Role for RAD18 in Homologous Recombination in DT40 Cells[∇]

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Received 14 July 2006/Returned for modification 8 August 2006/Accepted 14 August 2006

RAD18 is an E3 ubiquitin ligase that catalyzes the monoubiquitination of PCNA, a modification central to DNA damage bypass and postreplication repair in both yeast and vertebrates. Although current evidence suggests that homologous recombination provides an essential backup in vertebrate *rad18* mutants, we show that in chicken DT40 cells this is not the case and that RAD18 plays a role in the recombination reaction itself. Gene conversion tracts in the immunoglobulin locus of *rad18* cells are shorter and are associated with an increased frequency of deletions and duplications. *rad18* cells also exhibit reduced efficiency of gene conversion induced by targeted double-strand breaks in a reporter construct. Blocking an early stage of the recombination reaction by disruption of XRCC3 not only suppresses immunoglobulin gene conversion but also prevents the aberrant immunoglobulin gene rearrangements associated with RAD18 deficiency, reverses the elevated sister chromatid exchange of the *rad18* mutant, and reduces its sensitivity to DNA damage. Together, these data suggest that homologous recombination is toxic in the absence of RAD18 and show that, in addition to its established role in postreplication repair, RAD18 is also required for the orderly completion of gene conversion.

DNA replication is very sensitive to the quality of the DNA template. Damaged or missing bases can stall the replicative polymerases, which may, in turn, result in unreplicated gaps (29). To prevent such gaps, cells frequently opt to bypass DNA damage during replication, leaving it for repair at a later stage. In all organisms there are two basic strategies for bypass. The first, translesion synthesis, temporarily replaces the stalled replicative polymerases with specialized translesion polymerases that are able to synthesize across damaged bases directly (reviewed in reference 27). Translesion synthesis has the potential to be mutagenic both because of the noninstructional nature of the lesion and the error-prone nature of the translesion polymerases (reviewed in reference 25). The second strategy involves a transient switch by the replicative polymerases to use an alternative undamaged template, a recombination-like mechanism that is poorly characterized in higher organisms but which generally results in accurate bypass (19, 46).

RAD18 is central to both these DNA damage tolerance pathways in yeast (reviewed in reference 9). It is an E3 ubiquitin ligase that, with the E2 ubiquitin-conjugating enzyme RAD6, is responsible for the monoubiquitination of PCNA (3, 20). This modification facilitates the recruitment of translesion synthesis through ubiquitin-binding motifs located in the translesion polymerases (6) and also acts as a platform for the formation of a noncanonical K63-linked polyubiquitin chain by RAD5/MMS2/UBC13 (20). This latter modification controls error-free bypass by an as-yet-unidentified mechanism thought to be a form of template switch (19). RAD18 is conserved in

higher eukaryotes in which it also catalyzes the monoubiquitination of PCNA (21), although, in chicken DT40 cells at least, it is not the sole enzyme responsible for this modification (33). Further, there is evidence that RAD18 does not exert the same dominant controlling influence over lesion bypass as does its yeast counterpart. The available genetic evidence from DT40 suggests that RAD18 is not epistatic, in terms of DNA damage tolerance, to at least three components of the TLS machinery: DNA polymerase κ (24), REV1 (28), and REV3 (37). However, it is unlikely that this apparent independence is complete. The recombination-independent mutagenic repair of DNA interstrand cross-links in DT40 requires not only REV1 and REV3 but also the ubiquitination of PCNA (32). Further, studies in human cells points to a requirement of RAD18 for the full function of DNA polymerase κ in lesion bypass (5).

As well as sensitivity to a broad range of mutagens, a prominent feature of *rad18* dysfunction in chickens, mice, and humans is an increase in spontaneous and damage-induced sister chromatid exchange (41, 43). This latter phenotype has been explained in terms of DNA lesions being channeled from disabled postreplication repair into homologous recombination. This idea is supported by the inviability of a *rad18/rad54* mutant of DT40 (43), which also suggests that the *rad18* mutant relies on recombination for viability.

