation was performed under yellow light and the plates were kept in the dark during incubation to avoid photoreactivation of UV-induced DNA damage.

MUTAGENESIS ASSAYS

Spontaneous mutagenesis

To determine the rate of spontaneous mutations, yeast strains were first inoculated in liquid YPD and incubated overnight at 30 °C. Cells were counted and resuspended in liquid YPD to a cell density of 1×10^2 cells per ml. Eleven 1 ml-cultures, each containing 100 cells were incubated for 3 days at 30 °C. After incubation, cells were pelleted by centrifugation, washed once with sterile water, resuspended in 1 ml sterile water and transferred to 1.5-ml Eppendorf tubes. Ten µl from each of the 11 tubes were added to 890 µl sterile water and used to make further dilutions for determining viability after plating on SC plates. Appropriate dilutions from each of the eleven tubes were spread onto SC plates lacking arginine but containing canavanine (SC –arg +can) for determining the rate of *can1*^{r} mutations. Plates were incubated for 3 to 5 days at 30 °C prior to counting of yeast colonies. Mutation rates were calculated by the method of the median of Lea and Coulson (70).

UV-induced mutagenesis

To determine the frequency of UV-induced mutations, yeast strains were grown in liquid SC medium overnight at 30 °C. Cells were then counted, sonicated to disperse cell clumps when necessary, pelleted by centrifugation, and resuspended in sterile water to a cell density of 2×10^8 cells per ml. Appropriate dilutions were spread onto SC plates to measure viability and onto SC –arg +can for determining the frequency of *can1*^{r} mutations. The plates were then UV irradiated at the appropriate doses, followed by an incubation period in the dark of 3 to 5 days at 30 °C. After the incubation period, yeast colonies were counted and the mutation frequencies (mutants per 10^7 viable cells) calculated.

TABLE 2.1. *S. cerevisiae* strains used for epistasis experiments

Strain	Genotype
EMY74.7	*MAT* a *his3 -Δ1 leu2-3 ,112 trp1 Δ ura3-52*
YRP727	EMY74.7 *nse1-101*
YRP728	EMY74.7 *nse1-102*
YRP787	EMY74.7 *nse1* C274A
YMMS21-46	EMY74.7 *mms21* C200A H202A
YR5-51	EMY74.7 *rad5 Δ*
YR5-326	EMY74.7 *nse1-101 rad5 Δ*
YR5-232	EMY74.7 *nse1-102 rad5 Δ*
YR5-390	EMY74.7 *nse1* C274A *rad5 Δ*
YR5-388	EMY74.7 *mms21* C200A H202A *rad5 Δ*
YR6-284	EMY74.7 *rad6 Δ*
YR6-305	EMY74.7 *nse1-101 rad6 Δ*
YR6-285	EMY74.7 *nse1-102 rad6 Δ*
YR14-43	EMY74.7 *rad14 Δ*
YR14-185	EMY74.7 *nse1-101 rad14 Δ*
YR14-183	EMY74.7 *nse1-102 rad14 Δ*
YR14-217	EMY74.7 *nse1* C274A *rad14 Δ*
YR14-215	EMY74.7 *mms21* C200A H202A *rad14 Δ*
YR18-148	EMY74.7 *rad18 Δ*
YR18-131	EMY74.7 *nse1-101 rad18 Δ*
YR18-117	EMY74.7 *nse1-102 rad18 Δ*
YR18-152	EMY74.7 *nse1* C274A *rad18 Δ*
YR18-150	EMY74.7 *mms21* C200A H202A *rad18 Δ*
YR30-2	EMY74.7 *rad30 Δ*
YR30-219	EMY74.7 *nse1-101 rad30 Δ*
YR30-192	EMY74.7 *nse1-102 rad30 Δ*
YR52-90	EMY74.7 *rad52 Δ*
YR52-117	EMY74.7 *nse1-101 rad52 Δ*
YR52-105	EMY74.7 *nse1-102 rad52 Δ*
YR52-121	EMY74.7 *nse1* C274A *rad52 Δ*
YR52-123	EMY74.7 *mms21* C200A H202A *rad52 Δ*
YMMS2-6	EMY74.7 *mms2 Δ*
YMMS2-92	EMY74.7 *nse1-101 mms2 Δ*
YMMS2-76	EMY74.7 *nse1-102 mms2 Δ*
YRP551	EMY74.7 *ubc13 Δ*
YRP772	EMY74.7 *nse1-101 ubc13 Δ*
YRP743	EMY74.7 *nse1-102 ubc13 Δ*
YPCNA41	EMY74.7 *pol30-119*
YPCNA101	EMY74.7 *nse1-101 pol30-119*
YPCNA103	EMY74.7 *nse1* C274A *pol30-119*
YPCNA106	EMY74.7 *mms21* C200A H202A *pol30-119*
YR52-51	EMY74.7 *pol30-119 rad52 Δ*
YREV3-14	EMY74.7 *rev3 Δ*
YREV3-126	EMY74.7 *nse1-101 rev3 Δ*
YREV3-115	EMY74.7 *nse1-102 rev3 Δ*

POSTREPLICATION REPAIR STUDIES

Generation of ρ^0 strains

To avoid confusion between low molecular size nuclear DNA synthesized after UV irradiation and mitochondrial DNA, ρ^0 strains lacking mitochondrial DNA were used for postreplication repair (PRR) experiments. To generate strains lacking mitochondrial DNA, a dense suspension of yeast cells was spread on YPD plates. After the plates were dried, a sterile filter disc (12.5 mm x 12.5 mm) was placed in the middle of each plate and approximately 30 to 40 µl of 10 mg/ml ethidium bromide were added to each disc. The plates were incubated in the dark for 3-4 days at 30 °C. Cells growing around the disc were subcloned on YPD plates, and the subclones were screened for their ability to grow on YPD but not on yeast extract-peptone-glycerol (YPG) media. Cells lacking mitochondrial DNA are not able to grow on YPG plates whereas ρ^+ cells can grow on both YPD and YPG media. Under the experimental conditions used where cells are grown with glucose as the carbon source, there is no difference in physiology between ρ^+ vs. ρ^0 cells. Cells unable to grow on YPG were tested further to confirm the absence of mitochondrial DNA by examination of the sedimentation pattern in alkaline sucrose gradients of uniformly radiolabeled total DNA. Clones yielding little or no low molecular weight DNA were used for further studies. All the strains used for PRR experiments are listed in Table 2.2.

TABLE 2.2. *S. cerevisiae* strains used for PRR experiments

Strain	Genotype
YR1-65	EMY74.7 *rad1* Δ ρ^0
YR1-373	EMY74.7 *nse1-101 rad1* Δ ρ^0
YR1-118	EMY74.7 *rad1* Δ *rad5* Δ ρ^0
YR1-398	EMY74.7 *nse1-101 rad1* Δ *rad5* Δ ρ^0
YR1-231	EMY74.7 *rad1* Δ *rad52* Δ ρ^0
YR1-394	EMY74.7 *nse1-101 rad1* Δ *rad52* Δ ρ^0

Gradient preparation

To prepare the gradients, 5.7 ml of 15 % alkaline sucrose solution (15 grams DNase-free, high-grade sucrose per 100 ml alkaline sucrose buffer; 0.3 M NaOH, 0.7 M NaCl, 20 mM EDTA, pH 9, 1 % sarkosyl) were added to the right chamber of the gradient apparatus (Figure 2.1), and 5.6 ml of 30 % alkaline sucrose solution (30 grams DNase-free, high-grade sucrose per 100 ml alkaline sucrose buffer) were added to the left chamber. The magnetic stirrer was turned on and the interchamber valve opened to start mixing the sucrose solutions. After starting the polystaltic pump, the main valve was opened slowly (Figure 2.1). The needle which had been attached to the other end of the tube was inserted into the bottom of a polyallomer centrifuge tube (Beckman Coulter, Inc.); as the sucrose solution started to flow inside the tube, the needle was slowly pulled out until the solution reached the top. The alkaline sucrose gradients were kept at room temperature until the samples were ready for centrifugation.

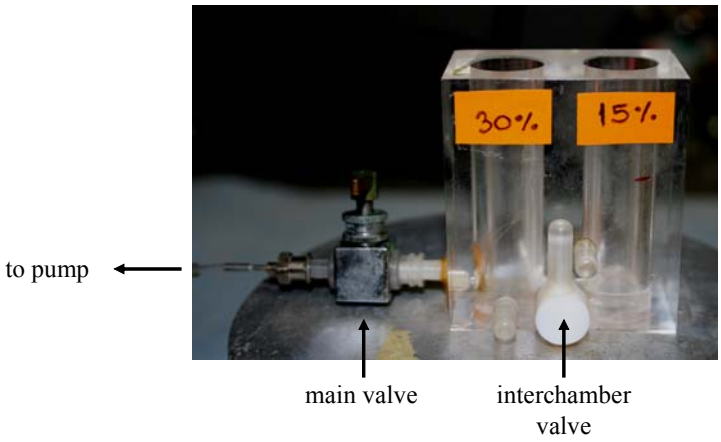

FIGURE 2.1. GRADIENT GENERATOR APPARATUS. The apparatus consists of two separate chambers sitting on a magnetic stirrer that allows mixing of the sucrose solutions. The flow between chambers is controlled by a valve, labeled "interchamber valve", and the flow between the apparatus and a polystaltic pump is manually controlled via a second valve, labeled "main valve". The newly mixed sucrose solution runs from the left chamber into a tube that passes through the pump via a flexible tube and ends in a fine needle placed in a polyallomer centrifuge tube in which the alkaline sucrose gradient forms.

UV irradiation and sedimentation in alkaline sucrose gradients

Yeast strains were grown overnight at 30 °C in 100 ml SC medium lacking uracil but containing 5 µg/ml uridine (SC –ura +UdR). When the asynchronously growing cells reached a density of 0.5 to 1 x 10^7 cells per ml, cells were concentrated to 1 x 10^8 cells, transferred to sterile 150- by 20-mm Petri dishes, and UV irradiated in the same growth medium with constant stirring at room temperature at 3.5 J/m^2 (dose rate of 0.1 J/m^2/s). All operations during and after UV irradiation were performed in yellow light to prevent photoreactivation. After UV irradiation, cells were collected onto GS 0.22 µm filters by filtration under vacuum. The filters were placed into sterile plastic Petri dishes and the cells were resuspended with 1 ml fresh SC –ura +UdR medium, after which they were transferred to sterile 15-ml Sarstedt tubes. For pulse-labeling of DNA, 100 µCi of [6'-^{3}H] uracil (20-25 Ci/mmol, 1 mCi/ml; Moravek Biochemicals and Radiochemicals) were

added to 1 ml of cells, followed by vigorous shaking for 15 minutes at 30 °C. The pulse labeling period was terminated by the addition of high-uracil medium (SC medium containing 1.67 mg/ml uracil), after which cells were collected by filtration, resuspended in 10 ml high-uracil medium and incubated for an additional 30 minutes or 6 hours at 30 °C or at 37 °C. After incubation, cells were collected by filtration and washed with sterile water. The filters were transferred to plastic Petri dishes, the cells were resuspended in 1 ml sterile water followed by an additional wash with 1 ml sterile water. For conversion of cells to spheroplasts, 100 µl 0.5 M EDTA, pH 9, and 25 µl *β*-mercaptoethanol were added for a 5-minute incubation period at room temperature, following which cells were pelleted and resuspended in 1 ml SE buffer (1.1 M sorbitol, 0.1 M EDTA, pH 7.5). After adding 100 µl of the yeast lytic enzyme zymolyase (2.5 mg/ml; Zymo Research Corporation), cells were incubated for 5 minutes at 37 °C, following which 300 µl of the spheroplast suspension were gently added to 200 µl of lysis buffer (0.79 M sorbitol, 0.066 M EDTA, pH 9, 2.5 % sarkosyl, 0.3 M NaOH, 0.35 M NaCl) which had been layered on top of the gradient. Gradients were kept at 4 °C for at least 20-30 minutes before centrifugation in an SW 41 Ti rotor for 15.5 hours at 21,000 rpm, 4 °C, in a Beckman Optima XL-100K ultracentrifuge.

Sample processing

Fractions of approximately 300 µl each were collected in disposable glass tubes from the bottom of the gradient using a polystaltic pump, followed by addition of 300 µl 2 M NaOH. After covering the tubes with parafilm and aluminum foil, they were incubated overnight at 37 °C or for 1.5-2 hours at 65 °C to allow for alkaline hydrolysis of RNA. After this incubation, the tubes were placed in an ice-water bath. 50 µl of 1 mg/ml herring sperm DNA were added to each fraction, the tubes vortexed and incubated for an additional 3-5 minutes in the ice-water bath, followed by addition of 3 ml acid

precipitation solution (APS; 1 M HCl, 0.1 M sodium pyrophosphate). After 30 minutes, the fractions were collected by filtration, washed four times with 3 ml ice-cold APS solution and once with ice-cold 95 % ethanol. After the filters were dried with an Infra-rediator apparatus (Fisher Scientific, Inc.) for 15-20 minutes, they were transferred to liquid scintillation (LS) vials and 3 ml Filter-Count LSC cocktail (PerkinElmer, Inc.) were added to each vial. After a 20-minute incubation period at room temperature, vials were placed in an LS 6500 Multiple-Purpose Scintillation Counter (Beckman Coulter, Inc.) for sample analysis. Data obtained from the LS counter were analyzed using Sigma Plot (Systat Software, Inc.).

RECOMBINATION ASSAYS

Strains used for spontaneous and UV-induced recombination experiments were isogenic to the wild type strain RSY6 (*MAT*a *ura3-52 leu2-3,112 trp5-27 ade2-40 ilv1-92 arg4-3 his3*Δ*5'-pRS6-his3*Δ*3'*) (117). This strain contains two genomic copies of the *his3* gene, one with a terminal deletion at the 3' end and the other with a terminal deletion at the 5' end, separated by the *LEU2* gene and plasmid DNA sequences. The two copies share about 400 base pairs of homology, and thus an intrachromosomal recombination event leads to *HIS3*+ reversion and loss of the *LEU2* marker (117, 118). A possible mechanism by which *HIS3*+ recombinants can be generated is by unequal sister chromatid conversion (117). It has been previously shown that deletion of genes involved in recombination (i.e., *RAD52*) significantly lowers the rate of mitotic intrachromosomal recombination (1, 118, 119). To study the role of *NSE1* and *MMS21* in recombination, the *nse1-101* and *mms21* RING mutations were introduced in this genetic background by genomic integration using the lithium acetate method, generating

strains YRP854 and YMMS21-56, respectively. The strains used in these recombination studies are listed in Table 2.3.

