MOLECULAR AND CELLULAR BIOLOGY, Apr. 2005, p. 2547–2557 Vol. 25, No. 7
0270-7306/05/$08.00+0 doi:10.1128/MCB.25.7.2547–2557.2005

Brh2-Dss1 Interplay Enables Properly Controlled Recombination in *Ustilago maydis*†

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Received 19 November 2004/Returned for modification 8 December 2004/Accepted 27 December 2004

Brh2, the BRCA2 homolog in *Ustilago maydis*, functions in recombinational repair of DNA damage by regulating Rad51 and is, in turn, regulated by Dss1. Dss1 is not required for Brh2 stability in vivo, nor for Brh2 to associate with Rad51, but is required for formation of green fluorescent protein (GFP)-Rad51 foci following DNA damage by gamma radiation. To understand more about the interplay between Brh2 and Dss1, we isolated mutant variants of Brh2 able to bypass the requirement for Dss1. These variants were found to lack the entire C-terminal DNA-Dss1 binding domain but to maintain the N-terminal region harboring the Rad51-interacting BRC element. GFP-Rad51 focus formation was nearly normal in *brh2* mutant cells expressing a representative Brh2 variant with the C-terminal domain deleted. These findings suggest that the N-terminal region of Brh2 has an innate ability to organize Rad51. Survival after DNA damage was almost fully restored by a chimeric form of Brh2 having a DNA-binding domain from RPA70 fused to the Brh2 N-terminal domain, but Rad51 focus formation and mitotic recombination were elevated above wild-type levels. The results provide evidence for a mechanism in which Dss1 activates a Brh2-Rad51 complex and balances a finely regulated recombinational repair system.

BRCA2 is the product of a major breast cancer susceptibility gene in humans functioning in homology-directed repair of DNA through association with the Rad51 recombinase that provides the essential activity required for DNA strand exchange. In vertebrate systems, regulated interaction between Rad51 and BRCA2 is critical for repair of DNA double-strand breaks (31). There is support for two primary levels of control. First, evidence has accumulated pointing to a role for BRCA2 in nuclear localization of Rad51 (10, 24, 30). Second, assembly of Rad51 into its catalytically active form, the nucleoprotein filament generated through Rad51 polymerization on single-stranded DNA, is regulated by BRCA2 (10, 30, 32, 43, 44). Filament assembly is balanced on the one hand by the inherent ability of Rad51 monomers to self-associate coupled with BRCA2's activity initiating the association and on the other hand by attenuation of assembly and destabilization of the filament through interference with a crucial contact between Rad51 subunits via BRCA2's BRC elements (32, 36, 43). The latter are a series of eight related sequences of about 30 residues each that mimic a Rad51 motif at the polymerization interface. Studies on the dynamics of green fluorescent protein (GFP)-tagged protein trafficking in the nuclei of living cells after photobleaching suggest that Rad51 is sequestered by BRCA2 in an immobilized form awaiting activation by DNA damage (44). The emerging model features BRCA2 as a governor of Rad51 nuclear localization and Rad51-mediated homology-directed repair (31), although evidence linking BRCA2 deficiency to abnormal centrosome formation (24, 40) and cytokinesis (9) suggests that this view may be oversimplified.

BRCA2 has a DNA-binding domain (DBD) contained within its C-terminal region that is composed of a tandem array of three OB (oligosaccharide/oligonucleotide-binding) folds comparable to those found in the single-stranded DNA-binding protein RPA (42). There is also a helix-loop-helix DBD similar to those found in the site-specific recombinase family exemplified by the γδ resolvase and Hin recombinase supported by twin antiparallel helices. In addition to the interaction with Rad51 that is mediated by the BRC repeats, there is a second, unrelated type of element located at the extreme C terminus of BRCA2 (35, 38).

The recent discovery of DSS1 as a newly recognized interacting partner of BRCA2 opens the possibility of exploration into the mechanism of BRCA2-RAD51 regulation. DSS1 is a small acidic protein ubiquitous in eukaryotes (28) that interacts with the DBD of BRCA2, associating through extensive contacts with OB1 and OB2, as well as with the α-helix-rich domain just proximal to the array of OB folds (42). The significance of the DSS1 interaction was at first uncertain because it had originally been considered a candidate gene for the developmental disorder split hand-split foot malformation (8). However, the issue was resolved by genetic studies on the ortholog in *Ustilago maydis*, a yeast-like fungus in which the BRCA2-Rad51-DSS1 system is recapitulated (19). Not only is the Rad51 ortholog of *U. maydis* extremely similar in sequence to human RAD51 (13), but there is also a BRCA2 homolog, Brh2 (21), and an interacting Dss1 partner (23). Mutants generated by disruption of any of the three structural genes *BRH2* (encoding the BRCA2 homolog), *RAD51*, and *DSS1* are phe-

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† Supplemental material for this article may be found at http://mcb.asm.org/.

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notypically nearly identical, exhibiting extreme deficiency in DNA repair and recombination, genome instability, and abortive meiosis.

Brh2 is a streamlined version of the mammalian BRCA2 protein (21). It has 1,075 amino acid residues containing a single N-terminal BRC element, a C-terminal region with extended similarity to the BRCA2 DBD (DNA-DSS1 binding domain) (42), and two putative nuclear localization signals (NLSs), one (NLS1) near the middle and the other (NLS2) near the C terminus. The sequence of the Brh2 C-terminal region with the highest similarity to the BRCA2 DBD corresponds to part of the helical domain (HD) and extends through the OB1 and OB2 folds. Within this region in the BRCA2 DBD, 40 residues contact DSS1 with 24 being identical in Brh2 and 12 conservatively substituted. The essential role of Dss1 in the homology-directed repair system of *U. maydis* suggests that it serves as a positive activator of Brh2 (23). Support for this interpretation has come recently from studies on cultured human cells and mouse embryonic stem cells in which it was shown that depletion of DSS1 by RNA interference (RNAi) resulted in a phenotype similar to that of BRCA2 deficiency, likewise implicating DSS1 in DNA double-strand break repair and cancer susceptibility (15).

The BRCA2-RAD51-DSS1 interplay poses a fascinating molecular puzzle whose elucidation is decisive for clarifying the mechanism of BRCA2-driven recombination. Conservation of the BRCA2 paradigm in *U. maydis* offers the possibility of a deeper understanding of this interplay through both biochemical (43) and molecular genetic experimentation (19). In the work described here, we investigated whether it is possible to obtain Brh2 mutant variants free of Dss1 control. To do so, we searched for mutant alleles of *BRH2* that could suppress the radiation sensitivity of the *dss1* mutant. Indeed, suppressors were obtained but it was surprising to discover that they were all due to truncating mutations predicted to encode only the N-terminal BRC-containing domain but with the entire C-terminal DBD deleted. These observations suggest that the N-terminal BRC-containing domain has an inherent ability to contribute in a positive manner to DNA repair, presumably by organizing and activating Rad51. Such a conclusion contrasts with reports on mammalians systems in which essentially negative effects have been observed upon expression of individual BRC elements in mammalian systems (6, 37). By domain swapping, we found that a hybrid protein composed of the N-terminal BRC domain of Brh2 and the DBD from single-stranded DNA-binding protein RPA70 was highly effective in promoting survival after DNA damage in either the *brh2* or the *dss1* mutant. But in addition, the frequency of spontaneous GFP-Rad51 focus formation was substantially elevated and recombination was highly unregulated in cells expressing the hybrid, a situation indicative of elevated genomic instability. Collectively, the results argue that Dss1 serves to activate an already assembled Brh2-Rad51 complex and suggest that Brh2-Dss1 interplay is developed to elaborate a tightly regulated recombinational repair system in *U. maydis*.