The study presented here also makes use of DT40. In addition to being a genetically tractable vertebrate cell line (11), an added advantage for the study of DNA damage bypass and recombination is its constitutively diversifying immunoglobulin loci (10, 22, 30). Immunoglobulin diversification in DT40 is initiated by abasic sites formed by the concerted action of activation-induced deaminase (AID) and uracil DNA glycosylase (UNG): AID acts on dC to form dU, which is then removed by UNG to produce an abasic site (1, 13, 14, 17). This abasic site may initiate a recombination-based gene conversion

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[∇] Published ahead of print on 21 August 2006.

TABLE 1. DT40 mutants used in this study

Cell line	Resistance cassette(s) ^a				Source or reference
	<i>rad18</i>	<i>xrcc3</i>	DR-GFP	YFP-hRAD18	
<i>rad18</i>	his/bsr				43
<i>xrcc3:loxP-hXRCC3-IRES GFP</i>		neo*/bsr/his			45
<i>xrcc3:loxP-hXRCC3-IRES GFP/rad18</i>	puro/hyg	neo*/bsr/his			This study
<i>xrcc3/rad18:hRAD18</i>	puro/hyg (puro excised)	neo*/bsr/his		puro	This study
<i>rad18:hRAD18</i>	his/bsr			puro	This study
DTDR17			puro		8
<i>rad18-DRGFP</i>	his/bsr		puro		This study
<i>rad18-DRGFP:hRAD18</i>	his/bsr		puro	neo	This study

^a Resistance cassette abbreviations: his, histidinol; bsr, blasticidin; puro, puromycin; hyg, hygromycin; and neo, neomycin. An asterisk indicates the neo cassette used in the hXRCC3-IRES-GFP transgene. The excision of loxP-flanked selection cassettes was performed by transient transfection with pGKCre, followed by replica plating and selection for loss of resistance.

between upstream pseudogenes and the expressed immunoglobulin variable gene. Alternatively, it can be bypassed by translesion synthesis, which may result in a point mutation. Thus, the pattern of mutations in the immunoglobulin genes can provide clues to the relative use of recombination and translesion synthesis in processing replication-stalling DNA lesions (30). Disruption of *rad54*, which is thought to play a relatively late role in the recombination reaction (reviewed in reference 40), results in a fivefold decrease in immunoglobulin gene conversion (4). Mutants that disrupt the early stages of the reaction by reducing the efficiency of loading RAD51 onto single-stranded DNA, for example, the RAD51 paralogues or BRCA2 (44), also result in the inhibition of immunoglobulin gene conversion (18). However, this reduction in recombination is accompanied by an increase in nontemplated point mutations (18, 31). This indicates that inhibition of recombination at a very early stage results in the initiating abasic sites being left as a substrate for translesion synthesis (30). We have previously argued that this difference may be explained by RAD54 acting beyond a point of commitment in recombination, possibly reflecting resection of the lesion, after which translesion synthesis is ineffective (34).

Translesion synthesis of abasic sites in the immunoglobulin loci of DT40 is dependent on REV1 (34). Based on the yeast paradigm, we had originally expected that it would also be dependent on RAD18. However, we have previously reported that there is substantial RAD18-independent point mutation in the immunoglobulin loci of DT40 (35), suggesting that RAD18 and REV1 have largely, but perhaps not completely, independent roles in vertebrate translesion synthesis (28, 35). While studying the role of RAD18 in immunoglobulin diversification, we noted an increase in the frequency of aberrant rearrangements, principally deletions and duplications, in the immunoglobulin light-chain variable-region gene segment (VL) of *rad18* cells (35). This observation suggested to us that RAD18 might also be involved in the recombination mechanism leading to immunoglobulin gene conversion. Here we report the results of experiments that suggest that RAD18 does indeed play a role in gene conversion in addition to its well-established role in postreplication repair.