Spontaneous intrachromosomal recombination

To determine the rate of spontaneous intrachromosomal recombination, yeast strains were subcloned on YPD plates for 2-3 days at 30 °C. Five individual colonies were inoculated into 5 ml of SC medium lacking leucine (SC –leu) and incubated overnight at 30 °C. Cells were pelleted by centrifugation, washed once with 1 ml sterile water and transferred to 1.5-ml Eppendorf tubes (sample A). Cells were spun down a second time and resuspended in 0.5 ml sterile water. A 10-fold dilution was then prepared from sample A, and labeled sample 10^0. Subsequent 10-fold serial dilutions were prepared from the 10^0 sample. Appropriate dilutions were plated on SC and SC lacking histidine (SC –his) media to determine viability and recombination, respectively. Plates were incubated for 3 to 5 days at 30 °C prior to counting colonies, and the *HIS3*+ recombination rates were calculated by the method of the median of Lea and Coulson (70).

UV-induced intrachromosomal recombination

To determine the frequency of UV-induced intrachromosomal recombination, yeast strains were grown overnight at 30 °C in liquid SC –leu medium. Cells were counted, sonicated to disperse cell clumps when necessary, and resuspended in sterile water to a cell density of 2×10^8 cells per ml. Appropriate dilutions were plated on SC and on SC –his medium to determine viability and recombination, respectively. The plates were UV irradiated at a dose rate of 1 $J/m^2/s$, followed by incubation for 3 to 5 days at 30 °C.

TABLE 2.3. *S. cerevisiae* strains used for recombination assays

Strain	Genotype
RSY6	*MAT* a *ura3-52 leu2-3,112 trp5-27 ade2-40 ilv1-92 arg4-3 his3* Δ*5'-pRS6-his3* Δ*3'*
YRP854	RSY6 *nse1-101*
YMMS21-56	RSY6 *mms21* C200A H202A

EXPRESSION AND PURIFICATION OF *S. CEREVISIAE* NSE1 PROTEIN

Cloning of the *S. cerevisiae NSE1* gene

The full-length open reading frame (ORF) of the *S. cerevisiae NSE1* gene was obtained from genomic DNA of the wild type strain EMY74.7 by PCR amplification using oligonucleotides LP790 (5'-CGCCATGCATGCAGGATCCTAAATAACGTATACG C CCTCTGT-3') and LP791 (5'-GCGGAGGTACCGGATCCACATATGGAGGTACATGAAG AGCAGGTC-3'). The ORF was then directionally cloned into plasmid pUC19, generating plasmid pPM1203. For expressing the Nse1 protein, the wild type *NSE1* gene was cloned as an N-terminal fusion with glutathione *S*-transferase (GST) under the control of the *S. cerevisiae* galactose-inducible phosphoglycerate kinase (PGK) promoter using the expression vector pBJ842, which resulted in plasmid pPM1204. pBJ842 is a 2-μm-based vector with a seven amino acid leader peptide (GPGGDPH) attached to the C terminus of GST for PreScission protease (Amersham Pharmacia) cleavage, and that carries the yeast *leu2-d* allele, which lacks a complete promoter (57). Hence, yeast cells harboring pBJ842 acquire an increased copy number of the plasmid in order to maintain growth on media lacking leucine, thereby leading to increasing expression of the fusion protein.

Expression and purification of Nse1 protein

The GST-Nse1 expression vector was transformed into the protease-deficient yeast strain YRP654 (*MATα ura3-52 trp1Δ leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R GAL*) (57). The transformed cells were grown overnight in liquid YPD at 30 °C before plating on SC –leu. Leu+ transformants were inoculated in liquid SC –leu medium, grown overnight at 30 °C, and then diluted 1:20 in SC –leu containing 3 % glycerol and 2.5 % DL-lactate, but lacking dextrose, and incubated for 16-24 hours at 30 °C. Protein expression was induced by the addition of 2 % galactose, 1 % peptone, and 0.5 % yeast extract, followed by an additional incubation period of 6-7 hours. Cells were harvested by centrifugation and stored as yeast paste at – 70 °C.

The frozen yeast paste was resuspended in a total of 2.5 volumes of 1x cell breakage buffer (CBB) (50 mM Tris-HCl, pH 7.5, 10 % sucrose, 1 mM EDTA) to which 500 mM NaCl, 10 mM β-mercaptoethanol, 0.5 mM benzamide-HCl, 0.5 mM phenyl-methysulfonyl fluoride, and one complete protease inhibitor tablet (Roche) were added. The cell suspension was passed through a 40K French press cell (Spectronic Instruments) twice at 20,000 psi at 4 °C to achieve cell breakage. Cell debris was removed by centrifugation at 10,000 rpm for 10 minutes, and the resulting supernatant was ultracentrifugated at 60,000 rpm for 1 hour in a Beckman type 70 Ti rotor. The extract, harvested from the clear, middle portion of the sample, avoiding any white floating lipid and the very bottom layer, was passed slowly over a glutathione-Sepharose column. The column was washed three times with buffer containing 50 mM Tris-HCl, pH 7.5, 10 % glycerol, 1 mM EDTA, 1 M NaCl, 10 mM *β*-mercaptoethanol, and 0.01 % NP-40. The column matrix was equilibrated with elution buffer containing 50 mM Tris-HCl, pH 7.5, 10 % glycerol, 1 mM EDTA, 150 mM NaCl, 5 mM dithiothreitol (DTT), and 0.01 % NP-40. The matrix was then removed from the column and transferred to an appropriately

sized tube. The GST-Nse1 protein was eluted by incubating the matrix with an equal volume of elution buffer containing 40 mM glutathione for 10 minutes on ice. The untagged form of the Nse1 protein (with the seven amino acid leader peptide) was obtained by cleavage directly off the beads following treatment with PreScission protease. The matrix was resuspended in an equal volume of elution buffer containing 50 units protease/ml and rocked slowly overnight at 4 °C. The proteins were analyzed by SDS-PAGE and Coomassie staining. Aliquots (10-20 µl) of purified proteins were flash frozen in liquid nitrogen and stored at – 70 °C.

BIOCHEMICAL CHARACTERIZATION OF NSE1 PROTEIN

DNA binding activity

DNA binding was examined using the electrophoretic mobility shift assay (EMSA). PreScission-cut Nse1 protein was incubated for 10 minutes at 30 °C in DNA binding buffer (50 mM Tris-HCl, pH 7.5, 100 µg/ml BSA, 1 mM DTT, 10 % glycerol) with ^{32}P-labeled single-stranded or double-stranded DNA (32-mer oligonucleotide and 32-mer annealed to a 52-mer template, respectively; 20 nM final concentration) in the presence or absence of ATP and $MgCl_2$. ATP and $MgCl_2$ were added to 1 mM and 5 mM when present, respectively. After the incubation period, the samples were loaded on 12 % polyacrylamide gels. The gels were subsequently dried and the DNA binding activity visualized by autoradiography.

ATPase activity

ATP hydrolysis was measured using thin layer chromatography (TLC) on polyethyleneimine-cellulose paper. Purified Nse1 protein was incubated at 37 °C for 30 minutes in 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 100 µg/ml BSA, 1 mM DTT, 0.5 mM

ATP, and 5 μCi of γ-^{32}P-labeled ATP (3000 Ci/mmol; Amersham) in the presence or absence of 100 ng of single-stranded M13mp18 DNA or double-stranded pUC19 DNA. After incubation, a 1-μl aliquot was spotted onto polyethyleneimine–cellulose TLC paper and developed for 15-20 minutes in a solution containing 0.3 M LiCl and 1 M formic acid. The TLC paper was then dried and the ATPase activity visualized by autoradiography.

***In vitro* ubiquitination assay**

In vitro ubiquitination reactions were carried out in 12.5 μl of ubiquitination buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 100 μg/ml BSA, 1 mM DTT, 100 μM ATP) in the presence of 125 ng Uba1 (E1 Ub-activating enzyme), 1 μg ubiquitin (Ub), 0.25 μg of Rad6 or 0.6 μg of Rad6-Rad18 complex (E2 Ub-conjugating enzyme), 0.4 μg Nse1 or GST-Nse1, and 100 ng histone H2B. Samples were incubated 1 hour at 30 °C, separated on denaturing polyacrylamide gels and visualized by Western blotting using anti-H2B (α-H2B) or anti-GST (α-GST) antibodies.

Protein-protein interaction experiments

The physical interactions between Nse1 or Mms21 and proteins that participate in the Rad6-Rad18-dependent lesion bypass pathway were determined by using a standard protein interaction protocol. Briefly, Nse1, Mms21, or their GST-tagged forms were incubated with the other proteins in buffer I (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM DTT, 0.01 % NP-40, 1 mM EDTA, and 10 % glycerol) in a 20-μl reaction mixture at 4 °C for 30 minutes, followed by 15 minutes at 25 °C. Glutathione-Sepharose beads (20 μl) were added to the mixtures, which were further incubated 2-3 hours with constant rocking at 4 °C. The beads were spun down, and the unbound protein was collected. The beads were then washed three times with 10 volumes of buffer I. Finally, the bound

proteins were eluted with 20 µl of sodium dodecyl sulfate (SDS) loading buffer. Various fractions were resolved on 10 % denaturing polyacrylamide gels, followed by Coomassie blue staining.

CHAPTER 3: *NSE1* IS INVOLVED IN POSTREPLICATION REPAIR[1]

The *S. cerevisiae NSE1* gene encodes an essential protein of 336 amino acids that localizes primarily to the nucleus (24). Nse1 was the first non-SMC subunit of the Smc5-Smc6 complex to be identified, and *nse1* mutants are sensitive to DNA damaging agents and exhibit abnormal cellular morphologies (24, 83). Epistasis analyses in fission yeast have suggested that Nse1 and Nse2, another non-SMC subunit of the complex, function together with the recombinational protein Rhp51 (Rad51 in budding yeast) in the homologous recombinational repair of DSBs following ionizing irradiation (83). Interestingly, Nse1 contains a conserved C_4HC_3 RING finger-like domain at its C terminus, which is characteristic of E3 ubiquitin ligases (Figure 3.1A). However, no such function has yet been identified for Nse1.

In this study, we have isolated *nse1* mutants in budding yeast by random mutagenesis. One of these mutants, *nse1-101*, is particularly sensitive to UV light and MMS at 30 °C, and shows a growth defect at 37 °C. We have used this mutant for genetic analyses and PRR experiments to better understand the role of *NSE1* in the repair of UV-damaged DNA.

ISOLATION OF *NSE1* MUTANTS

Because the *NSE1* gene is essential for viability, the plasmid shuffle method (13) to isolate *nse1* mutants by random mutagenesis was used. For this purpose, plasmid pPM1162, which contains the wild type *NSE1* gene on a *CEN LEU2* plasmid, was treated with hydroxylamine hydrochloride to introduce random mutations in the DNA.

[1] Santa Maria, S.R., et al. 2007. Mol. Cell. Biol. 27:8409-8418. Used with permission.

Mutagenized plasmids were introduced in the yeast strain YRP723, which contains a genomic deletion of the *NSE1* gene but is viable because it carries plasmid pPM1168 (*NSE1 CEN URA3*), by transformation to Leu+ and subsequent growth on FOA, and transformants were screened for their sensitivity to DNA damaging agents and growth at the non-permissive temperature (37 °C). Of all the mutants obtained, two, *nse1-101* and *nse1-102*, were of particular interest. Both mutants were sensitive to UV light and MMS at 30 °C, but in addition, *nse1-101* was temperature sensitive (*ts*) at 37 °C (Figure 3.1C). The *nse1-101* mutant allele encodes three amino acid substitutions, later confirmed by sequencing to be G175E, S207T, and G332D changes (Figure 3.1A). The *nse1-101* cells held at 37 °C arrested in the cell cycle as large doublets, indicative of arrest at the G_2/M boundary, and even at the permissive temperature (30 °C) the mutant cells showed a preponderance of larger, multibudded cells compared to those of the wild type strain EMY74.7 (Figure 3.1B).

To investigate if *NSE1* plays any significant role in mutagenesis, we examined the rates of spontaneous mutations in the *nse1-101* and *nse1-102* mutants, and the frequency of UV-induced mutations in the *nse1-102* mutant using the $CAN1^S$ to $can1^r$ forward mutation system. Forward mutations were measured by selection for canavanine-resistant colonies which originate from mutation of the $CAN1^S$ wild type allele to $can1^r$. As shown in Table 3.1, the rates of spontaneous mutations were about the same in the wild type and *nse1* mutant strains. In addition, the frequency of UV-induced mutations for the *nse1-102* mutant showed no significant increase (or decrease) compared to wild type (data not shown).

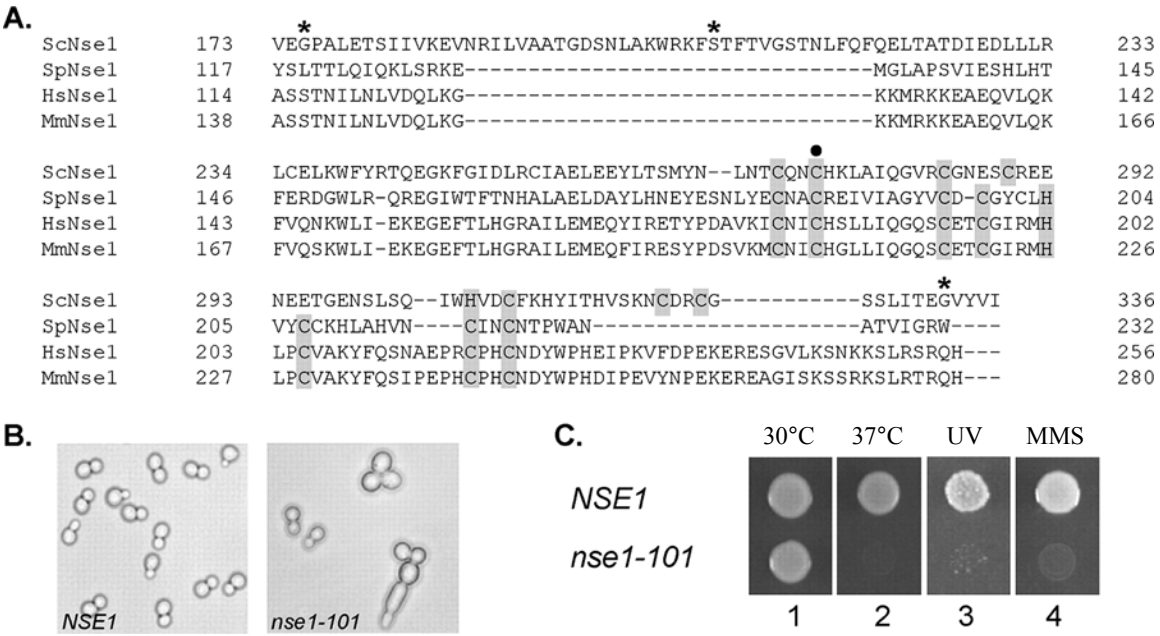

FIGURE 3.1. ISOLATION OF THE *S. CEREVISIAE NSE1-101* MUTANT. (A) Sequence alignment of the C termini of Nse1 proteins from various organisms. Multiple sequence alignment was performed with CLUSTAL W version 1.82. Sequences aligned are *S. cerevisiae* Nse1 (ScNse1; 336 amino acids), *S. pombe* Nse1 (SpNse1; 232 amino acids), *Homo sapiens* Nse1 (HsNse1; 256 amino acids), and *Mus musculus* Nse1 (MmNse1, 280 amino acids). Shaded residues correspond to the C_4HC_3 RING finger-like domain. 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* C274A 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 37 °C. (3) Cells were spotted on a YPD plate, UV irradiated at 80 J/m^2, and incubated for 2 days at 30 °C. (4) Cells were spotted on a YPD plate containing 0.035 % MMS and incubated at 30 °C for 2 days. This figure is adapted with permission from reference 115.