MATERIALS AND METHODS

***U. maydis* strains and methods.** Manipulations; haploids strains UCM350 (*pan1-1 nar1-6*), UCM342 (*rad51-1 ade1-1 leu1-1*), UCM565 (*brh2-2 pan1-1 nar1-6*), and UCM591 (*dss1-2 met1-2 nar1-1*); culture methods; gene transfer procedures; and survival after irradiation have been described previously (23). Allelic recombination was measured in UCM33 (*nar1-1*/*nar1-6 pan1-1*/+ *met1-2*/+) or UCM593 (*dss1-1*/*dss1-2 nar1-1*/*nar1-6 pan1-1*/+ *met1-2*/+) transformed derivatives as Nar^+ prototroph formation after plating of cultures on minimal medium containing nitrate as the sole source of nitrogen (20). Strains expressing Brh2 from a self-replicating plasmid (pCM973), Rad51 (pCM1030), Brh2 ΔDBD (pCM1016), or Brh2-RPA70 (pCM1034) were tested, and rates were established by fluctuation analysis from two to five independent determinations, each performed with nine cultures as described previously (22). Plasmid-by-chromosome recombination assays were performed by transformation with nonreplicating plasmid pCM1039 made linear by cutting with DraIII, which cleaves once within the CBX^R gene. Protoplasts were plated on medium containing 4 μg of carboxin per ml for selection. Cbx^R transformants were purified by one round of streaking on nonselective medium. Isolates were tested for integration of the plasmid by patching on medium containing 2 μg of carboxin per ml and then 40 μg of nourseothricin (NAT) per ml as previously described (20). Transformation efficiency was controlled for by parallel transformations with autonomously replicating plasmid pCM619 expressing CBX^R as the standard. GFP fluorescence microscopy and differential interference contrast imaging of live cells were performed as previously described (25) on cells harboring pCM1001 or pCM1036, self-replicating a plasmid expressing GFP-Rad51 and hygromycin resistance or carboxin resistance, respectively, as a marker. Live images were captured at 100-fold magnification with a cooled charge-coupled device camera mounted on a Zeiss Axioplan II microscope with an acquisition time of 2,500 ms and a 10% neutral-density filter to reduce photobleaching. Cells were grown in yeast synthetic complete medium supplemented with hygromycin at 100 μg/ml to a density of 0.5×10^7 to 1×10^7/ml. When required, cells were exposed to gamma rays delivered from a GammaCell 220 ^{60}Co source (Nordion International, Kanata, Ontario, Canada). Approximately 200 cells of each genotype were inspected for focus formation before and at each time point after irradiation.

Plasmid construction and procedures. The gene expressing a blue-shifted GFP derivative was obtained from pCRGFP2 (4), modified suitably at the 3′ end by addition of a sequence with a multiple cloning site, and fused in frame with the 5′ end of the gene expressing Rad51. Self-replicating plasmids were used to express the GFP-Rad51 fusion under control of the *gap* (glyceraldehyde 3-phosphate dehydrogenase) promoter with either *HPH* (hygromycin resistance, pCM1001) or CBX^R (carboxin resistance, pCM1036), respectively, as a selectable marker. Shuttle vector pCM973 containing the *BRH2* gene driven by the *gap* promoter was randomly mutagenized by passage through *Escherichia coli* mutator strain XL-1 red (Stratagene, La Jolla, Calif.) as described by the manufacturer. The plasmid DNA recovered was used to transform *U. maydis* UCM591. Hyg^R transformants were plated on medium containing 0.005% methyl methane sulfonate (MMS). Surviving colonies were replica plated and irradiated with UV light at 120 J/m^2. Radiation-resistant survivors were tested for dependence on maintenance of the plasmid after 20 generations of nonselective growth. DNA sequence analysis was performed on plasmid DNA recovered from appropriate candidates. pCM1016 is a derivative of pCM973 in which an SphI fragment encoding the C-terminal DBD of Brh2 was removed. pCM1017 is similar but has the proximal SphI fragment encoding the N-terminal domain of Brh2 removed. The RPA70 coding sequence was amplified from a *U. maydis* cDNA library by PCR with primers 5′-TCATATGTCGCTCAACGACCTGTCG and 5′-GCGC CCGCTTTACATATAGG. pCM1040 is similar to pCM973 but has the RPA70 structural gene driven by the *gap* promoter. pCM1023 is similar to pCM1016 but has a 1.2-kbp SphI fragment encoding the DBD from RPA70 joined to the region encoding the N-terminal Brh2 fragment through the SphI site and the *HPH* gene encoding hygromycin resistance. pCM1034 is similar to pCM1023, but the *HPH* gene has been exchanged with CBX^R, a gene encoding carboxin resistance from plasmid pCM619 (20). pCM1033 and pCM1035 are self-replicating plasmids with the CBX^R gene for selection expressing Brh2 and Rad51, respectively, from the *gap* promoter. pCM1039 is a nonreplicating plasmid derivative of pCM691 (20) with the CBX^R and *NAT1* (NAT resistance) genes for selection.

RESULTS

Brh2 stability and association with Rad51 are not dependent on Dss1. In principle, the crucial function of Dss1 could be explained by a stabilizing role for Brh2. Suggestive findings for the requirement of Dss1 in enabling production of a soluble form of Brh2 in insect cells (23), as well as the mammalian BRCA2 DBD (42), clearly argue for this notion. However, more attractive roles for Dss1 are also conceivable (19). For-

mally, Dss1 might control the ability of Brh2 to associate with Rad51 or to activate an already formed Brh2-Rad51 complex. As our experimental work addressing these issues was in progress, Ashworth's laboratory (15) reported relevant findings on DSS1 in mammalian cells by Western blotting, coimmunoprecipitation, and immunofluorescence microscopy to determine its role in maintaining the steady-state level of BRCA2 and association with RAD51. By using RNAi methodology to deplete cells of DSS1, these investigators found no evidence of a DSS1 requirement in BRCA2 stability or in its interaction with RAD51 and, together with additional experimentation, concluded that DSS1 may be required for the BRCA2/RAD51 complex to become associated with sites of DNA damage. By a conceptually similar strategy, we posed similar questions regarding the role of Dss1 in *U. maydis*. Our line of attack was to measure steady-state levels of Brh2 and test its association with Rad51 in the complete absence of Dss1 (see the supplemental material). To answer the question of Brh2 stability, we conducted Western blotting of *U. maydis* cells devoid of Dss1 through complete deletion of the structural gene (Fig. S1 in the supplemental material). To determine whether Dss1 is required for Brh2-Rad51 association, we performed pulldown assays after coexpressing Brh2 (as a maltose-binding protein fusion) and Rad51 in *E. coli*, which has no Dss1 homolog (Fig. S2 in the supplemental material). By both procedures, we found no role for Dss1 in Brh2 stability or association with Rad51. These findings agree with the conclusions drawn about the role of DSS1 in mammalian cells (15) and thus strengthen and extend the common paradigm developing from investigations in these two systems.

Rad51 nuclear focus formation after DNA damage is Dss1 dependent. It has been well established in other systems that Rad51 assembles in intranuclear foci along with other recombinational repair proteins such as Rad52 and Rad54 in response to DNA damage and that GFP-Rad51 responds similarly (11, 27, 29, 44). The foci are thought to mark sites of active recombinational repair following UV damage, ionizing radiation, or endonuclease-induced DNA double-strand breaks (25, 29, 39) and presumably contain nucleoprotein filaments of Rad51, although it should be emphasized that there is no unequivocal evidence to support this point. On the basis of the above finding, we inferred that Dss1 must act at some stage in the Brh2-Rad51 interplay other than in promoting their direct association. One possibility, as mentioned above, is that Dss1 could be considered to be a regulatory protein that somehow activates a dormant Brh2-Rad51 complex, making it competent for loading Rad51 molecules onto DNA in preparation for recombination reaction. At the other extreme is the possibility that Dss1 has a crucial role in disassembly of the established nucleoprotein filaments after recombination is completed. Assuming that there is a physical link between aggregates of Rad51 manifested in foci and assembly of Rad51 nucleoprotein filaments, then a way that might be used to distinguish between the above possibilities is to test the influence of Dss1 on the formation and maintenance of Rad51 nuclear foci. If the role of Dss1 precedes or involves nucleoprotein filament formation, then there would be no Rad51 foci formed in the *dss1* mutant. Conversely, if Dss1 participates in the disassembly of the nucleoprotein filament, it would follow that the time course of Rad51 focus disappearance should be lengthened and the frequency of their formation should be elevated.