MATERIALS AND METHODS

Cell culture and gene disruption. The culture, transfection, and screening of DT40 and mutants have been described previously (34). The constructs and

selection cassettes used in the generation of the mutants in the present study have also been described previously and are summarized in Table 1.

hRAD18 expression construct. The human RAD18 open reading frame was cloned as a SalI-NotI fragment into pXPSN (28) cut with HindIII-NotI in a three-way ligation with enhanced yellow fluorescent protein (eYFP; Clontech) as a HindIII-SalI fragment. This fuses YFP to the N terminus of RAD18. The β -actin promoter-YFP-RAD18 cassettes were subsequently cloned into pLoxPuro or pLoxNeo (2).

Immunoglobulin gene diversification analysis. Immunoglobulin gene diversification analysis has been described in detail elsewhere (30, 31). Briefly, sequence changes in the immunoglobulin light-chain locus of DT40 are categorized as templated (i.e., a donor sequence of ≥ 9 bp exists in the V pseudogene array) or nontemplated. This provides a measure of the relative use of translesion synthesis (nontemplated point mutations) and recombination (gene conversions) in response to AID-induced abasic sites (30). The analysis is shown for all sequences (see Fig. 6A) and for mutated sequences only (Fig. 6B). In the latter analysis (Fig. 6B), clonally related changes are excluded by counting a gene conversion or point mutation from a given cellular subclone only once.

Western blotting. Western blotting for hXRCC3 was carried out by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis of whole-cell lysates followed by transfer to nitrocellulose and blotting with rabbit anti-human XRCC3 antiserum (Novus Biologicals NB100-165A2), followed by anti-rabbit immunoglobulin-horseradish peroxidase. Antibody binding was visualized by using enhanced chemiluminescence (Amersham).

Immunofluorescence. For immunofluorescence staining, DT40 cells were spun onto polylysine-coated glass coverslips, fixed in 4% paraformaldehyde in phosphate-buffered saline, and blocked in phosphate-buffered saline containing 0.1% Triton X-100 and 0.02% sodium dodecyl sulfate. Primary and secondary antibodies were diluted in the same buffer. The primary antibodies were anti-RAD51 (rabbit polyclonal H-92; Santa Cruz) and anti-phospho-H2AX (mouse monoclonal JBW103; Upstate). Fluorescence-labeled secondary antibodies were obtained from Molecular Probes.

Analysis of SCE. Staining metaphase chromosomes for sister chromatid exchange (SCE) was performed as previously described (34). Slides were scored by investigators blinded to the genotype or treatment. DNA damage was induced with 0.2 ng of 4-nitroquinoline-1-oxide (4NQO) (Sigma)/ml.

Monitoring I-SceI-induced double-strand break repair. The RAD18 gene was disrupted in DT40 (DTDR17) cells (8) stably expressing a single copy of DR-green fluorescent protein (GFP) (from Maria Jasin) (26). Two resulting clones were complemented with hRAD18 as an N-terminal fusion with YFP. A total of 3×10^6 cells were transfected with 30 μ g of pCBASce or the control vector pmaxGFP in 100 μ l of Nucleofector T solution by using the Amaxa Nucleofection system and program B23. After incubation of the cells in 5 ml of medium for 48 h, GFP expression was quantified by using a MoFlo cytometer (Dako). GFP fluorescence and YFP fluorescence were separated by using a 510/20 filter for GFP detection, a 550/30 filter for YFP detection, and a 525 SP dichroic mirror (Omega Optical, Inc.). The same instrument setup and compensation settings were used for cell lines that did not express YFP.

Colony survival. The method for determining colony survival on methylcellulose-containing medium has been described previously (34). UV light (254 nm) was delivered from a benchtop lamp (UVP, Inc.) whose output had been equilibrated for 10 min and measured with a UV radiometer (UVP, Inc.). Cisplatin and methylmethane sulfonate were obtained from Sigma.