TABLE 3.1. Analysis of the rate of spontaneous mutations in *NSE1*, *nse1-101*, *nse1-102*, and *mms21* RING strains

		can1^r mutants per 10^7 viable cells	
Strain	Genotype	Spontaneous (mean ± SD)	Fold increase
EMY74.7	*NSE1*	6.45 ± 1.38	1
YRP727	*nse1-101*	4.99 ± 0.40	0.8
YRP728	*nse1-102*	10.38 ± 2.27	1.6
YMMS21-46	*mms21* C200A H202A	14.94 ± 1.05	2.3

EPISTASIS ANALYSES OF *NSE1-101* FOR UV SENSITIVITY

Epistasis experiments using the serial dilution method were performed to better understand the role of *NSE1* in DNA repair. To determine the epistasis relationship of the *nse1* mutants with genes that function in different DNA repair pathways for UV damage, we combined the *nse1-101* and *nse1-102* mutations with the *rad14*Δ, *rad18*Δ, or *rad52*Δ mutation. Cells carrying deletions of the *RAD14* and *RAD52* genes are defective in NER and recombination, respectively. *RAD6* and *RAD18* encode proteins that form a complex with E2 ubiquitin conjugating and DNA binding activities, and mutations of these genes render cells defective in the promotion of replication through DNA lesions. Unfortunately, double mutants carrying an *nse1* mutation and *rad6*Δ grew poorly, and therefore gave inconclusive results. As shown in Figure 3.2, both the *nse1-101 rad14*Δ and *nse1-101 rad18*Δ double mutant strains exhibited higher degrees of UV sensitivity than the corresponding *rad14*Δ and *rad18*Δ strains, whereas the UV sensitivity of the *nse1-101 rad52*Δ strain is more similar to that of the *rad52*Δ strain. Similar results were

obtained for the *nse1-102* mutant (data not shown). These observations suggest that Nse1 functions in the repair of UV-damaged DNA together with the Rad52-dependent recombinational repair pathway.

To verify that Nse1 promotes repair of UV lesions independently of the Rad6-Rad18-dependent pathway, we combined the *nse1* mutations with the *rad5*Δ, *mms2*Δ, or *ubc13*Δ mutation. Rad5 is a RING finger protein that interacts with Rad18, and this interaction brings together the ubiquitin conjugating functions of Rad6-Rad18 and Mms2-Ubc13 to promote error-free PRR of UV-damaged DNA (138). Thus, a deletion of any of these three genes inactivates the error-free PRR pathway controlled by the Mms2-Ubc13-Rad5 branch of the Rad6-Rad18 pathway. As shown in Figure 3.3, *nse1-101 rad5*Δ, *nse1-101 mms2*Δ, and *nse1-101 ubc13*Δ double mutants were more sensitive to UV light than the corresponding *rad5*Δ, *mms2*Δ, and *ubc13*Δ single mutants. In addition, we also examined the UV sensitivity conferred by the *nse1* mutations in combination with the *rad30*Δ or *rev3*Δ, which inactivate error-free and mutagenic TLS of UV-damaged DNA, respectively. Whereas *RAD30* encodes Polη, which promotes error-free bypass of UV lesions, *REV3* encodes the catalytic domain of the mutagenic TLS polymerase Polζ. As in the case of Rad5, Mms2, and Ubc13, an increase in the UV sensitivity occurred in the double mutant strains compared to the single mutants (data not shown). Thus, these results further support a role for Nse1 in a pathway that acts independently of the Rad6-Rad18-dependent TLS and error-free PRR pathways.

Interestingly, the UV sensitivity of the *pol30-119* mutant is greatly increased in the presence of the *nse1-101* mutation (Figure 3.3). In the absence of SUMO modification at the lysine 164 of PCNA, as occurs in the *pol30-119* mutation, the Rad52-dependent recombinational pathway becomes activated (37). Thus, the enhanced sensitivity observed in the *nse1-101 pol30-119* double mutant further supports a role for

Nse1 in the Rad52-dependent repair pathway. A probable role for the E3 ligase functions of Nse1 and Mms21 in this pathway is discussed in Chapter 4.

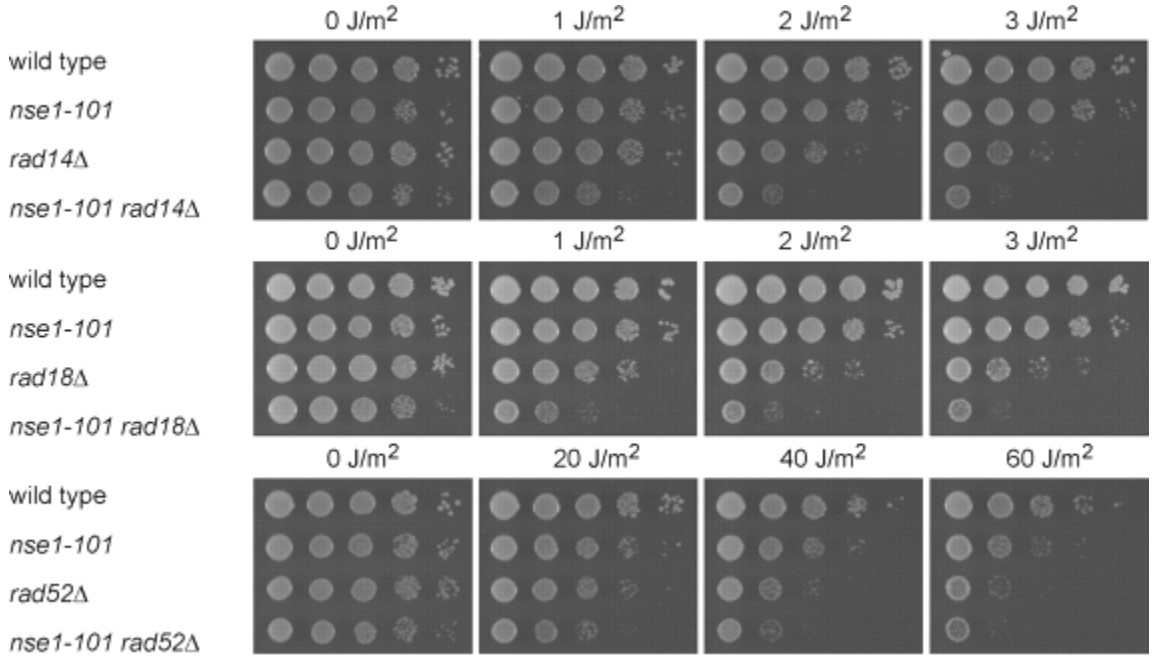

FIGURE 3.2. EPISTASIS ANALYSIS OF THE *NSE1-101* MUTANT AND MEMBERS OF DIFFERENT DNA REPAIR PATHWAYS. The *nse1-101* mutation enhances the UV sensitivity of the *rad14*Δ and *rad18*Δ mutants, but not of the *rad52*Δ mutant, suggesting that Nse1 functions in conjunction with the Rad52-dependent repair pathway. 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. This figure is adapted with permission from reference 115.

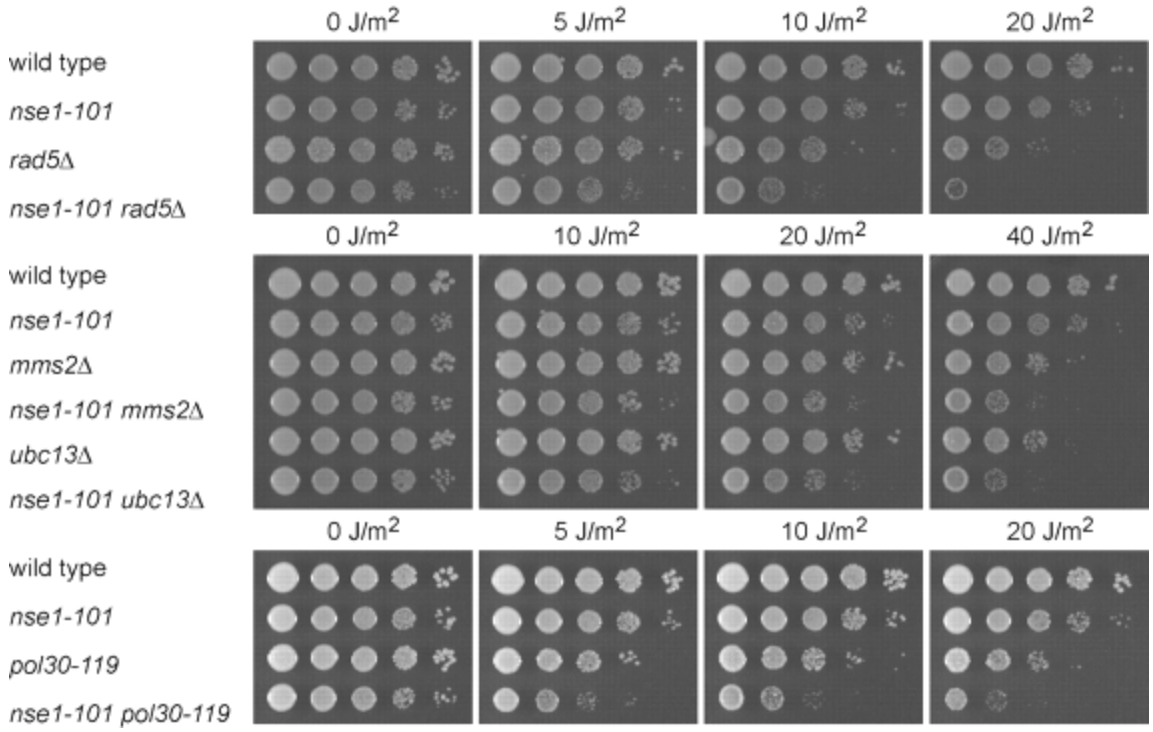

FIGURE 3.3. EPISTASIS ANALYSIS OF THE *NSE1-101* MUTANT AND MEMBERS OF THE RAD6-RAD18 PATHWAY. The *nse1-101* mutation enhances the UV sensitivity of the *rad5*Δ, *mms2*Δ, and *ubc13*Δ mutants, adding further support for a role of Nse1 in a pathway independent of Rad6-Rad18. The UV sensitivity of the *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. This figure is adapted with permission from reference 115.

PURIFICATION AND CHARACTERIZATION OF THE NSE1 PROTEIN

We purified the Nse1 protein from yeast and characterized its properties in order to gain an understanding of its role *in vivo*. For this purpose, we cloned the open reading frame (ORF) of the wild type *NSE1* gene in N-terminal fusion with GST using the expression vector pBJ842 (57). The GST-Nse1 fusion protein was expressed in the protease-deficient yeast strain YRP654 (*MAT*α *ura3-52 trp1*Δ *leu2*Δ*1 his3*Δ*200 pep4::HIS3 prb1*Δ*1.6R GAL*) by induction with galactose, and purified by affinity

chromatography on immobilized glutathione. Nse1 protein was obtained after cleavage with PreScission protease. After elution, the resulting fractions were analyzed by SDS-PAGE and Coomassie blue staining, and the molecular weight of the resulting bands checked using protein standards. As shown in Figure 3.4, both GST-Nse1 fusion protein (~ 65 kDa) and Nse1 protein (38 kDa) band sizes are consistent with their predicted molecular weight.

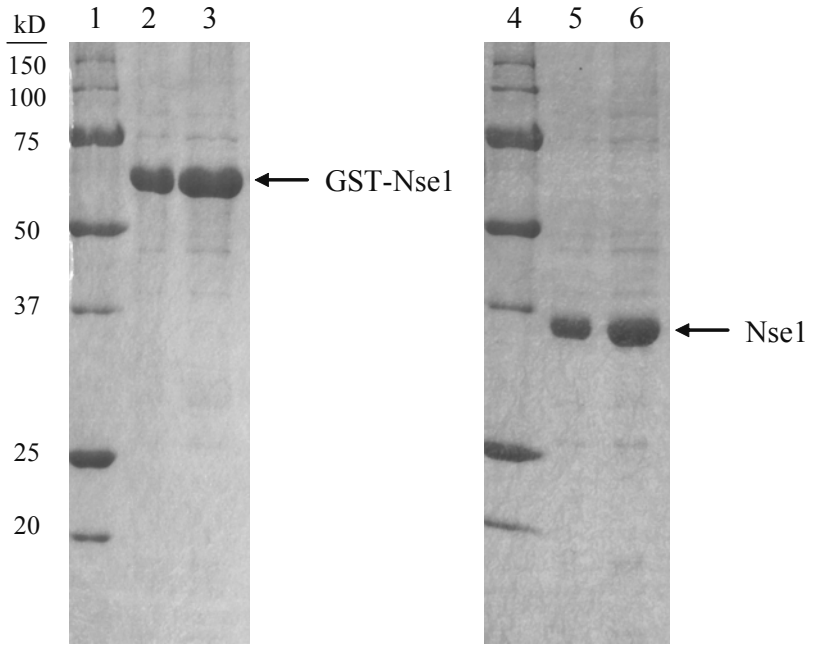

FIGURE 3.4. EXPRESSION AND PURIFICATION OF NSE1 PROTEIN. Coomassie brilliant blue-stained 12 % denaturing polyacrylamide gel. Lanes 1 and 4, molecular weight standards. Lanes 2 and 3, fractions containing purified GST-Nse1 fusion protein. Lanes 5 and 6, purified Nse1 protein after cleavage with PreScission protease.