As Rad51 focus formation had not yet been reported before in *U. maydis*, we were interested in developing this methodology as a tool for assessing the genetic dependence and dynamics of the Rad51 nucleoprotein filament in vivo. Since nuclear spreading techniques in *U. maydis* are undeveloped and since antibodies to *U. maydis* Rad51 were not available, we used epifluorescence microscopy to track GFP-Rad51 in living cells. This modification slightly compromises the biological activity of Rad51, as evidenced by a modest loss of the ability to restore the DNA repair proficiency to a *rad51* null mutant compared to the unmodified protein but imparts no significant negative effect on a repair-proficient strain when expressed ectopically (Fig. S3 in the supplemental material). DNA damage was introduced by using gamma radiation from a ^{60}Co source. This method was chosen as a matter of convenience since cells in liquid culture can be directly irradiated with high doses of gamma rays in a short period of time so as to enable one to follow focus formation easily. The response in focus formation from gamma rays is most likely a general representation of the response following DNA damage because *dss1* mutants, like *rad51* and *brh2* mutants, are extremely sensitive not only to gamma rays but also to UV light and to genotoxic chemicals. Thus, the pathway through which *U. maydis* normally channels lesions resulting from these and other damaging agents proceeds through a common recombinational repair system.

In the absence of exogenous DNA damage, GFP-Rad51 displayed diffuse nuclear localization in the wild type and seemed little different in the *brh2* and *dss1* null mutants (Fig. 1A). On the other hand, the GFP-Rad51 response in the mutants was markedly different compared to that of the wild type after DNA damage. In wild-type cells, GFP-Rad51 relocalized to form bright subnuclear foci after delivery of a dose of gamma radiation estimated as sufficient to introduce approximately three double-strand breaks per haploid cell (26). Foci formed in the majority of the cells after 2.5 h and dissipated thereafter (Fig. 1B). However, foci did not form in either the *brh2* or *dss1* deletion mutant, and furthermore the background of faint Rad51 staining observed in the *brh2* and *dss1* mutants did not change after irradiation. If one accepts the assumption that there is a physical relationship between Rad51 focus formation and assembly of Rad51 nucleoprotein filaments, then the results suggest that Brh2 and Dss1 are both needed for nucleoprotein filament formation at sites requiring DNA repair but that neither is required for maintenance of Rad51 in the nucleus. The latter point contrasts with results obtained with mammalian cells, where there is evidence based on subcellular fractionation to suggest that Rad51 nuclear localization depends on BRCA2 (10, 24, 30). The requirement for Dss1 in focus formation is in agreement with the finding on mammalian cells depleted of DSS1 by RNAi (15) and supports the idea that Dss1 functions prior to nucleoprotein filament formation, presumably activating a dormant Brh2-Rad51 complex.

Brh2 variants active in the absence of Dss1. In keeping with the hypothesis that the specific action of Dss1 is to awake a dormant Brh2-Rad51 complex, we reasoned that it should be possible to obtain Brh2 mutant variants free of Dss1 control. Our reasoning was based upon the assumptions that the Brh2

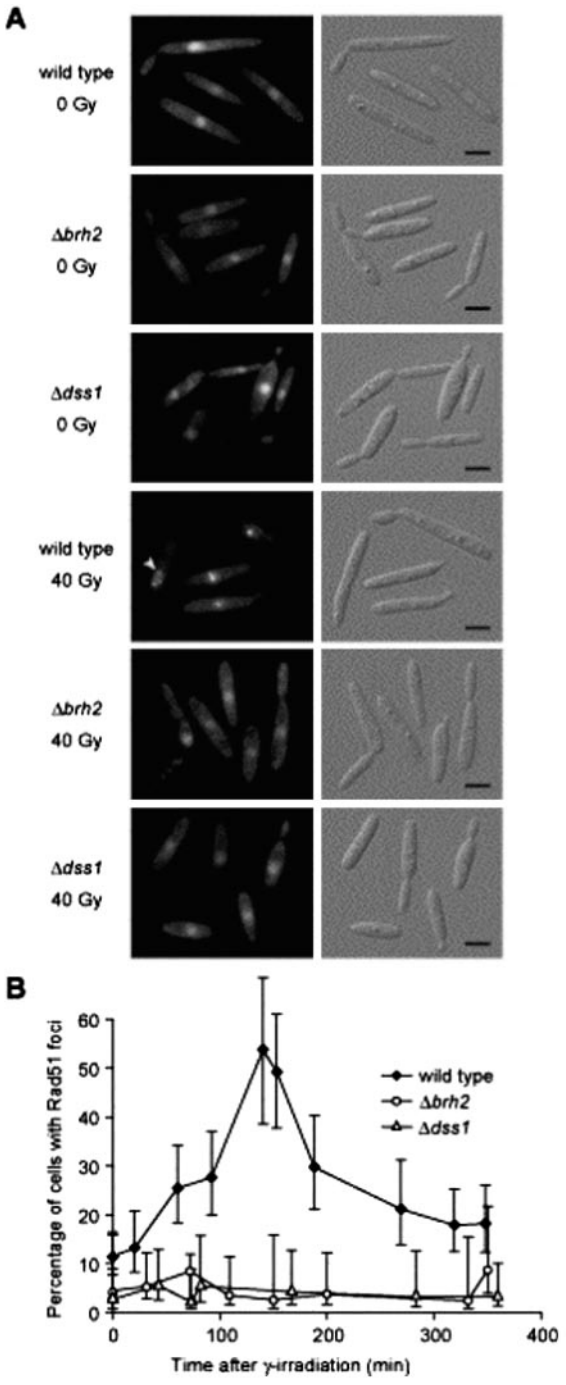

FIG. 1. GFP-Rad51 focus formation. (A) Wild-type (UCM350) or *brh2* (UCM565) or *dss1*(UCM591) mutant cells expressing GFP-Rad51 were viewed by differential interference contrast imaging or fluorescence microscopy without fixation. Cells were examined for focus formation 90 min after irradiation with gamma rays (40 Gy). The arrow shows an example of a nucleus with three foci. Scale bar, 5 μm. (B) The fraction of cells with foci at each time point was determined after counting approximately 200 cells.

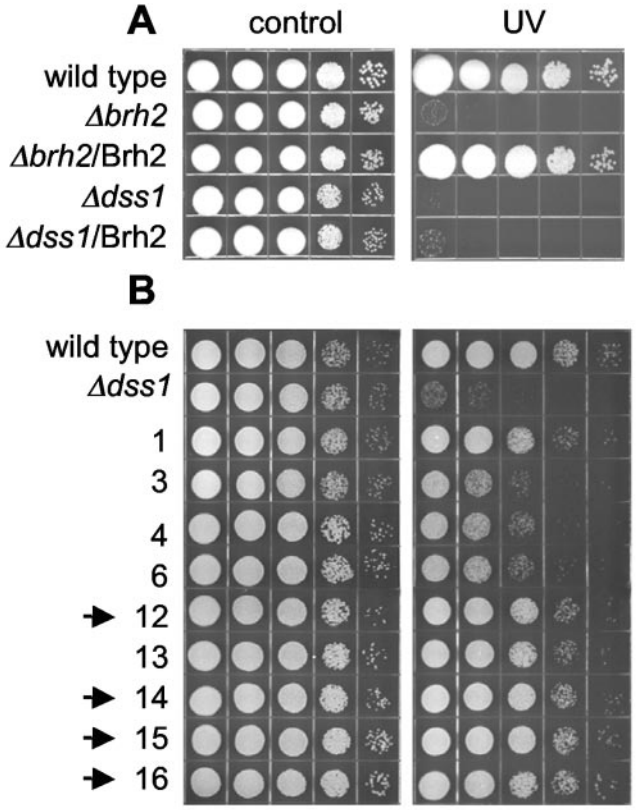

FIG. 2. Isolation of Brh2 variants that suppress the UV sensitivity of *dss1*. (A) Controls illustrating the UV sensitivity of wild-type (UCM350) and Δ*brh2* (UCM565) and Δ*dss1* (UCM591) mutant strains. Ten-fold serial dilutions of cell suspensions were plated and irradiated with UV light at 120 J/m^2. Brh2 was expressed ectopically by introducing plasmid pCM973 into *brh2* and *dss1*. (B) pCM973 containing the *BRH2* gene was mutagenized by passage through hypermutator strain *E. coli* XL-1 red and used to transform UCM591. Serial dilutions and irradiations were performed as described above except that the UV dose was 90 J/m^2. Nine candidates isolated that were partially active in suppressing the UV sensitivity of *dss1* are shown. Plasmid DNA was successfully recovered from those indicated by the arrows, and the sequence of *BRH2* was determined.

molecule contains a domain(s) involved in locking the Brh2-Rad51 complex and that disruption of this domain would not affect the association of Brh2 with Rad51. Thus, the class of variants of interest was imagined to be composed of Brh2 mutants that are able to recognize and bind Rad51 but are defective in locking Rad51 in an inactive complex, free of Dss1 control.