Next, we examined the predicted ATP hydrolysis and DNA binding activities of Nse1 by thin layer chromatography (TLC) and electrophoretic mobility shift assays (EMSA), respectively. These functions had been indicated using *ab initio* structure

prediction models (Yeast Resource Center, University of Washington); however, we were not able to detect any ATPase or DNA binding activity for Nse1 under a variety of conditions tested (Figures 3.5 and 3.6). *In vitro* protein-protein interaction experiments were also performed, but as expected from the genetic analyses, no evidence for the physical or functional interaction of Nse1 (or GST-Nse1) with members of the Rad6-Rad18-dependent pathway was found (data not shown).

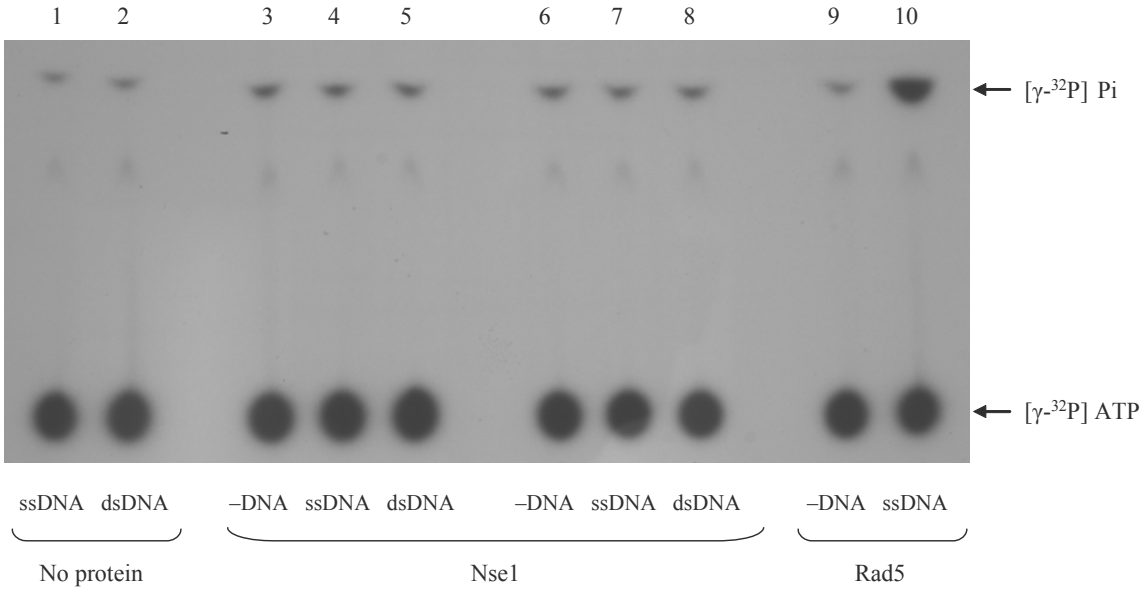

FIGURE 3.5. ATP HYDROLYSIS ACTIVITY OF NSE1. Purified Nse1 or Rad5 protein was incubated at 37 °C for 30 minutes in 50 mM buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 100 µg/ml BSA, 1 mM DTT, 0.5 mM ATP) using γ-^{32}P-labeled ATP in the presence or absence of 100 ng of single-stranded M13mp18 DNA or double-stranded pUC19 DNA. A 1-µl aliquot was then spotted onto TLC paper and developed for 15-20 minutes. The ATPase activity was visualized by autoradiography.

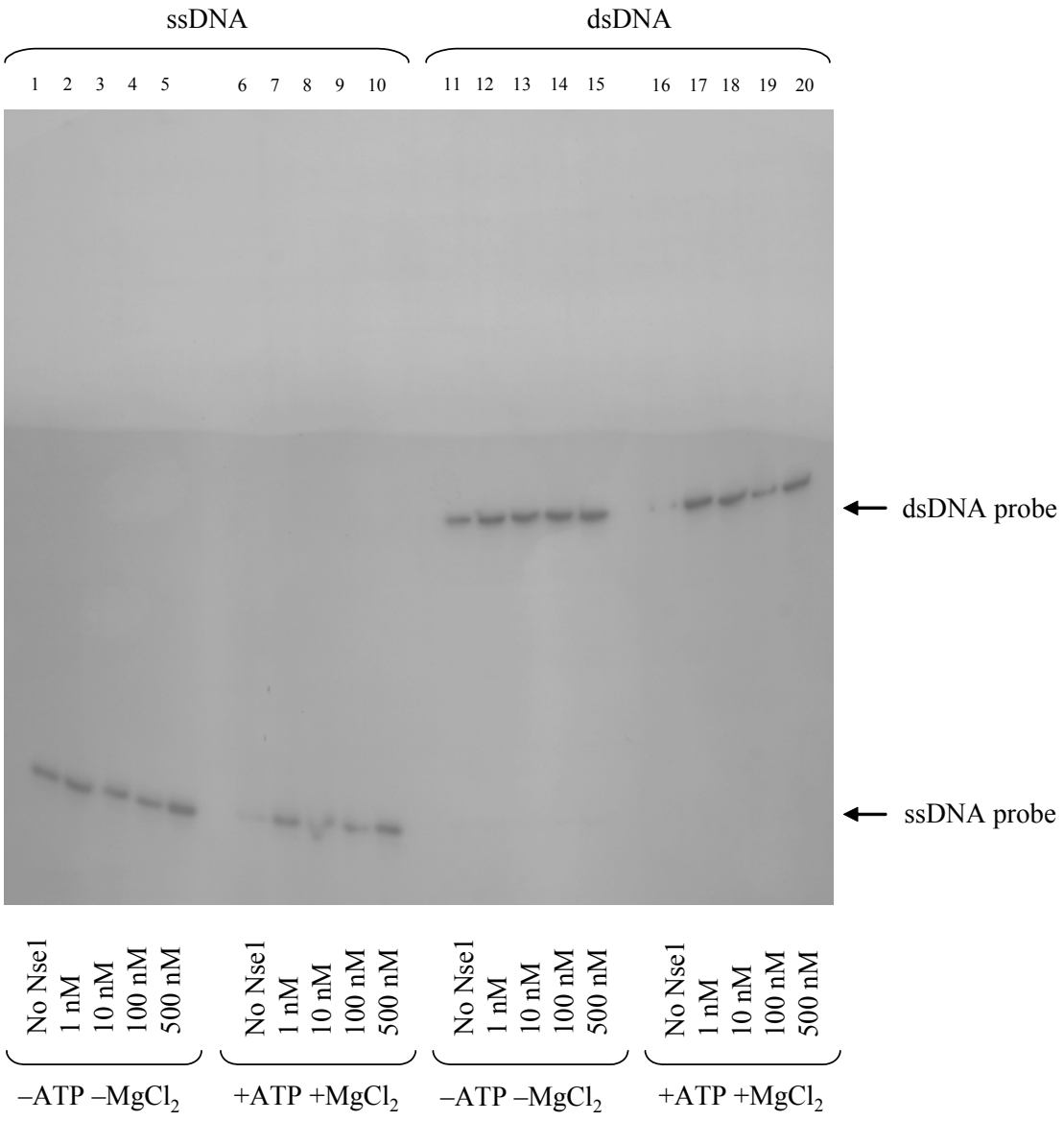

FIGURE 3.6. DNA BINDING ACTIVITY OF NSE1. PreScission-cut Nse1 protein was incubated for 10 minutes at 30 °C in DNA binding buffer with ^{32}P-labeled single-stranded (lanes 1-10) or double-stranded DNA (lanes 11-20) in the presence or absence of ATP and $MgCl_2$. The samples were then loaded on a 12 % polyacrylamide gel and the DNA binding activity visualized by autoradiography.

IMPAIRED PRR IN THE *NSE1-101* MUTANT

PRR has been defined as the process by which cells are able to restore to high molecular weight DNA synthesized from damaged DNA after UV irradiation. 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 *rad1*Δ *nse1-101* mutant cells following UV irradiation by alkaline sucrose gradient sedimentation.

Cells carrying a deletion of the *RAD1* gene are defective in NER. Because of the absence of excision repair, UV-induced DNA damage is not removed, and replication through such lesions is dependent upon the various lesion bypass processes. When *rad1*Δ cells are UV irradiated at 3.5 J/m^2 at 30 °C and the size of the newly synthesized DNA is examined by pulse-labeling of DNA with [^{3}H] uracil for 15 minutes, followed by a chase for 30 minutes at 30 °C, the DNA sediments toward the top of the alkaline sucrose gradient, indicative of the presence of discontinuities or gaps in the newly synthesized DNA. Further incubation for 6 hours at 30 °C restores the DNA to high molecular weight, indicating that the postreplicative gap filling processes are functional and have restored normal-size DNA (Figure 3.7A). On the other hand, when *rad1*Δ *nse1-101* cells are UV irradiated at 30 °C and then given a 15-minute pulse and a 6-hour incubation at 30 °C, normal-size DNA is not fully reconstituted; instead, the size attained is intermediate between low molecular weight DNA from cells UV irradiated with no period of repair and DNA from unirradiated cells given a 6-hour incubation period (Figure 3.7B). Commensurate with its *ts* phenotype at 37 °C, the *rad1*Δ *nse1-101* mutant displays a higher degree of the PRR defect at 37 °C than at 30 °C (Figure 3.7B). In the unirradiated control, however, the *rad1*Δ *nse1-101* mutant showed normal-size DNA in cells incubated at 37 °C. Since no evidence was found for a defect in DNA replication in unirradiated *rad1*Δ *nse1-101* cells at the non-permissive temperature and normal-size

DNA was formed in unirradiated controls, the persistence of low molecular weight DNA after UV irradiation must result from a defect in PRR.

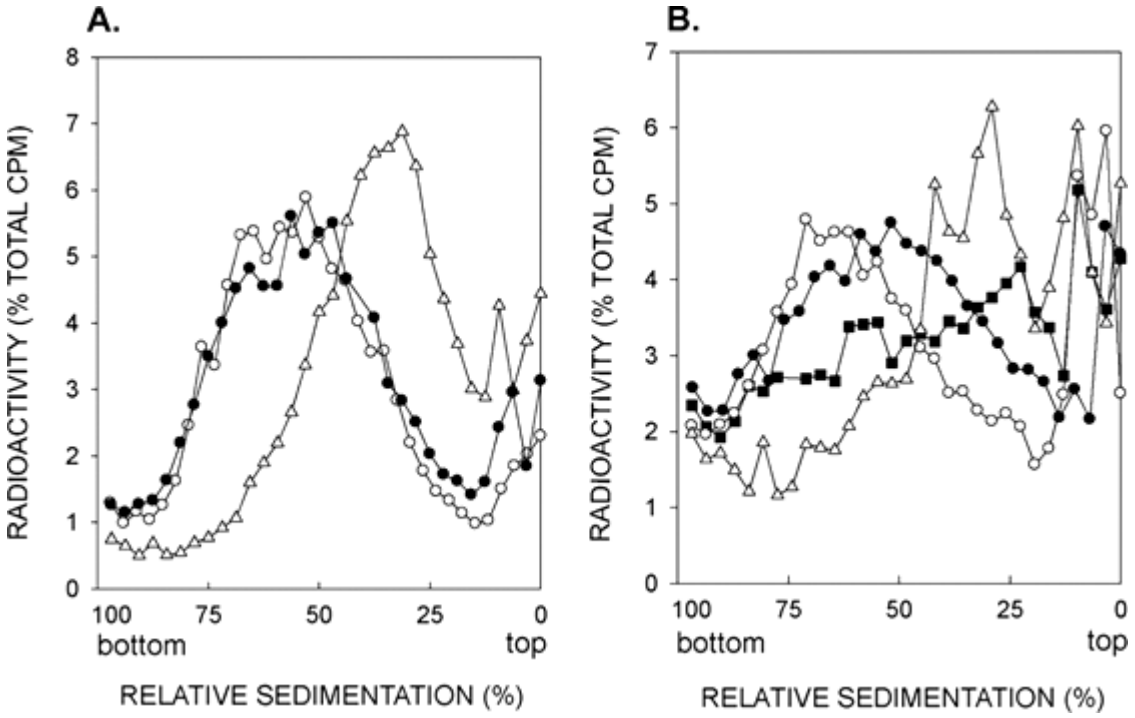

FIGURE 3.7. REQUIREMENT OF THE *NSE1* GENE FOR PRR OF UV-DAMAGED DNA. Sedimentation in alkaline sucrose gradients of nuclear DNA from cells incubated for different periods following UV irradiation. The *rad1*Δ (A) and *rad1*Δ *nse1-101* (B) strains were UV irradiated at 3.5 J/m^2 and then pulse-labeled with [^{3}H] uracil for 15 minutes, followed by a 30-minute chase (Δ), a 6-hour chase at 30 °C (●), or a 6-hour chase at 37 °C (■) in high-uracil medium. DNA synthesized in unirradiated cells was pulse-labeled with [^{3}H] uracil for 15 minutes, which was followed by a 6-hour chase (○) at 30 °C for the *rad1*Δ strain and at 37 °C for the *rad1*Δ *nse1-101* strain; in unirradiated *rad1*Δ *nse1-101* cells that were treated similarly but kept for 6 hours at 30 °C, normal-size DNA was reconstituted in a manner similar to that seen in cells held at 37 °C. This figure is reproduced with permission from reference 115.

INVOLVEMENT OF NSE1 IN RAD52-DEPENDENT PRR

In addition to the Rad5-Mms2-Ubc13-dependent pathway, recent evidence has indicated a role for Rad52-dependent recombination as an alternate pathway for PRR of

UV-damaged DNA (26). Thus, replication through UV lesions in yeast cells is mediated by Rad6-Rad18-dependent lesion bypass processes and by a Rad52-dependent pathway that acts independently of Rad6 and Rad18. Genetic studies in budding yeast have suggested that the Rad5-dependent and Rad52-dependent PRR pathways act predominantly in a noncompeting manner (25, 26, 128, 146), in which the Rad5-dependent PRR pathway promotes replication when the UV lesion is on the leading strand, and that the Rad52-dependent pathway promotes replication when the lesion is on the lagging strand.