To identify such variants, we conducted a search for mutant alleles of *BRH2* that could suppress the DNA damage sensitivity of a strain with *DSS1* completely deleted. As ectopic expression of the *BRH2* gene completely complements the DNA repair deficiency of Δ*brh2* mutants but has no ability to suppress Δ*dss1* mutants (Fig. 2A), the cloned *BRH2* gene in a self-replicating shuttle vector was mutagenized by passage through a hypermutator *E. coli* strain and the resulting library was screened for alleles that could promote survival of *dss1* following DNA damage. Approximately 40,000 *dss1* clones transformed with the mutagenized vector were screened for

survival after two successive rounds of killing by MMS, followed by UV light. A cohort of 14 of the most MMS-resistant, UV-resistant survivors was culled from about 200 candidates. Nine of these were found to be dependent on maintenance of the transforming plasmid for elevated resistance to DNA damage (Fig. 1B), and plasmid DNA was successfully recovered from four. DNA sequence determination revealed that all contained chain-terminating mutations confined to a locus approximately in the middle of the *BRH2* gene just proximal to the region encoding the DBD but downstream of the first presumed NLS. Three (no. 14 to 16 in Fig. 2B) contained the same C1609T transition creating a nonsense mutation at codon 537, and the fourth (no. 12 in Fig. 2B) contained a C1591T transition creating a nonsense mutation at codon 531.

These mutant alleles are predicted to encode truncated Brh2 variants with an intact BRC region and NLS1 but lacking the entire C-terminal sequence containing the Dss1 interaction region and the DNA-binding OB folds (Fig. 3A). An additional allele prepared in vitro by cutting the gene at a unique restriction site at a site 14 codons downstream (SphI at codon 551), thereby removing the entire C-terminal encoding region (the protein from this allele is referred to hereafter as Brh2 ΔDBD and is used as the representative Brh2 N-terminal BRC-containing domain in the experiments to be presented), behaved virtually the same in partially suppressing the UV sensitivity of *dss1* (Fig. 2B). All of these alleles were also partially functional in complementing the UV sensitivity of the *brh2* null mutant (only representative Brh2 ΔDBD is shown in the bottom part of Fig. 3B). Thus, the repair capacity in rescuing UV sensitivity afforded by the truncated alleles appeared to be about the same whether in the *brh2* null mutant or in the *dss1* null mutant. The distal C-terminal sequence alone (designated Brh2 ΔN-term in Fig. 3) had no ability to rescue activity, indicating that the N-terminal BRC-containing domain is a necessary participant in Rad51-mediated repair. Furthermore, it was remarkable to observe that GFP-Rad51 focus formation could be restored to nearly normal levels in cells expressing Brh2 ΔDBD in place of endogenous Brh2 (see Fig. 5A). The time course of focus formation and disappearance after irradiation was similar to that in the wild type, although the rate of disappearance was slower, probably reflecting the constitutive expression of Brh2 ΔDBD driven by the heterologous promoter (see Fig. 5B). If indeed foci represent the accumulation of Rad51 at sites of repair on DNA, as is generally supposed, then on the basis of this finding it seems that Dss1 is not needed for Rad51 to be loaded onto DNA. Rather, the N-terminal BRC-containing domain itself is sufficient to organize Rad51 on DNA, but repair so enabled in the absence of the Brh2 DBD is not completely efficient or appropriate. While these findings are provocative, additional study is required before a firm conclusion can be drawn about a possible role for Dss1 at this step. In summary, the substantial ability of the N-terminal BRC-containing region of Brh2 to promote survival after DNA damage in the absence of Dss1 is evidence that Dss1 is not required for BRC-Rad51 partnering.

Deletion analysis of Brh2 defines a narrow region permitting Dss1 independence. The clustering of Brh2 variants rendered independent of Dss1 within a stretch of 20 amino acid residues in the middle of the protein sequence was surprising. This suggested the presence of some type of feature(s) or

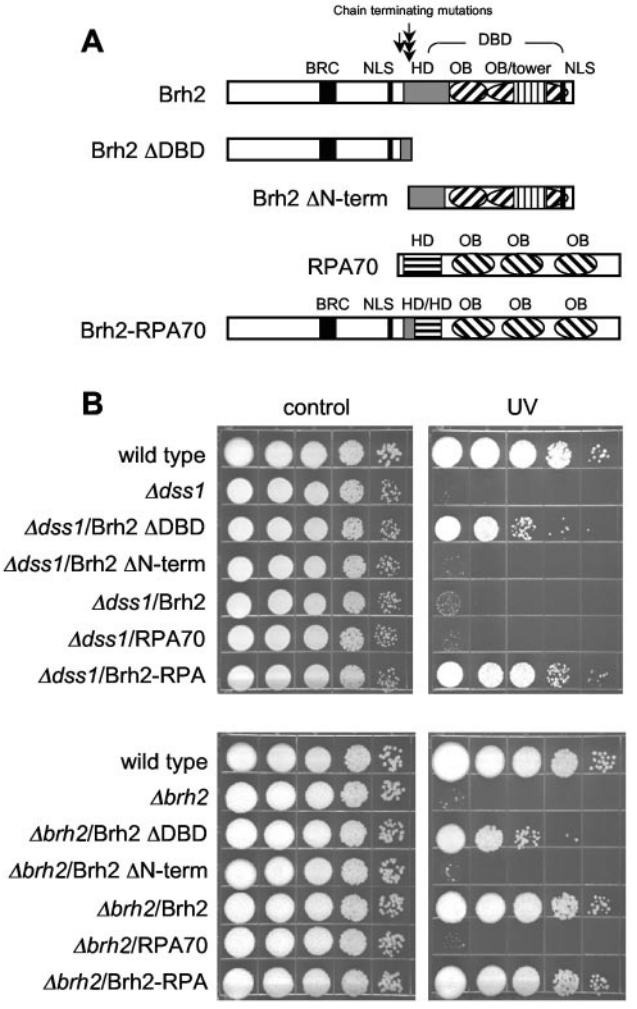

FIG. 3. Brh2 variants rescue DNA repair proficiency in *dss1* and *brh2* mutants. (A) Domain structure of Brh2 and RPA70. Brh2 schematic representation of the 1,075-residue protein features a single BRC motif (black box), an HD (gray box), tandem OB folds (hatched ovals slanted right) with the distal one containing a tower domain (vertical stripes), and two putative NLSs (vertical bars). The arrows show the sites of chain termination in the mutant alleles that suppress the DNA repair sensitivity of *dss1*. These occur at codons 531 (candidate 12) and 537 (candidates 14 to 16). The region of the protein corresponding to residues in BRCA2 that interact with DSS1 (23) is indicated by the bracket. Brh2 ΔDBD is the representative Brh2 variant of 551 residues prepared by cleavage within the coding region at the unique SphI site. The *U. maydis* RPA70 (GenBank accession number AY669151) has a predicted size of 623 amino acid residues and by BLAST is 36% identical with 55% similarity to human RPA70 over the entire length. It is arranged with an N-terminal HD (horizontal stripes) and three sequential OB folds (hatched ovals slanted left). The Brh2-RPA70 hybrid contains the N-terminal 551 residues from Brh2 fused to the C-terminal 444 residues from RPA70. (B) Survival was determined after irradiating 10-fold serially diluted cells with UV light at 120 J/m^2. At the top are shown wild-type (UCM350) and *dss1* mutant (UCM591) strains expressing Brh2 (from pCM973), Brh2 ΔDBD (pCM1016), Brh2 ΔN-terminal (pCM1017), RPA (pCM1040), and Brh2-RPA70 (pCM1034). Similarly, at the bottom are *brh2* mutant (UCM565) derivatives expressing the same set.

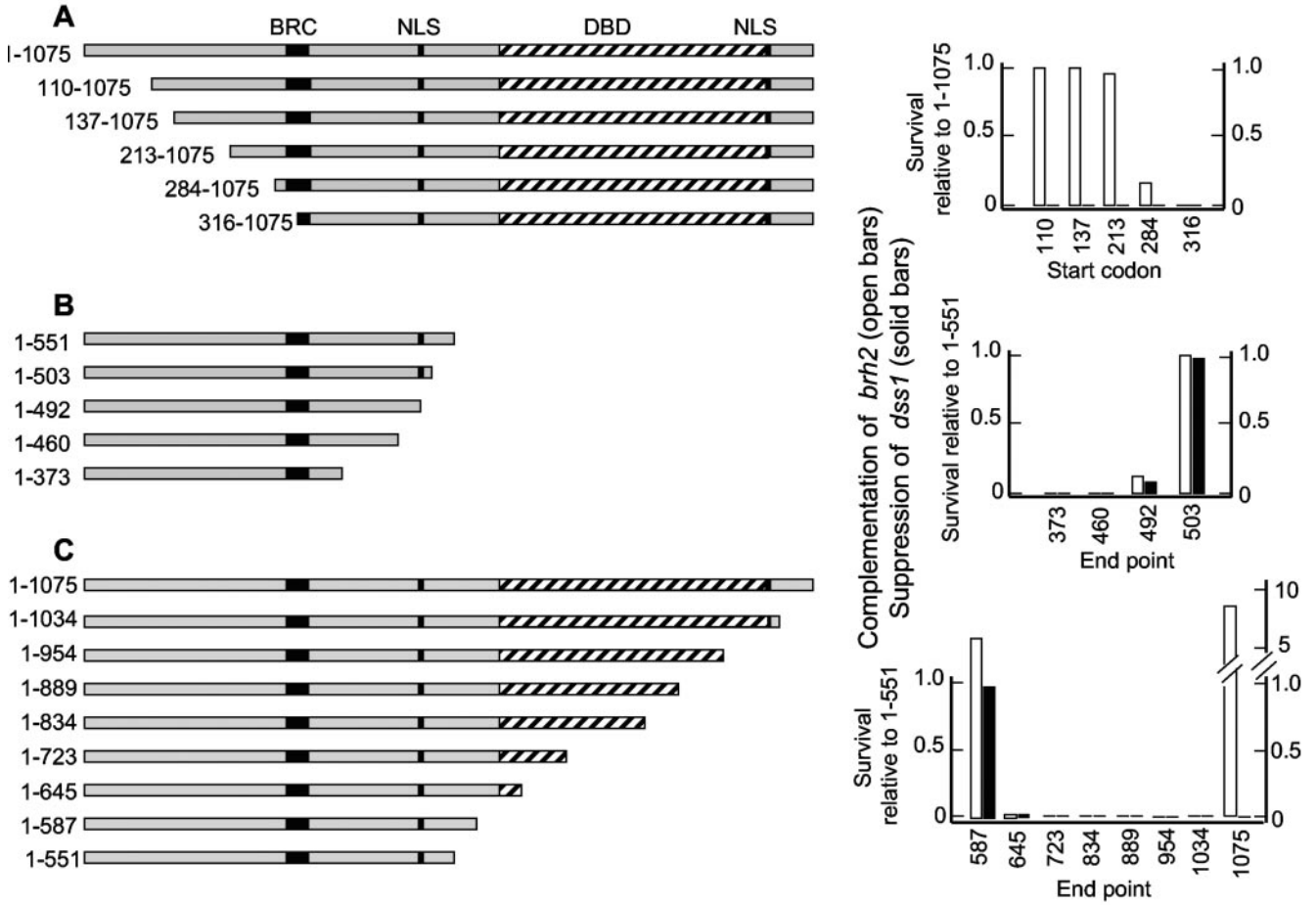

FIG. 4. Deletion mapping of Brh2. Brh2 is shown schematically with the single BRC element, the DBD, and two putative NLSs. (A) A Brh2 N-terminal deletion series was constructed by sequentially removing fragments from the 5′ end of the open reading frame so as to bring the next ATG start codon into register as shown. Each deletion was expressed from the *gap* promoter on a self-replicating plasmid and tested for complementation activity (open bars) when introduced into *brh2* (UCM565). The level of survival with pCM973 ($Brh2^{1-1075}$) as the standard was tested after preparing a dilution series and irradiating it with UV light at 120 J/m^2 as described in the legend to Fig. 2. (B) N-terminal fragments (with end points noted) created by removing sequentially larger segments from pCM1016 beginning at the SphI site in the open reading frame were tested for the ability to complement *brh2* and suppress the UV sensitivity of *dss1* (UCM591). Survival relative to that conferred upon expression of pCM1016 is shown as black bars. (C) C-terminal fragments created by removing sequentially larger segments from the full-length molecule were tested for the ability to complement *brh2* and suppress the UV sensitivity of *dss1* (UCM591).

element(s) both proximal and distal to the locus that constrained or confined the chain termination events to that precise region of the molecule. A deletion analysis of Brh2 was performed to localize the element(s) and define what properties of the Brh2 variants are necessary for promoting survival after DNA damage. Three deletion series were constructed. First, the 5′ end of the full-length gene was trimmed to define the point at which DNA repair complementing activity was lost (Fig. 4A). This was performed by progressively shortening the 5′ end of the open reading frame so as to juxtapose the promoter sequence with successive ATG codons (start codon in the graph). Biological activity was measured with the survival assay described above as the ability to complement the UV sensitivity of the Δ*brh2* mutant relative to full-length Brh2 after a dose of 120 J/m^2. Complementing activity was undiminished with deletions removing the first 212 amino acids but fell sharply when the deletion end point was just proximal to the BRC element ($Brh2^{213-1075}$) and dropped to null upon expression of $Brh2^{316-1075}$, which cuts into the BRC element. In a second series, the DNA fragment encoding Brh2 ΔDBD was trimmed back from the 3′ end to produce deletions in which the N-terminal Brh2 domain was progressively shortened at its carboxy end (Fig. 4B). In this case, biological activity was measured by the ability to suppress the UV sensitivity of the Δ*dss1* mutant and is expressed relative to the activity of representative Brh2 ΔDBD ($Brh2^{1-551}$). Activity remained high when the deletion end point stopped at residue 503 but dropped sharply when it receded past the putative NLS KRPR (located at residues 496 to 499). In the third series, the ability to suppress the UV sensitivity of the Δ*dss1* mutant was measured as the deletion end point was extended from residue 551 toward the C terminus (Fig. 4C). Again, activity in suppressing the UV sensitivity of Δ*dss1* is expressed relative to that of representative Brh2 ΔDBD ($Brh2^{1-551}$). In this case, activity was retained when the end point was extended to residue 587 but dropped to null when the end point was residue 645, the

next point tested. It is apparent upon examining the sequence in this region that the former deletion removes all of the conserved Dss1-interacting residues evident from BLAST alignment with those in the human BRCA2 DBD, while the latter deletion leaves behind a stretch that includes six highly conserved Dss-interacting residues (637 to 641, WKLAAM). Thus, survival activity appears to be abruptly eliminated when the protein's C-terminal tail extends a modest number of residues past some critical demarcation. It should be mentioned that an almost identical survival response was observed when these deletions were expressed in the *brh2* mutant. Thus, with the exception of full-length Brh2, the presence or absence of Dss1 makes no difference in the complementing ability of these C-terminal deletions.