Here, we examined whether, as suggested by the epistasis analysis of the *nse1-101* mutation with genes involved in different DNA repair processes (Figure 3.2), Nse1 functions together with the Rad52 group of recombinational proteins in PRR. As shown in Figure 3.8C, compared to that in the *rad1*Δ *nse1-101* (Figure 3.7B) and *rad1*Δ *rad5*Δ (Figure 3.8A) cells, the PRR defect is greatly enhanced in *rad1*Δ *rad5*Δ *nse1-101* cells that are UV irradiated, pulse-labeled for 15 minutes, and then incubated for 6 hours at 37 °C. In fact, no residual PRR was observed in the mutant cells, suggesting that Nse1 works independently of the Rad5-dependent pathway. To verify that Nse1 is involved in Rad52-dependent PRR, we examined PRR in *rad1*Δ *rad52*Δ *nse1-101* mutant cells (Figure 3.8D), and found that the PRR defect observed in these cells was not greater than the defect seen in *rad1*Δ *nse1-101* (Figure 3.7B) and *rad1*Δ *rad52*Δ (Figure 3.8B) cells. Since normal-size DNA was reconstituted in unirradiated *rad1*Δ *rad5*Δ *nse1-101* and *rad1*Δ *rad52*Δ *nse1-101* mutant cells which were pulse-labeled for 15 minutes and then incubated for 6 hours at 37 °C (Figures 3.8C and 3.8D), the persistence of small molecular size DNA in these mutants when they are UV irradiated and held at 37 °C reflects the PRR defect. Together with our epistasis experiments, these observations further confirm a role for Nse1 in Rad52-dependent PRR of UV-damaged DNA.

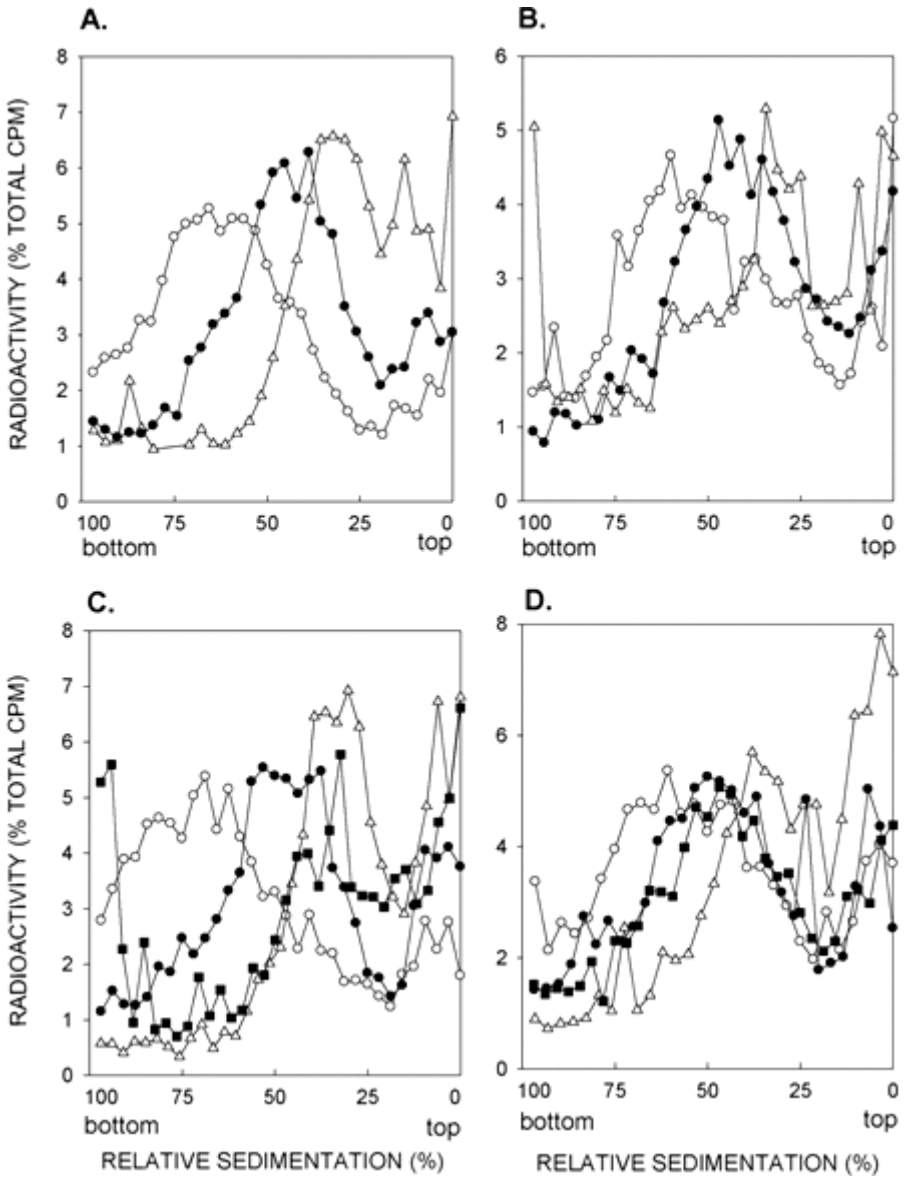

FIGURE 3.8. INVOLVEMENT OF *NSE1* IN THE *RAD52*-DEPENDENT PRR PATHWAY. Sedimentation in alkaline sucrose gradients of nuclear DNA from cells incubated for different periods following UV irradiation. The *rad1*Δ *rad5*Δ (A), *rad1*Δ *rad52*Δ (B), *rad1*Δ *rad5*Δ *nse1-101* (C), and *rad1*Δ *rad52*Δ *nse1-101* (D) strains were UV irradiated at 3.5 J/m^2 and then pulse-labeled with [^{3}H] uracil for 15 minutes, followed by a 30-minute chase (Δ), a 6-hour chase at 30 °C (●), or a 6-hour chase at 37 °C (■) in high-uracil medium. DNA synthesized in unirradiated cells was pulse-labeled with [^{3}H] uracil for 15 minutes, which was followed by a 6-hour chase (○) at 30 °C for the *rad1*Δ *rad5*Δ and *rad1*Δ *rad52*Δ strains and at 37 °C for the *rad1*Δ *rad5*Δ *nse1-101* and *rad1*Δ *rad52*Δ *nse1-101* strains. This figure is reproduced with permission from reference 115.

DISCUSSION

In eukaryotes, SMC proteins play essential roles in chromosome dynamics, regulation of gene expression, and DNA repair. In comparison to the well characterized roles of the condensin and cohesin complexes in chromosome condensation and sister chromatid cohesion, respectively, the Smc5-Smc6 complex is particularly required for a coordinated response to DNA damage and for normal chromosome integrity. Although the major components of this complex have already been identified, their functional roles in DNA repair remain unclear. The *S. cerevisiae* Smc5-Smc6 complex is composed of eight essential subunits: Smc5, Smc6, Nse1, Mms21, Nse3, Nse4, Nse5, and Nse6. Hypomorphic mutants of different members of the Smc5-Smc6 complex have been isolated and found to be sensitive to a variety of DNA damaging agents, including UV and gamma irradiation, and the alkylating agent MMS (6, 24, 83, 86, 93, 95, 96, 125, 147). A role for Smc5-Smc6 in the repair of DSBs was inferred from epistasis analyses of *S. pombe smc6* mutants with mutations in the *RAD52* gene epistasis group. Furthermore, these mutants showed a defect in the repair of DSBs resulting from ionizing radiation damage (22, 75, 141). Although these studies indicate a possible role for Smc6 and probably the entire complex in the recombinational repair of DSBs following gamma irradiation, they fail to explain the UV and MMS sensitivities observed in the Smc5-Smc6 complex mutants, since base damage and not DSB is the predominant lesion formed by these DNA damaging agents.

Of the non-SMC subunits of the Smc5-Smc6 complex, the Nse1 and Mms21 proteins are of particular interest since they contain sequence domains characteristic of E3 ubiquitin ligases and E3 SUMO ligases, respectively, and Mms21 has been shown to promote the sumoylation of different subunits of the Smc5-Smc6 complex and other proteins involved in DNA repair in both yeast and humans (6, 103, 147). The *MMS21*

gene was identified in a screen for genes affecting resistance to MMS more than 30 years ago (106). The isolated mutation (*mms21-1*) conferred sensitivity to UV, MMS, and gamma rays, and a growth defect at 37 °C. In the absence of DNA damaging agents, the mutant cells also exhibited poor growth, a preponderance of large multibudded cells, an increase in the rate of spontaneous mutations, and a hyper-recombinational phenotype (85, 108). It was later found that the *mms21-1* mutation results in the deletion of the C terminus of Mms21, which contains the RING motif necessary for its enzymatic activity. Unfortunately, and because of its poor growth and associated phenotypes, this mutant could not be used to further investigate the role of Mms21 in the repair of UV-damaged DNA.

In this study we have identified a *ts* mutation of *NSE1* in *S. cerevisiae*, *nse1-101*, with which we have been able to determine a role for Nse1 in promoting resistance to UV damage. The *nse1-101* mutation confers sensitivity to UV irradiation as well as to MMS at the permissive temperature (30 °C), and the mutant cells exhibit a preponderance of larger, multibudded cells compared to those of a wild type strain (Figure 3.1). From epistasis analyses with different genes involved in repair processes, we have inferred a role for Nse1 in Rad52-dependent repair of UV lesions. Consistent with this, double mutant combinations of the *nse1-101* mutation with mutations of genes involved in NER and in Rad6-Rad18-dependent lesion bypass processes were more UV sensitive than the corresponding single deletion mutants. Our PRR experiments further support a role for *NSE1* in Rad52-dependent lesion bypass. We found that whereas at 30 °C the proficiency of PRR following UV irradiation is impaired to only a modest degree by the *nse1-101* mutation, a much higher degree of impairment in PRR occurs at 37 °C. Since Rad5 and Rad52 function in alternate PRR pathways for UV damage, we examined whether Nse1 is involved in Rad52-dependent PRR, as suggested by our epistasis

experiments. From these studies, we provided evidence that Nse1 functions in PRR in a Rad5-independent but Rad52-dependent manner.

Interestingly, we observed that newly synthesized DNA in the UV-irradiated *rad1*Δ *rad52*Δ *nse1-101* cells held at 37 °C shows more of a shift to a larger molecular size than is seen in the *rad1*Δ *nse1-101* mutant cells (compare Figure 3.7B with Figure 3.8D). This accumulation of low molecular weight DNA in the *rad1*Δ *nse1-101* mutant suggests that in the absence of Nse1 function, the DNA structures that are generated from the action of Rad52 and other recombinational proteins are more prone to nucleolytic attack and degradation, and implies a role for Nse1 and the entire Smc5-Smc6 complex in stabilizing these recombinogenic intermediate structures and protecting them from nucleolytic degradation.

We have purified the Nse1 protein in yeast, and as we expected from our genetic and PRR analyses, we found no evidence for the physical or functional interaction of Nse1 with different members of the Rad6-Rad18-dependent lesion bypass processes. In addition, we were unable to detect any DNA binding or ATPase activity. The E2 ubiquitin conjugating enzyme with which Nse1 probably collaborates as an E3 ubiquitin ligase and the protein substrates that are ubiquitinated by this enzyme complex remain to be identified.

CHAPTER 4: REQUIREMENT OF NSE1 AND MMS21 LIGASE FUNCTIONS FOR RAD52-DEPENDENT PRR[2]

Nse1 and Mms21 were the first two non-SMC subunits of the Smc5-Smc6 complex to be identified in both budding and fission yeast (6, 24, 83, 147). Hypomorphic *nse1* and *mms21* mutant cells exhibit increased sensitivity to different DNA damaging agents and display abnormal cellular morphologies, and epistasis analyses have indicated a role for Nse1 and Mms21 (and presumably for the entire Smc5-Smc6 complex) in Rad52-dependent recombination repair. Most importantly, examination of the primary sequences of Nse1 and Mms21 in yeast and humans reveals the presence of conserved RING finger-like and SP-RING motifs that are found in E3 ubiquitin and E3 SUMO ligases, respectively. It was subsequently reported that Mms21 in yeast and humans functions as a SUMO ligase that modifies different subunits of the Smc5-Smc6 complex, as well as other proteins involved in DNA repair, and that this activity is required for a normal DNA damage response (6, 103, 147). Hence this nuclear SMC complex potentially combines ubiquitin and SUMO ligase activities in addition to its role in chromosome organization.

In this study, we have constructed mutations in the RING finger motifs of both Nse1 and Mms21 to examine whether the putative ubiquitin ligase activity of Nse1 and the SUMO ligase function of Mms21 contribute to the Rad52-dependent PRR pathway for UV-damaged DNA.

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[2] Santa Maria, S.R., et al. 2007. Mol. Cell. Biol. 27:8409-8418. Used with permission.

ISOLATION AND GENETIC CHARACTERIZATION OF THE *NSE1* RING MUTATION

Since the *nse1-101* mutation does not involve any alteration in the C_4HC_3 RING finger-like motif of Nse1 (Figure 3.1), we constructed a mutation in this domain to analyze whether the putative E3 ubiquitin ligase activity of Nse1 contributes to the Rad52-dependent PRR pathway. For this purpose, we changed the cysteine 274 present in this motif to alanine by site-directed mutagenesis of plasmid pPM1162 (*NSE1 CEN LEU2*), and introduced the plasmid containing the mutation in the *nse1*Δ strain using the plasmid shuffle method (13). We examined the effect of the *nse1* C274A mutation on cell growth and sensitivity to DNA damaging agents.

Similar to the *nse1-101* mutation, cells carrying the *nse1* C274A mutation exhibit a *ts* phenotype at the restrictive temperature (37 °C) (Figure 4.1B); cells stop division as large, multibudded cells, indicative of G_2/M checkpoint arrest, and even at 30 °C, the mutant cells show a preponderance of large, multibudded cells that tend to form large clusters (Figure 4.1A). The mutant cells also show sensitivity to UV irradiation and the alkylating agent MMS (Figure 4.1B). To examine the DNA repair role of the putative ligase function of Nse1, we performed epistasis experiments with mutations in genes affecting different DNA repair pathways. As shown in Figure 4.1C, the UV sensitivity resulting from the *rad14*Δ, *rad18*Δ, and *rad5*Δ mutations is enhanced significantly when they are combined with the *nse1* C274A mutation, whereas the *nse1* C274A *rad52*Δ double mutant displays nearly the same extent of UV sensitivity as the *rad52*Δ single mutant. These observations also suggest a role for the putative E3 ubiquitin ligase function of Nse1 in Rad52-dependent PRR of UV damage. Although these mutant cells are viable, their poor growth characteristics did not allow us to perform PRR experiments, and thus we were unable to directly examine the effect of this mutation on PRR of UV lesions.