From these studies two conclusions emerge. First, it seems evident that there is no element proximal to the BRC element that is important in complementing the radiation sensitivity of *brh2*. Second, the sequence represented by the putative upstream NLS appears to define a proximal boundary in the ability of Brh2 truncated variants to suppress *dss1*, while sequences represented by the Dss1-interacting residues appear to represent a distal boundary. This accounts for the narrow sequence window in which the mutant variants in the screen were isolated. The basis for the loss of complementing activity by variants whose C-terminal tail extends into the Dss1-interacting domain is unknown. It is possible that the stability of the truncated proteins is altered or that Rad51's interaction with the N-terminal domain is somehow compromised. These points are being investigated.

Brh2-RPA70 hybrid effectively promotes Dss1-independent survival after DNA damage. The restoration of DNA repair proficiency by the BRC-containing N-terminal domain (Brh2 ΔDBD) was unexpected in view of the evidence of a negative role played by BRC elements in dissociating Rad51 nucleoprotein filaments (10). But given the obvious significance of the C-terminal DBD, and the requirement for Dss1 as an activator for Brh2, a simple notion for how Dss1 might function can be envisioned. We suggest that Brh2 facilitated by its DBD can deliver Rad51 to a DNA site requiring repair but subject to appropriate regulation. Once the Brh2-Rad51 complex is "modulated" or "unlocked" by interaction with Dss1, Rad51 is imagined to be brought under a state of proper governance for controlled homologous pairing. Consequently, it might be predicted that substitution of the Brh2 DBD with a similar DBD lacking the Dss1-interacting residues could enhance the activity of the BRC-containing N-terminal domain in repair, but such a chimeric molecule would no longer be subject to Dss1 regulation.

To test this idea, a hybrid Brh2 variant was constructed by fusing its N-terminal BRC-containing domain to the DBD from the *U. maydis* ortholog of the RPA70 subunit (single-stranded DNA-binding replication protein A). This protein was chosen as a suitable DBD donor since it has features in common with the Brh2 DBD (3). The primary sequence of RPA70 contains a tandem arrangement of an N-terminal HD followed by an array of three OB folds (Fig. 3A), the first two of which are responsible for high-affinity DNA binding (41). Therefore, RPA70 was considered to be an excellent substitute for the Brh2 DBD but at the same time is devoid of corresponding Dss1-interacting residues, as deduced from sequence alignment and from threading of the sequence onto the three-dimensional structure of the BRCA2 DBD (not shown).

Introduction of the Brh2-RPA70 hybrid effectively revived the DNA repair capacity of *brh2* (Fig. 3B). This was evident not only in the complementation of the radiation sensitivity of *brh2* but also in elevating the spontaneous level of GFP-Rad51 focus-forming activity and in promoting recombination (see below). Moreover, in contrast to the case in which ectopically expressed Brh2 had no activity in rescuing *dss1*, the Brh2-RPA70 hybrid was highly effective (Fig. 3B). Expression of the RPA70 subunit by itself had no ability to rescue DNA repair proficiency (Fig. 3B). These results are thus consistent with the prediction from the simple model.

Elevated recombination in the absence of Dss1 regulation. While the Brh2-RPA70 hybrid was active in promoting survival of *brh2* or *dss1* mutant cells after DNA damage, the absence of an essential element for regulating Brh2-promoted recombination would not be expected to be without consequence regarding genome stability. It seemed obvious upon inspection of colony morphology that expression of the Brh2-RPA70 hybrid in diploids was detrimental as the colonies were slow growing and somewhat irregular in size and shape (data not shown). Indeed, the spontaneous level of Rad51 foci formed in cells expressing the Brh2-RPA70 hybrid was elevated, raising the consideration that recombination was active but unchecked (Fig. 5). After irradiation, the level of GFP-Rad51 focus formation was much more exaggerated compared to that of the wild type or to Brh2 ΔDBD by itself, again suggesting a loss of control of recombination.

To follow up the latter point, we measured recombination by two different means, our rationale being that if Dss1's normal function is to regulate Brh2, then cells expressing Brh2 variants free of Dss1 regulation should exhibit inappropriately high levels of recombination regardless of how the markers were configured. First we measured heteroallelic recombination at the nitrate reductase locus (*nar1*), which has been extensively characterized in studies on mitotic recombination in *U. maydis* and which is known from half-tetrad analysis to take place almost exclusively through gene conversion (16, 17). Recombination was determined as Nar^+ prototroph formation after plating of *nar1-1*/*nar1-6* diploids on minimal medium with nitrate as the sole source of nitrogen. Tester strains wild type for recombination functions expressing Rad51 or Brh2 ectopically from plasmids exhibited rates of spontaneous recombination at or marginally above the normal level (1.5 to 2.2 times, respectively). However, in cells expressing Brh2 ΔDBD or the Brh2-RPA70 hybrid, rates were elevated 3.5-fold and 40-fold, respectively (Table 1), likely reflecting a loss of the competence of these Brh2 variants to be regulated by Dss1. A comparable rate of recombination was also noted in the *dss1* diploid strain when the Brh2-RPA70 hybrid was expressed, reinforcing the notion that Dss1 serves to limit recombination, through interaction with Brh2, rather than to play a role in its stability or its interaction with Rad51.

It is clear from the data above that the level of recombination increased upon ectopic expression of the Brh2 variants free of Dss1 control, but the assay for heteroallelic recombination as performed does not provide insight into the basis for that increase. It could be that the elevated frequency is an indirect consequence of more breaks being introduced into the

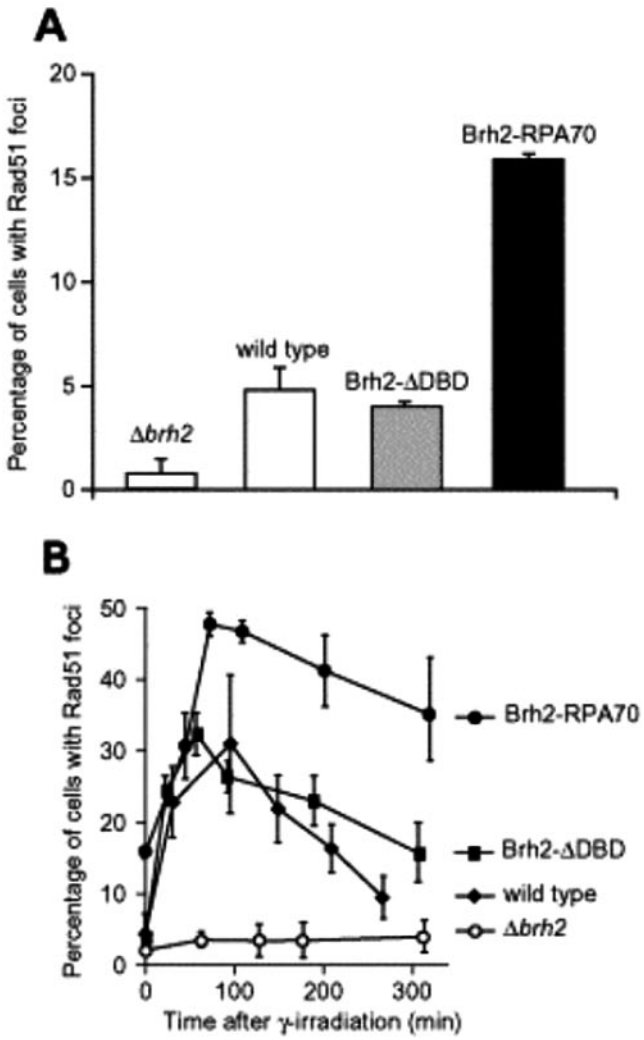

FIG. 5. GFP-Rad51 focus formation in cells expressing Brh2 variants. (A) Wild-type UCM350 and *brh2* mutant UCM565 cells expressing GFP-Rad51 and Brh2 ΔDBD or Brh2-RPA70 were viewed under a fluorescence microscope without fixation. Cells were examined for spontaneous GFP-Rad51 focus formation. Cells generally had one to three foci, with most having only a single focus. Error bars are standard deviations based on two or three independent determinations. The fraction of cells with GFP-Rad51 foci was determined after counting 200 cells. (B) Kinetics of GFP-Rad51 focus formation in wild-type (Brh2) and *brh2* mutant strains expressing the indicated Brh2 variant. Percentages at each time point were determined by counting 200 cells.

DNA by some unknown means upon expressing the Brh2 variants, or on the other hand it could be due to an inherently more active recombination system. To distinguish between these possibilities, we used a plasmid-by-chromosome assay similar to one used in the laboratory previously for measuring DNA double-strand break repair (12, 34). Our rationale was that if the elevated recombination observed upon ectopic expression of the Brh2 variants was an indirect consequence of increased nicks or breaks in DNA, then introducing a recombination substrate already precut should not result in any further elevation of recombination in cells expressing the Brh2 variants. On the other hand, if recombination were found to increase, it would imply that the system was inherently more active upon ectopic expression of the Brh2 variants.

TABLE 1. Heteroallelic recombination in cells expressing Brh2 variants

Strain	Nar^+ recombination rate, 10^{-7}/cell per generation
Wild type[a]	0.34 ± 0.05
+ Rad51	0.52 ± 0.1
+ Brh2	0.74 ± 0.2
+ Brh2 ΔDBD	1.2 ± 0.2
+ Brh2-RPA70	11 ± 3.2
dss1/dss1[b]	<0.003
+ Brh2-RPA70	7.2 ± 2.9

[a] Tested were strain UCM33 (*nar1-1/nar1-6 pan1-1/+ met1-2/+*) and derivatives expressing Rad51 (pCM1035), Brh2 (pCM1033), Brh2 ΔDBD (pCM1016), or Brh2-RPA70 (pCM1034).

[b] Tested were strain UCM593 (*dss1-1/dss1-2 nar1-1/nar1-6 pan1-1/+ met1-2/+*) and a derivative expressing Brh2-RPA70 (pCM1034).

The experimental design formulated was to measure recombination of a cloned *U. maydis* gene present in a nonreplicating plasmid, pCM1039, with its homologous sequence in the genome during transformation (Fig. 6). The chromosomal target chosen was the structural gene for the iron-sulfur protein subunit of succinic dehydrogenase (CBX^S), while the homologous gene on the plasmid, present on a 2-kbp fragment, was an allele of that same gene (CBX^R) altered by a single amino acid change, thereby conferring resistance to the fungicide carboxin (Cbx^R) (5). In *U. maydis* transformation, as is the case in yeast, introducing a plasmid cut within a cloned gene targets recombination to the endogenous locus. Therefore, to direct recombination events to the succinic dehydrogenase gene, pCM1039 DNA was cut within the CBX^R gene at the unique DraIII site, which is located 0.9 kbp from the mutation responsible for Cbx^R, leaving about 0.6 kbp of homologous sequence on the

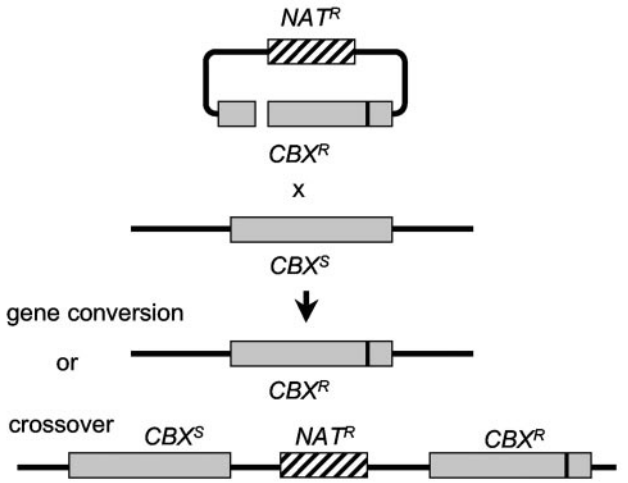

FIG. 6. Schematic view of plasmid-by-chromosome recombination. A representation is shown of recombination between chromosomal allele CBX^S (gray rectangle) and the homologous gene CBX^R (split gray rectangle) on plasmid pCM1039 cut with DraIII in the gene. The amino acid change corresponding to carboxin resistance is indicated (black bar). An additional NAT^R marker (hatched rectangle) is present on the plasmid.

TABLE 2. Plasmid-by-chromosome recombination in cells expressing Brh2 or variants

Strain[a]	Recombination frequency[b] (10^{-3})	Crossover/conversion[c]
Wild type	2.8	24/32 (0.75)
+ Brh2	6.9	39/48 (0.81)
+ Brh2 ΔDBD	7.8	20/22 (0.91)
+ Brh2-RPA70	22.1	40/48 (0.83)

[a] Tested were wild-type (for recombination functions) strain UCM350 (*nar1-6 pan1-1 a1 b1*) and derivatives expressing Brh2 ΔDBD (pCM1016), Brh2 (pCM973), or Brh2-RPA70 (pCM1023).

[b] Recombination was determined by transformation to Cbx^R with 10 μg of DraIII-cut linear DNA. Transformation efficiencies were standardized by parallel transformation with 10 ng of pCM619 DNA. Recombination frequencies were calculated by dividing the number of Cbx^R transformants per microgram of pCM1039 by the number of Cbx^R transformants per microgram of pCM619 DNA.

[c] Crossover/conversion fractions were obtained by measuring the frequency of Nat^R among Cbx^R transformants. Values in parentheses are the fractions of Cbx^R recombinants that were Nat^R.

proximal side of the cleavage site and 1.4 kbp on the distal for pairing. Recombination frequencies were standardized by comparing the number of Cbx^R transformants obtained to the number resulting from the introduction of an autonomously replicating plasmid so as to control for cellular competence and DNA uptake. Consistent with previous findings, analysis of recombination with the plasmid-by-chromosome system revealed that the overall frequency in cells expressing Brh2, Brh2 ΔDBD, or Brh2-RPA70 was elevated. In the latter case, the frequency was up about eightfold. Thus, the results support the notion that the inherent recombination activity of cells not tightly regulated in Brh2 expression or expressing Brh2 variants free of Dss1 control is elevated.

An additional important feature in the design of this plasmid-by-chromosome recombination system was the arranged configuration of marker and DNA double-strand break that weighted the outcome in favor of crossover events. During mitotic recombination in yeast, there is evidence to suggest that crossovers associated with conversion become more frequent as the length of the conversion tracts increases, reaching a maximum for tracts of ~1 kbp (1, 2). Therefore, we tried to bias our system toward crossing over by positioning the double-strand break 0.9 kbp from the mutation conferring Cbx^R. Transformation to Cbx^R can occur by gene conversion of the endogenous allele or by integration of the plasmid at the endogenous locus through crossing over (Table 2). Since the plasmid used for the analysis (pCM1039) also contained another marker (*NAT*^{R}) of bacterial origin expressing resistance to the antibiotic NAT (Nat^R), it was possible to confirm that the plasmid had become integrated into the *U. maydis* genome simply by plating Cbx^R transformants on medium containing NAT and scoring for Nat^R. Thus, Cbx^R transformants that were Nat^S were categorized as having undergone recombination by gene conversion while Chx^R transformants simultaneously Nat^R were considered crossovers. Indeed, the recombinants were found to be biased toward crossing over as there was a strong preference for integration of the plasmid DNA evident from the high ratio of Nat^R to Cbx^R. In cells expressing Brh2 or the variants, the bias in the distribution of recombinants remained strongly in favor of crossing over.