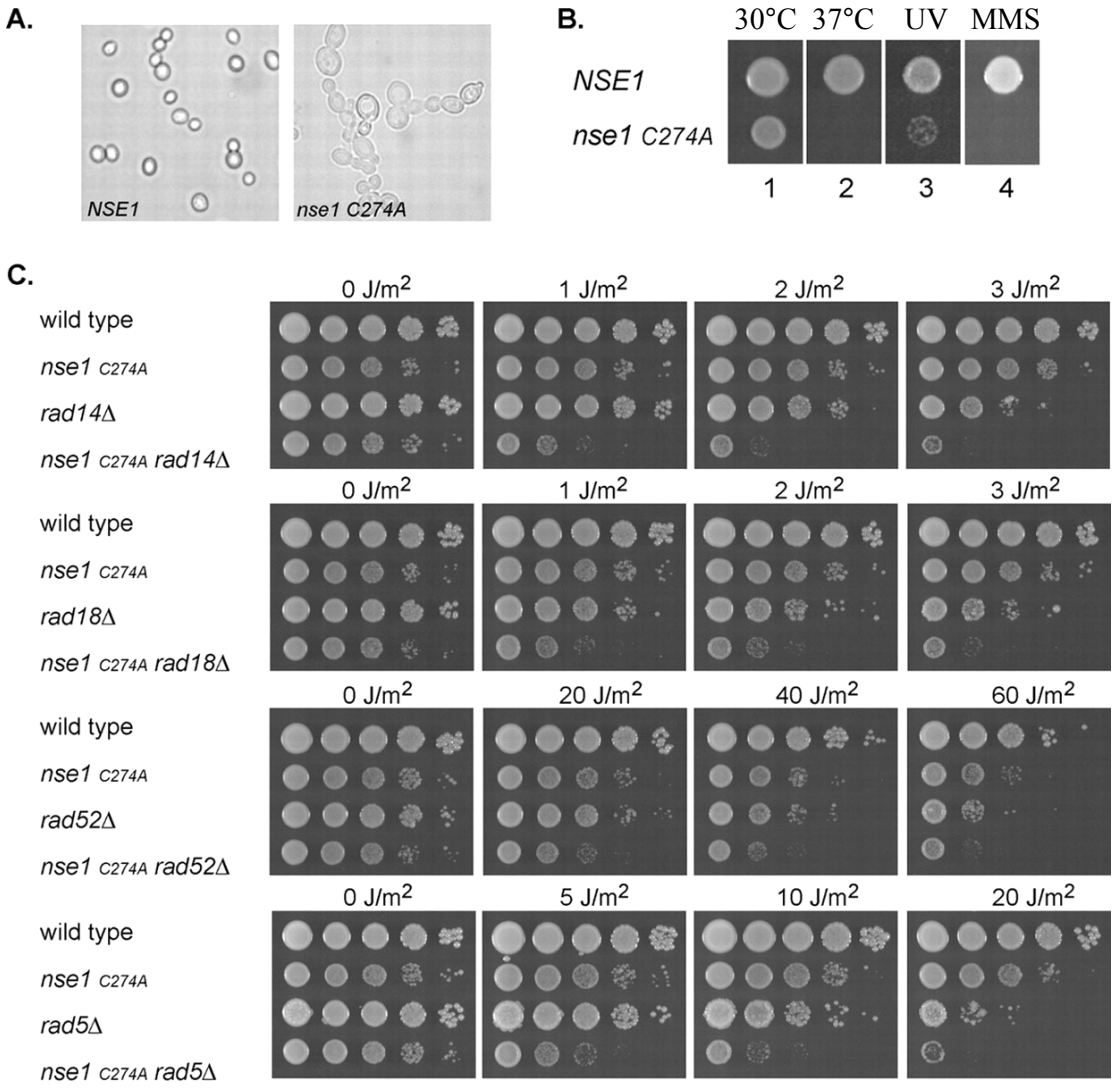

FIGURE 4.1. EPISTASIS ANALYSIS OF THE *NSE1* C274A MUTATION. (A) Photomicrograph of *nse1* C274A mutant cells showing abnormal morphology compared to that of wild type cells at 30 °C. (B) Sensitivity of the *nse1* C274A mutant to DNA damaging agents. (1) Growth on a YPD plate at 30 °C for 2 days. (2) Growth deficiency on a YPD plate at 37 °C. (3) Cells were spotted on a YPD plate, UV irradiated at 80 J/m^2, and incubated for 2 days at 30 °C. (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 *rad14*Δ, *rad18*Δ, and *rad5*Δ mutants, but not of the *rad52*Δ 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. This figure is adapted with permission (115).

Because Nse1 carries a RING finger-like motif common in E3 ligases for ubiquitin conjugation, we decided to study its putative ligase activity using an *in vitro* ubiquitination assay (Figure 4.2). Using purified GST-Nse1 protein as a possible ubiquitin ligase, we investigated whether ubiquitin is transferred to Nse1 in a reaction mix containing the E1 ubiquitin-activating enzyme Uba1, and Rad6 or Rad6-Rad18 complex (Figure 4.2B) or Mms2-Ubc13 (data not shown) as possible E2 ubiquitin-conjugating enzymes. However, we did not detect any ubiquitin ligase activity for Nse1 under these conditions. The most logical explanation for these results is that if Nse1 is indeed an E3 ubiquitin ligase, it probably coordinates ubiquitin conjugation of target proteins with E2s other than the ones involved in the Rad6-Rad18 pathway. In addition, not all ubiquitin ligases interact directly with ubiquitin but rather interact with the E2 enzyme or the target protein to increase conjugation efficiency. Furthermore, the protein substrates that may be ubiquitinated by Nse1 *in vivo* or *in vitro* are not known.

EFFECT OF A MUTATION IN THE E3 SUMO LIGASE FUNCTION OF MMS21

To investigate whether the SUMO ligase function of Mms21 is also involved in Rad52-dependent PRR, we constructed a mutation in the SP-RING motif of Mms21 by changing the cysteine 200 and histidine 202 residues to alanines (Figure 4.3A). These mutations were produced by site-directed mutagenesis of plasmid pMMS21-44, and integrated directly into the genome using the gene replacement method. The resulting mutation, *mms21* RING, confers a growth defect at the non-permissive temperature (37 °C), and cells stop division with a terminal morphology of large, multibudded cells even at 30 °C (Figure 4.3B and C). As with the *nse1* mutants, *mms21* RING mutant cells exhibit sensitivity to UV irradiation and MMS at 30 °C (Figure 4.3C).

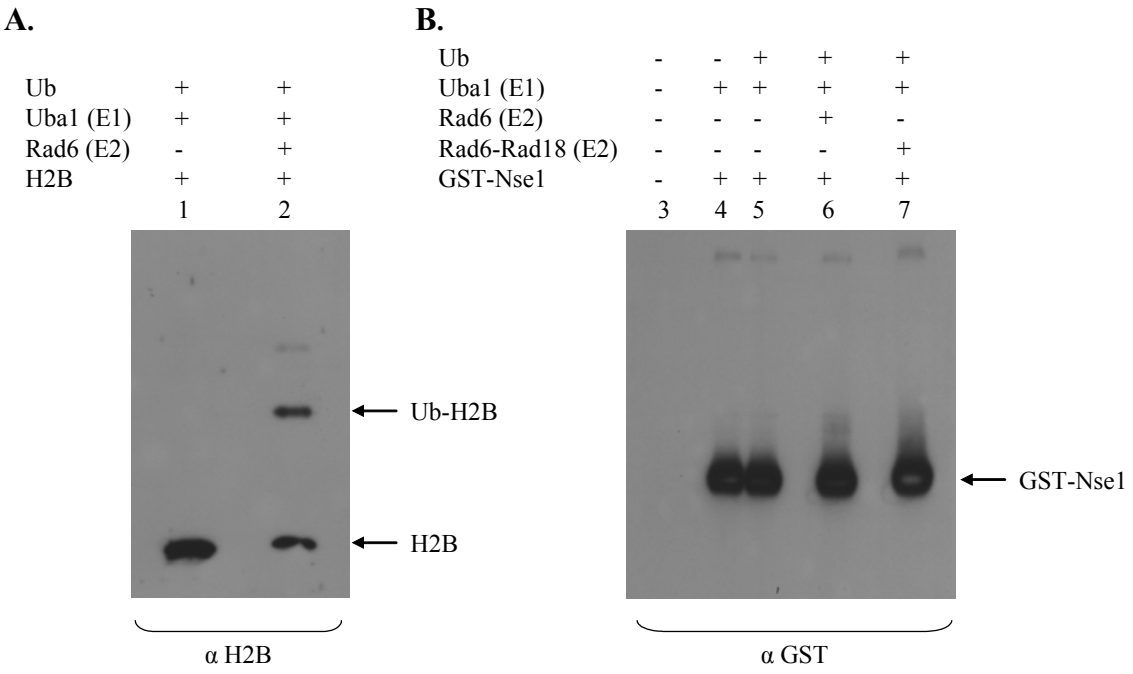

FIGURE 4.2. *IN VITRO* UBIQUITINATION ASSAYS. *In vitro* ubiquitination reactions were carried out in 50 mM ubiquitination buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 100 μg/ml BSA, 1 mM DTT, 100 μM ATP) in the presence of Uba1 protein (E1), ubiquitin (Ub), and in the presence or absence of Rad6 (E2), Rad6-Rad18 (E2), histone H2B, and GST-Nse1. Samples were incubated 1 hour at 30 °C, separated on denaturing polyacrylamide gels and visualized by Western blotting using anti-H2B (α-H2B) or anti-GST (α-GST) antibodies. (A) Ubiquitination of histone H2B by the E2 Ub-conjugating enzyme Rad6. Uba1, Ub, H2B were incubated in the absence (1) or presence (2) of Rad6 protein. (B) Ubiquitination reactions using GST-Nse1 as the E3 Ub ligase. Uba1 and GST-Nse1 were incubated in the absence (4) or presence (5) of Ub. No transfer of Ub to Nse1 was observed, even upon addition of Rad6 (6) or Rad6-Rad18 (7).

The role of the Mms21 SUMO ligase in DNA repair was examined by epistasis analysis using the serial dilution method. We constructed double mutant combinations of the *mms21* RING mutation with mutations of genes involved in different DNA repair pathways. As shown in Figure 4.3D, the UV sensitivity resulting from the *mms21* RING mutation is greatly enhanced when combined with the *rad14*Δ and *rad18*Δ mutations, whereas with increasing UV fluence, the UV sensitivity of the *mms21* RING *rad52*Δ double mutant increases to nearly the same extent as that of the *rad52*Δ mutant. Similar

to what occurs with the *nse1-101* and *nse1* C274A mutations, the epistasis of the *rad52*Δ and *mms21* RING mutations suggests an involvement of the E3 SUMO ligase activity of Mms21 in Rad52-dependent PRR. However, because of the poor growth impairment of the *mms21* RING mutant, we have been unable to directly examine its effects on PRR of UV-damaged DNA.

To determine whether Nse1 and the E3 SUMO ligase function of Mms21 play any role in recombinational repair processes, we examined the rates and frequencies of spontaneous and UV-induced recombination in the *nse1-101* and *mms21* RING mutants, respectively. For this purpose, we introduced the *mms21* C200A H202A and *nse1-101* mutations in the genomic DNA of yeast strain RSY6, which carries an intrachromosomal duplication of the *his3* gene (117, 118). In this duplication, one copy of the *his3* gene bears a terminal deletion at the 3' end and the other a terminal deletion at the 5' end, separated by the *LEU2* gene and plasmid DNA sequences. The two copies share about 400 base pairs of homology, and thus can recombine with each other to revert to the *HIS3*+ allele, which also results in loss of the *LEU2* marker (117, 118). As shown in Table 4.1, the rates of spontaneously arising *HIS3*+ recombinants in the *nse1* and *mms21* mutants were similar to that observed in the wild type strain. UV-induced frequencies in the *his3* duplication at different doses did not show any significant difference either. We also examined the rate of spontaneous mutations in the *mms21* RING mutant using the *CAN1*^{S} to *can1*^{r} forward mutation system; however, we observed only a slight increase in the rate of spontaneous mutations (2.3-fold increase) compared to wild type cells (Table 3.1). These results suggest that the SUMO ligase activity of Mms21, and probably Nse1 and the entire Smc5-Smc6 complex do not play a direct role in mutagenic or recombinational repair.

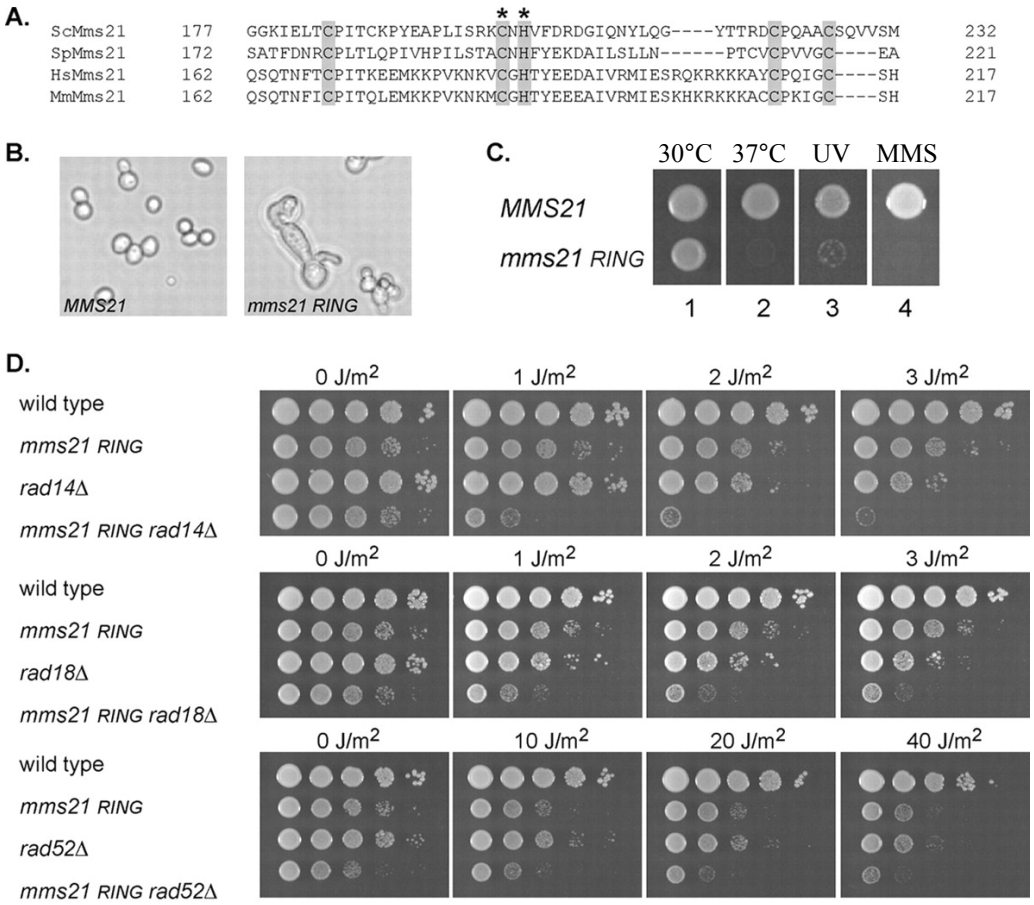

FIGURE 4.3. EPISTASIS ANALYSIS OF THE *MMS21* RING MUTATION. (A) Sequence alignment of the C-terminal SP-RING domain of Mms21 proteins from various organisms. Multiple sequence alignment was performed with CLUSTAL W version 1.83. Sequences aligned are *S. cerevisiae* Mms21 (ScMms21; 267 amino acids), *S. pombe* Mms21 (SpMms21; 250 amino acids), *H. sapiens* Mms21 (HsMms21; 247 amino acids), and *M. musculus* Mms21 (MmMms21, 247 amino acids). Shaded residues correspond to the zinc-binding residues of the SP-RING domain. Residues with an asterisk (C200A and H202A) are mutated in the *mms21* RING mutant. (B) Photomicrograph of *mms21* RING mutant cells showing abnormal 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 37 °C. (3) Cells were spotted on a YPD plate, UV irradiated at 80 J/m^2, and incubated for 2 days at 30 °C. (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 *rad14*Δ and *rad18*Δ mutants, but not of the *rad52*Δ 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. This figure is adapted with permission from reference 115.

TABLE 4.1. Spontaneous and UV-induced recombination of the *his3Δ3' his3Δ5'* duplication in *NSE1, nse1-101,* and *mms21* RING strains

		Frequency (% survival) of *HIS3* + recombinants/10^4 viable cells						
			UV-induced at (J/m^2):					
Strain	Genotype	Spontaneous (mean ± SD)	0	10	20	30	40	50
RSY6	*NSE1*	3.95 ± 0.29	5.1 (100)	10.8 (89)	13.7 (48)	15.5 (27)	21.5 (15)	26.5 (8.5)
YRP854	*nse1-101*	3.38 ± 0.37	4.5 (100)	10.7 (72)	13 (43)	17 (21)	23.7 (9)	32.6 (4)
YMMS21-56	*mms21* RING	5.50 ± 0.25	7.7 (100)	14.2 (63.4)	20.5 (23.6)	16.5 (12.1)	22.6 (6)	25.9 (2.8)

REQUIREMENT OF NSE1 AND MMS21 FUNCTIONS FOR RAD52-DEPENDENT PRR IN THE ABSENCE OF PCNA SUMOYLATION

Genetic and biochemical studies have indicated a very important role for PCNA modifications in the regulation of the Rad6-Rad18-dependent bypass processes. Whereas ubiquitin conjugation at lysine 164 of PCNA by Rad6-Rad18 activates TLS and/or error-free PRR, SUMO modification at this residue keeps the Rad52-dependent recombinational pathway in check by promoting the recruitment of the Srs2 helicase (94, 97). Epistasis analysis of the yeast *pol30-119* mutant in which the lysine 164 residue of PCNA has been changed to arginine (K164R) has suggested that this mutation inactivates all three Rad6-Rad18-dependent lesion bypass processes by inhibiting ubiquitin binding to PCNA. At the same time, this mutation also inhibits SUMO conjugation, thus activating the Rad52-dependent recombinational pathway (37). As a consequence, the sensitivity to DNA damaging agents conferred by the *pol30-119* mutation is greatly enhanced in the absence of Rad52 (37).

To investigate 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 constructed double mutant strains by combining the *nse1* and *mms21*

mutations with the *pol30-119* mutation of PCNA. As shown in Figure 3.3 and in Figure 4.4, the UV sensitivity of the *pol30-119* mutation is greatly enhanced in the presence of the *nse1-101*, *nse1* C274A, and *mms21* RING mutations, indicating a role for the Nse1 and Mms21 E3 ligase functions in the Rad52-dependent recombinational pathway.

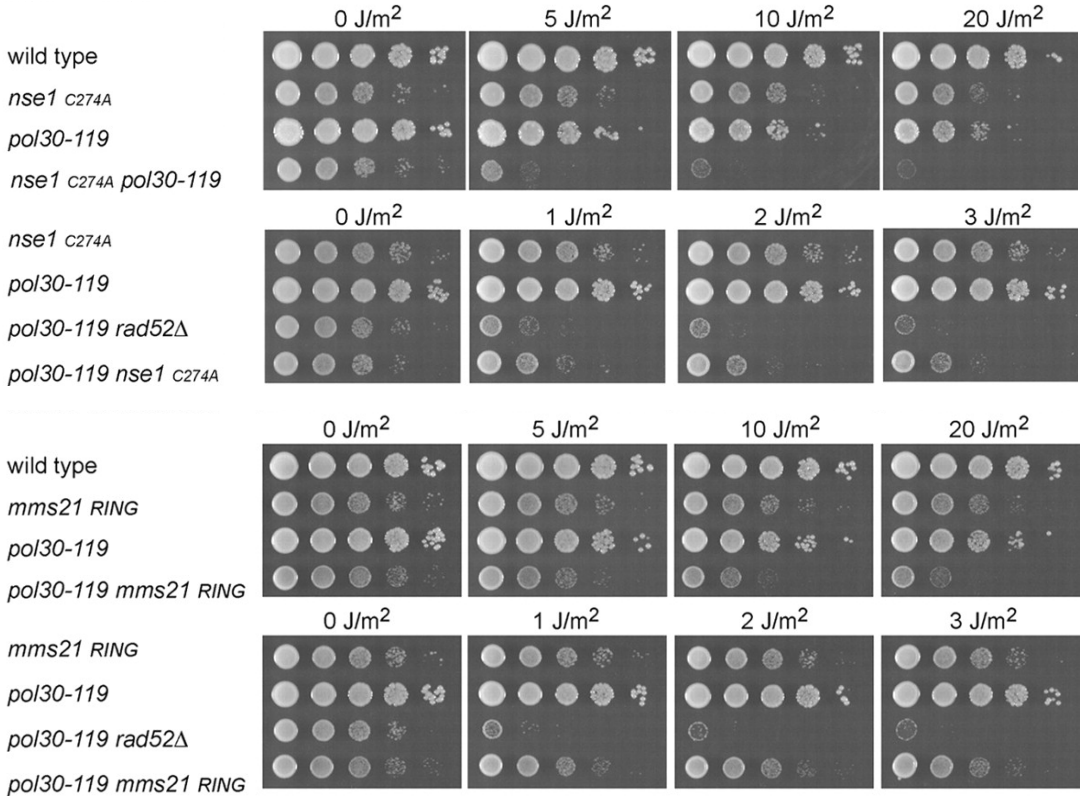

FIGURE 4.4. EPISTASIS ANALYSIS OF THE *NSE1* C274A AND *MMS21* RING MUTATIONS WITH THE PCNA *POL30-119* MUTATION. The UV sensitivity of the *pol30-119* mutation, defective in lysine 164 ubiquitination and the sumoylation of PCNA, is enhanced in the presence of the *nse1* C274A and *mms21* RING mutations. 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. This figure is adapted with permission from reference 115.

In the absence of functional Rad6-Rad18-dependent and Rad52-dependent lesion bypass processes, cells become extremely sensitive to DNA damaging agents, as observed in the *pol30-119 rad52*Δ mutant (37). To examine whether the ubiquitin ligase functions of Nse1 and Mms21 are required to the same extent as Rad52 activity, we compared the UV sensitivity of the *pol30-119 nse1* C274A and *pol30-119 mms21* RING mutant cells with the UV sensitivity of the *pol30-119 rad52*Δ mutant (Figure 4.4). Interestingly, the *pol30-119 nse1* C274A and *pol30-119 mms21* RING mutants are not as UV sensitive as the *pol30-119 rad52*Δ strain. These observations have suggested that the E3 ligase activities of Nse1 and Mms21 do function in Rad52-dependent recombinational repair, but that they are not absolutely required.

DISCUSSION

Covalent posttranslational modifications are rapid, energetically inexpensive mechanisms for reversibly altering protein function, and modifications such as phosphorylation, acetylation, and methylation participate in most cellular processes in a tightly regulated fashion (49). Entire proteins can also be attached covalently to protein substrates. The classic example of a protein that modifies other proteins is ubiquitin, a 76-amino acid polypeptide that is highly conserved in eukaryotes and targets substrate proteins for proteasomal degradation, but it can also have many other functions (98, 100, 101). More recently, several other ubiquitin-related proteins that also act as posttranslational modifications have been discovered. Although these ubiquitin-like proteins do not share high sequence similarity, they all possess essentially the same three-dimensional structure and conjugation mechanism (21, 63). Ubiquitin-like proteins are first activated by an E1 enzyme in an ATP-dependent manner. The second step involves the transfer of the ubiquitin-like protein to an E2 conjugating enzyme, which in turn

transfers it to the protein substrate with the assistance of an E3 ligase. Both, the E2 and the E3 ligase contribute to substrate specificity. One of the most interesting ubiquitin-like modifiers is SUMO. SUMOs constitute a highly conserved protein family found in all eukaryotes and are required for viability of most eukaryotic cells, including the budding yeast *S. cerevisiae* (53). As with ubiquitin, SUMO is involved in a broad spectrum of cellular processes, including signal transduction, subcellular localization, chromosome dynamics, and DNA repair.

An intriguing coincidence in the last step of the ubiquitin and SUMO conjugation cycles is the presence of the RING finger motif. The RING domain is a cysteine and histidine-rich domain found in many E3 ligases for ubiquitin conjugation. Canonical E3 RING domains contain eight conserved cysteine or histidine residues coordinating two zinc ions in a cross-brace arrangement that is thought to create a globular domain able to directly bind E2s (45, 116). Several variations on the classic RING have also been found in E3s. These include non-canonical RING variations and the Siz/PIAS RING (SP-RING) motif found in E3 SUMO ligases. Although the SP-RING lacks two of the cysteines that are conserved in RING E3s, it has been suggested that SP-RINGs function in sumoylation in a manner that is mechanistically similar to ubiquitin (45). RING E3s are thought to act by bringing the E2 and the substrate protein in close spatial proximity to allow transfer of the ubiquitin or ubiquitin-like modifier from the E2 to the lysine residue of the substrate (148).

Here we have investigated the possible involvement of the ubiquitin and SUMO ligase functions of Nse1 and Mms21, respectively, in Rad52-dependent PRR. Although the *nse1-101* mutant allele contains three amino acid changes within its sequence, none of them are located within the non-canonical C_4HC_3 RING finger-like motif of Nse1. We constructed a mutation in this motif by changing the second cysteine residue to alanine

(C274A), and used this mutation to study whether the putative E3 ligase function of Nse1 is involved in Rad52-dependent PRR. The *nse1* C274A mutation results in cells that are viable at the permissive temperature (30 °C) but that show a higher degree of growth defect compared with *nse1-101* mutant cells, and the *nse1* C274A cells attain a much larger size and abnormal cellular morphologies than the *nse1-101* cells (Figure 4.1A). As with *nse1-101*, in addition to a *ts* phenotype at 37 °C, *nse1* C274A mutant cells are also sensitive to UV irradiation and MMS (Figure 4.1B). Our observations that the UV sensitivity conferred by the *nse1* C274A mutation is greatly enhanced when combined with the *rad14*Δ, *rad18*Δ, or *rad5*Δ mutations, and that the UV sensitivity conferred by the *nse1* C274A *rad52*Δ double mutant combination is not more sensitive than the *rad52*Δ strain, suggest that the putative Nse1 ubiquitin ligase also contributes to the Rad52-dependent PRR pathway. We used purified Nse1 protein to further investigate its ligase function. As expected from our genetic and PRR studies with the *nse1-101* mutant, we did not observe any ligase activity when using the E2 Rad6 for ubiquitin conjugation. Nevertheless, our results are highly suggestive of a role for Nse1 as an E3 ligase in Rad52-dependent pathways that functions independently of Rad6-Rad18. The identification of the protein substrates for Nse1 and its E2 partners for ubiquitin conjugation will be important for understanding the role of the Smc5-Smc6 complex in DNA repair.

In addition to the possible involvement of the putative Nse1 ligase in Rad52-dependent PRR, we investigated the role of the E3 SUMO ligase activity of Mms21 in this pathway. We constructed a mutation in the SP-RING motif of Mms21 by changing the cysteine 200 and histidine 202 residues to alanines. The resulting *mms21* RING mutation renders cells that are sensitive to UV and MMS and that exhibit abnormal cellular morphologies at 30 °C (Figure 4.3). This mutation also confers a *ts* phenotype at

37 °C. Furthermore, our epistasis analyses suggest the involvement of the Mms21 SUMO ligase function in the Rad52-dependent PRR pathway. It has recently been reported that both yeast and human Mms21 proteins function as E3 SUMO ligases that sumoylate different subunits of the Smc5-Smc6 complex and other DNA repair proteins (6, 103, 147). Thus, it is possible that the Mms21-mediated sumoylation of the Smc5-Smc6 dimer and perhaps of the other subunits of this complex modulates the physical interactions of the complex with members of the Rad52-dependent repair pathway.