Thus, in both types of assay systems, one measuring primarily gene conversion events and the other measuring crossover events, recombination was found to be elevated upon expression of Brh2 ΔDBD and in particular Brh2-RPA70. These results are consistent with the notion that the stage at which Dss1 operates in regulating recombination is at the level of initiation, not at a later stage where resolution of intermediates might be directed. We have no unequivocal evidence that the elevated recombination frequencies observed are detrimental to the cell, but it certainly seems possible that inappropriate activation of recombination due to the absence of Dss1 control could lead to genomic instability resulting from increased loss of heterozygosity.

DISCUSSION

There are three principal conclusions to be drawn from this investigation. First, Dss1 is in dynamic interplay with Brh2, operating beyond the simple role as a static constituent in Brh2's architecture. Second, mutations in Brh2 liberating it from Dss1 control disclose a modular arrangement and functionally separable domains. Third, enhanced survival after DNA damage and runaway recombination upon substitution of RPA70 for the DBD of Brh2 reveal the governing role of Dss1 in endowing the Brh2-Rad51 system with activity and suggest that Brh2-Dss1 interplay is developed to elaborate tight regulation.

Recent work from the laboratory establishing the importance of Dss1 in recombination and repair rendered obsolete the simple model of an exclusive partnering of Brh2 and Rad51 (23). However, something other than a static interplay between Brh2 and Dss1 was not necessarily anticipated since the requirement for Dss1 in producing Brh2 in soluble form in insect cells suggested a permanent union of these two molecules. Thus, the more dynamic roles for Dss1 that had been considered, as a regulator, activator, modifier, or signal transducer, were done in an ad hoc manner (19). Nevertheless, the predictions from these attractive models were straightforward and certainly warranted further testing. Accordingly, the first key questions posed were whether the absence of Dss1 affected the stability of Brh2 or its ability to associate with Rad51. The answer to these questions was no, in agreement with the conclusions drawn about mammalian BRCA2 by the Ashworth laboratory (15). These investigators found that depletion of DSS1 by RNAi had no effect on the steady-state level of BRCA2 or on its ability to associate with RAD51. In view of the requirement for Dss1 in the formation of Rad51 foci following DNA damage, the inference is that Dss1 exerts its action at a step subsequent to Rad51's association with Brh2 but before properly coordinated assembly of Rad51 nucleoprotein filaments at sites requiring recombinational repair. Therefore, Dss1 may be thought of as a positive activator of the dormant Brh2-Rad51 complex, thereby enabling recombination repair to take place. Thus, the accumulating evidence suggests that the functional interplay among Brh2, Rad51, and Dss1 has been highly conserved during the course of evolution.

Starting from the premise that specific protein domains would be key in locking the hypothesized Brh2-Rad51 complex, we reasoned that these domains could be identified by isolating mutant forms of Brh2 able to suppress the radiation sensitivity caused by loss of Dss1 function. Our expectation of

this genetic approach was that it would offer a modest way to define residues at the Brh2-Dss1 interface important for the Dss1 interaction. However, we were surprised when it turned out that the mutant Brh2 variants obtained all arose from chain-terminating mutations predicted to eliminate the C-terminal DNA-DSS1 binding domain. Furthermore, the points of chain termination were confined to a narrow window so that the expressed N-terminal region included a putative NLS but lacked even a few Dss1-interacting residues. These findings suggest that the N-terminal region of Brh2 containing the BRC element crucial for interaction with Rad51 has an innate ability to organize Rad51, as witnessed by the near normal level of Rad51 focus formation upon its expression, and to enable promotion of a substantial level of DNA repair activity despite the complete absence of the DBD. It might well be that BRC contact with Rad51 alters it in such a way as to enhance or stabilize its inherent filament-forming capacity. Alternatively, there is the possibility that the N-terminal domain still has some remnant DNA-binding ability. Further discussion of the molecular basis that underlies this finding is best postponed until relevant biochemical evidence has been obtained. Nevertheless, it should be kept in mind that the fusion protein containing the DBD from RPA70 shows a larger difference in its behavior from that of the wild-type protein. Evidently, Dss1 is not required for communication between the BRC domain and Rad51.

The findings supporting a positive, active role of the BRC-containing N-terminal domain were unexpected in light of accumulated evidence pointing to an essentially negative role played by BRC peptides from mammalian BRCA2 in inhibiting DNA strand exchange and destabilizing Rad51 nucleoprotein filaments in vitro, and in interfering with Rad51 focus formation and inhibiting DNA repair and recombination in mammalian cells (6, 10, 37). However, recent in vitro studies showing that BRC peptide modified by phosphorylation loses the ability to depolymerize the Rad51 filament may indicate a role for the BRC elements in mammalian cells that is not strictly negative (38). On the other hand, the C-terminal domain provides some negative regulatory property, as is apparent from the inability of full-length Brh2 to support focus formation or DNA repair in the absence of Dss1.

Brh2's modular arrangement of an N-terminal domain with inherent Rad51 organizing activity plus an NLS together with a C-terminal DBD plus the presence of another NLS raises the speculation that these domains were once encoded separately but then became fused during the course of evolution. Perhaps through such an elaboration the innate Rad51 organizing activity could be harnessed to provide a finer level of control. The two NLSs might thus be remnants from the time when the genes were separate.

The inappropriately high level of recombination in cells expressing the Brh2-RPA70 hybrid clearly highlights the importance of Brh2-Dss1 interplay in ensuring that Brh2-driven recombination is properly controlled. Here Dss1 is unable to attenuate the hyperrecombination, implying that fine tuning is accorded by its interaction with the C-terminal DNA-DSS1 binding domain. The distribution of hyperrecombination events via the outcomes through gene conversion and crossing over remains unchanged from that in normal wild-type cells, but nevertheless the consequence is still an increase in the absolute frequency of combined conversion and crossover events. Thus, as has been documented in mammalian cells overexpressing Rad51 (33), there is increased risk for chromosomal rearrangements, loss of heterozygosity, and potentially damaging consequences that might ensue once recessive mutations are revealed. In conclusion, even though the molecular basis is not yet clear, it seems that a primary function of Dss1 is to ensure that Brh2-driven recombination is tightly regulated.

Finally, the above findings might provide an additional perspective to help account for the genomic instability reported to be associated with various hypomorphic BRCA2 alleles resulting from mutations causing premature chain termination (18). In mice the embryonic lethality of *Brca2* null mutations has been documented, but certain targeted mutations in BRC-encoding exon 11 result in premature truncations that are hypomorphic and homozygous mice bearing these alleles have been noted to survive to adulthood, although not without a wide range of defects, including an increased likelihood of tumor formation (7, 14). As these alleles are predicted to result in C-terminal truncations retaining several of the BRC repeats but with the DBD and the attendant DSS1-interacting domain deleted, it seems possible that the N-terminal BRC domain retains some biological activity, in keeping with the above findings. Of course, such an interpretation is complicated by the elimination of the extreme C-terminal Rad51-binding domain, as well as C-terminal NLSs. However, it remains a possibility that unregulated, DSS1-independent recombination resulting from expression of these mutant alleles could contribute to elevated genomic instability and possibly predispose to cancer even in the heterozygous state when a second normal allele is present.

In summary, the results here extend the previous conclusions that Dss1 is a key determinant of Brh2-driven recombination but in so doing pose a whole set of new questions. How the Brh2-Rad51 complex is rendered active and how precision in control of recombination is conferred by Dss1 are challenging mechanistic issues awaiting insightful experimentation.

ACKNOWLEDGMENTS

We are indebted to Rodney Rothstein for providing access to his fluorescence microscopy imaging facility and to the GammaCell 220 ^{60}Co irradiator at the Columbia University College of Physicians and Surgeons. We thank Shaneen Singh for performing the threading analysis of RPA70. Nikola Pavletich and Haijuan Yang offered instructive suggestions, and Lorraine Symington and Robin Holliday provided critical comments on the manuscript.

W.K.H. gratefully acknowledges support for this work from National Institutes of Health grant GM42482, Department of Defense Breast Cancer Research Program grant DAMD17-3-1-0234, and the William Randolph Hearst Foundation.

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