Replication through UV lesions in yeast cells can occur by Rad6-Rad18-dependent processes or by a Rad52-dependent pathway that acts independently of Rad6 and Rad18 (Figure 4.5A). In the Rad5-Mms2-Ubc13-mediated PRR pathway, the Mms2-Ubc13 E2 complex promotes polyubiquitination of PCNA following UV damage, and Rad5 acts as the E3 ubiquitin ligase. Since Rad5 has a DNA helicase function specialized for replication fork regression, it has been suggested that the Rad5-controlled pathway promotes lesion bypass when the UV lesion is located on the leading strand template (10). In this model, when replication on the leading strand is blocked by a lesion, lagging strand replication continues on. Rad5 then gains access to the asymmetric fork and promotes fork regression by unwinding both the leading and lagging nascent DNA strands from their respective templates and then anneals them with one another. Following that, the undamaged newly synthesized lagging strand is used as a template to bypass the lesion (10). Finally, the reversed fork is regressed and synthesis resumes beyond the lesion site. Thus, such a requirement for Rad5-dependent PRR in leading strand lesion bypass then relegates the Rad52-dependent PRR pathway to function when the lesion is located on the lagging strand template (26). Furthermore, since recombination is normally kept in check in wild type yeast cells by processes such as PCNA sumoylation and by the helicase functions of Srs2 and Sgs1 (27, 37, 71, 94, 97), it

is possible that the Rad52-mediated PRR pathway operates in a nonrecombinational manner, employing mechanisms such as those depicted in synthesis-dependent strand annealing (SDSA) models (64, 123) (Figure 4.6), wherein the Rad51-coated single-stranded nucleoprotein filament invades the DNA duplex on the leading strand side and the gap on the lagging strand is then filled in by DNA synthesis using the newly synthesized leading strand as a template.

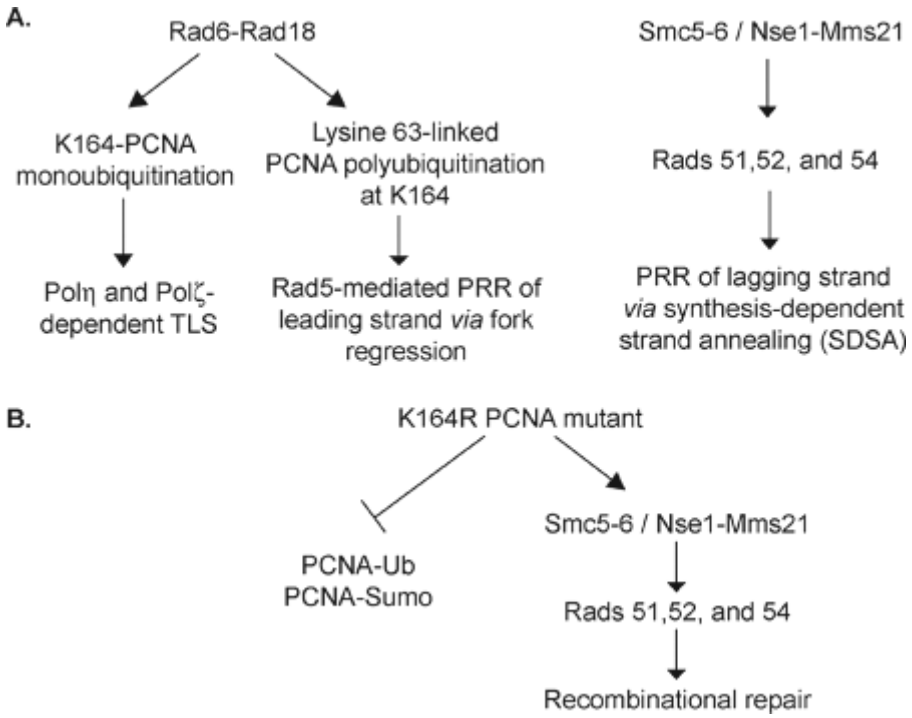

FIGURE 4.5. RAD6-RAD18-DEPENDENT AND SMC5-SMC6 / NSE1-MMS21-DEPENDENT PATHWAYS FOR THE REPLICATION OF UV-DAMAGED DNA IN YEAST. (A) Rad6-Rad18-dependent replication through UV lesions can occur either by TLS mediated by Polη or Polζ, or by Rad5-mediated fork regression and template switching. The Smc5-Smc6 complex functions in the alternate 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 might function here also. This figure is reproduced with permission from reference 115.

Our observations that Nse1 is required for Rad52-dependent PRR of UV-damaged DNA and that the ligase functions of Nse1 and Mms21 might also contribute to this pathway raise the strong possibility that the entire Smc5-Smc6 complex functions in promoting lesion bypass when the damage is located on the lagging strand. Since we expect little inhibition of replication fork progression when the lesion is on the lagging strand template (16, 17, 77, 124), we assume that the replication fork continues its advance and that the gap that is left behind from the inhibition of Okazaki fragment completion is then filled by Rad52-dependent PRR in a nonrecombinational manner. From previous studies with the other SMC complexes, condensin and cohesin (32, 42, 50), it is likely that the role of the Smc5-Smc6 complex in this repair mode is to hold the sister duplexes in close proximity by encircling them using the ring structure formed by the SMC dimer and the non-SMC subunits, thus allowing for lesion bypass using the proposed nonrecombinational SDSA model (Figure 4.6). In addition, the proposed ubiquitin ligase function of Nse1 and the Mms21 SUMO ligase can contribute to this process by mediating physical interactions between the Smc5-Smc6 complex, DNA, and the Rad52 group of recombinational proteins.

The enhanced UV sensitivity observed in the *nse1* and *mms21* mutants when combined with *pol30-119* implicates a role for Nse1, its putative E3 ligase function, and the Mms21 SUMO ligase in promoting Rad52-dependent recombinational lesion bypass. Since the *pol30-119* (K164R) mutation of PCNA inhibits the ubiquitination and sumoylation at lysine 164 and hence inactivates all Rad6-Rad18-dependent processes and activates the Rad52-dependent pathway, respectively, presumably all the lesion bypass becomes completely dependent upon the Rad52-dependent recombinational pathway (Figure 4.5B). Our observations that the *pol30-119 nse1* C274A and *pol30-119 mms21* RING mutants are not as UV sensitive as the bypass-deficient *pol30-119 rad52*Δ mutant

suggest that the Smc5-Smc6 complex, along with its associated ubiquitin and SUMO ligase activities, could function in the Rad52-dependent recombinational repair pathway in a manner similar to that proposed for its role in nonrecombinational repair.

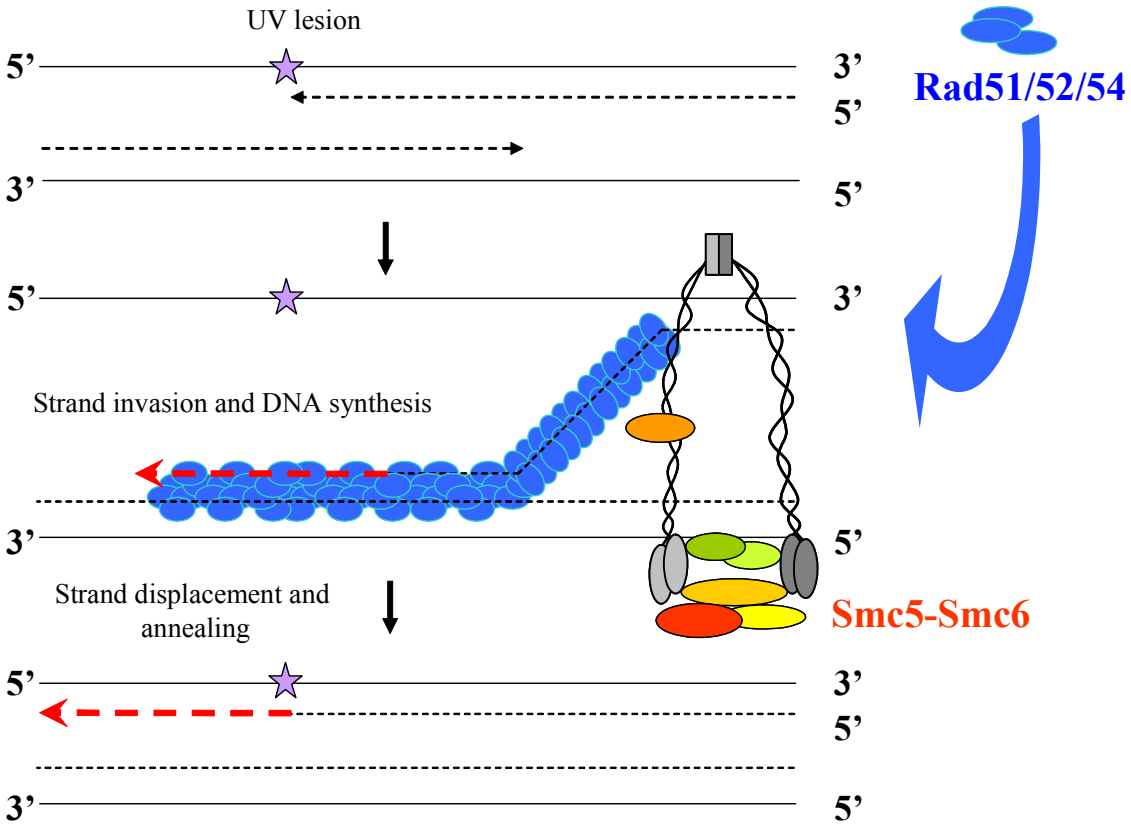

FIGURE 4.6. PROPOSED MODEL FOR THE ROLE OF THE SMC5-SMC6 COMPLEX IN NONRECOMBINATIONAL SYNTHESIS-DEPENDENT STRAND ANNEALING. Upon encounter of a UV lesion on the lagging strand (depicted here on top), the replication fork stops and recombinational proteins are recruited. These proteins form a single-stranded nucleoprotein filament that promotes strand invasion and DNA synthesis using the newly synthesized sister leading strand as a template. It is proposed that the Smc5-Smc6 complex holds the sister duplexes in close proximity allowing for bypass of the damaged site. The DNA synthesis is completed by strand displacement and annealing to the original template DNA. A role for the SUMO and ubiquitin ligase functions of the complex in the coordination and regulation of this nonrecombinational mechanism can also be envisioned.

CHAPTER 5: CONCLUDING REMARKS

The structural maintenance of chromosomes (SMC) complexes play essential roles in chromosome dynamics. While cohesin is involved in sister chromatid cohesion, condensin promotes chromosome condensation. A third complex, the Smc5-Smc6 complex, is involved in DNA repair and normal chromosome organization. Much insight into the study of condensin and cohesin has been provided by the characterization of their non-SMC subunits. The Smc5-Smc6 complex is composed of eight essential subunits. The Smc5 and Smc6 proteins form the core of the complex and bind to each other to form a V-shaped heterodimer with a hinge domain at one end and two ATP binding domains or heads at the other end, separated by two long coiled-coil motifs. SMC complexes are thought to encircle DNA using their V-shaped structure together with their non-SMC subunits. In the Smc5-Smc6 complex two non-SMC subunits are of particular interest in terms of their role in DNA repair, Nse1 and Mms21. Nse1 contains a C_4HC_3 RING finger-like motif at its C terminus, characteristic of E3 ubiquitin ligases. Mms21, on the other hand, has an SP-RING domain essential for its SUMO ligase activity.

In this study we have isolated *nse1* mutants by random mutagenesis and studied one of them to gain a better understanding of the role of the Smc5-Smc6 complex in DNA repair. The *nse1-101* mutation renders cells sensitive to UV irradiation and the alkylating agent MMS, and mutant cells exhibit growth defects and abnormal morphologies. Epistasis and PRR analyses with this mutation have indicated a role for Nse1 in Rad52-dependent PRR of UV-damaged DNA. We have also introduced mutations in the RING motifs of Nse1 (*nse1* C274A) and Mms21 (*mms21* C200A H202A) to investigate whether the ubiquitin and SUMO ligase functions of Nse1 and

Mms21 are also involved in Rad52-dependent PRR. Indeed, our genetic studies have also indicated a role for these ligase activities in Rad52-dependent processes.

It yeast cells, PRR is promoted by two alternate, noncompeting pathways. The Rad5-Mms2-Ubc13-dependent PRR pathway is activated by polyubiquitination of PCNA following DNA damage and initial monoubiquitination by the Rad6-Rad18 complex. Further, it has been proposed that this pathway promotes PRR when the lesion is located on the leading strand by a template switching and copy choice type of DNA synthesis. A second pathway, the Rad52-dependent pathway is involved in PRR independent of Rad6-Rad18 and PCNA ubiquitination. This pathway is thought to act in a nonrecombinational manner when the lesion is located on the lagging strand. Upon encountering the lesion, the Rad52 group proteins promote formation of a nucleoprotein filament that invades the leading strand duplex and uses the newly synthesized DNA as a template. We propose that the Smc5-Smc6 complex works in this type of bypass by holding both DNA duplexes in close proximity using the ring structure formed by the SMC dimer and the non-SMC subunits. Further, the E3 ligase functions of Nse1 and Mms21 might contribute to this mechanism by promoting the physical interaction between the Smc5-Smc6 complex, the DNA duplexes, and the Rad52 group of recombinational proteins. A role for the Smc5-Smc6 complex in recombinational repair is also supported from our genetic studies.

APPENDIX

GENE DELETION GENERATING PLASMIDS USED IN THESE STUDIES

Plasmid	Gene	Description
pR1.34	*RAD1*	Deletes from +37 to +3211 of the 3300 bp *RAD1* ORF Transforms to Trp+
pR5.15	*RAD5*	Deletes from +670 to +3445 of the 3507 bp *RAD5* ORF Transforms to Ura+
pR6.71	*RAD6*	Deletes the entire 516 bp *RAD6* ORF Transforms to Ura+
pR14.4	*RAD14*	Deletes from +40 to +581 of the 1113 bp *RAD14* ORF Transforms to Ura+
pR18.120	*RAD18*	Deletes from +129 to +1482 of the 1461 bp *RAD18* ORF Transforms to Ura+
pR30.295	*RAD30*	Deletes the entire 1896 bp *RAD30* ORF Transforms to His+
pR52.8	*RAD52*	Deletes the entire 1413 bp *RAD52* ORF Transforms to Trp+
pMMS2.2	*MMS2*	Deletes from +11 to +390 of the 411 bp *MMS2* ORF Transforms to Ura+
pPM1023	*UBC13*	Deletes from +31 to +360 of the 459 bp *UBC13* ORF Transforms to Ura+
pPCNA1.44	*POL30*	Integrates the *pol30-119* (K164R) mutation into the genome Transforms to Ura+
pREV3.75	*REV3*	Deletes from +436 to +3928 of the 4512 bp *REV3* ORF Transforms to Ura+

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VITA

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Publications

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