RESULTS

Disruption of RAD18 leads to aberrant immunoglobulin gene diversification. We have previously reported that, whereas the overall level of immunoglobulin diversification in *rad18* cells is little changed from that of the wild type, its pattern is perturbed (35). These results are summarized in Fig. 1A. The frequency of bona fide gene conversions was reduced, but the number of aberrant events, sequence deletions, and particularly duplications increased. Thus, the number of duplications, which are normally rare events, being found in less than 1 in 100 mutated sequences from wild-type cells, increased to around 1 in 10 mutated sequences in the *rad18* mutant. This increase in aberrant immunoglobulin gene sequences is a consequence of the loss of RAD18, since the expression of human RAD18, fused to YFP, reduced the levels of deletions and duplications detectable by sequencing to background levels (Fig. 1A).

While analyzing immunoglobulin diversification in the *rad18* mutant, we noticed that we were unable to amplify the rearranged light-chain genes using our standard PCR primers from a significant number of surface immunoglobulin (sIg)-negative *rad18* clones, suggesting that the primer binding sites had been lost. We therefore looked for more gross rearrangements in the rearranged light chain by Southern blotting of KpnI-digested DNA from sIg-negative clones. The probe we used was the rearranged VL itself, which therefore hybridized to both the rearranged and unrearranged light-chain variable regions, as well as to the array of pseudogenes that serve as donors during gene conversion (Fig. 1B). A relatively frequent gene conversion event introduces a KpnI site into VL and results in a hybridizing restriction fragment shift from about 9 to 6.5 kb (Fig. 1B) (12). Such gene conversion events were detected in both wild-type and *rad18* cells. However, in addition, *rad18* cells showed an ~6-fold increase in the loss of the rearranged light-chain band accompanied by aberrant hybridization patterns consistent with gross alterations in the structure of the locus around VL (Fig. 1B and C). Again, expression of human RAD18 reduced the frequency of such events to wild-type levels (Fig. 1C). Thus, RAD18 seems to be required for the orderly completion of immunoglobulin gene conversion.

Immunoglobulin gene conversion tracts are shorter in the absence of RAD18. We then looked at immunoglobulin gene conversion tracts in *rad18* cells and compared them to the wild type, as well as to *rev1* and *ube2v2* mutants. It is usually not possible to unambiguously determine the length of a gene conversion tract, since the actual start and end of the tract are not marked by sequence changes. To circumvent this problem, we examined a large database of gene conversion tracts derived from multiple subclones and from all parts of the rearranged light chain gene, taking the maximum tract length possible. The results are presented in Fig. 2. While long tracts are seen in *rad18* cells, the median tract length is decreased from 39 bp in wild-type cells to 22 bp in *rad18*, representing a highly significant ($P \leq 0.001$) reduction. *ube2v2* and *rev1* cells showed median tract lengths of 40 and 44 bp, respectively. In *rad18* cells reconstituted with human RAD18, the median gene conversion tract length was restored to 41 bp. Thus, disruption of RAD18 is associated with shorter gene conversion tracts, as

well as with an increased incidence of aberrant rearrangements.

Impaired double-strand break-induced gene conversion in *rad18* DT40. To examine whether loss of RAD18 had an impact on recombination outside the immunoglobulin loci, we took advantage of a DT40 strain, DTDR17 (8), that harbors a single copy of a GFP gene disrupted by a recognition site for the rare-cutting endonuclease I-SceI (26). A double-strand break introduced at this site can be repaired by gene conversion using a truncated copy of GFP located in *cis* (Fig. 3A). We disrupted RAD18 in this cell line and reconstituted RAD18 expression with hRAD18 as before. Two *rad18* clones and their hRAD18-complemented subclones were transiently transfected with a plasmid driving the expression of nuclear-localized I-SceI. GFP expression was monitored by cytometry at 48 h. As a control for transfection efficiency, each line was separately transfected with a plasmid driving GFP expression. Transfection efficiency in all experiments was 43 to 68%. The frequency with which the *rad18* mutant lines generated GFP-positive cells after I-SceI expression was consistently significantly lower than that with the wild type ($P = 0.003$ for clone #38 and $P = 0.026$ for clone #69), whereas expression of hRAD18 restored this defect (Fig. 3B). However, the recombination defect in the *rad18* mutant is considerably less striking than in a *brca1* mutant line (8) derived from the same background.

Viability of *rad18* cells after disruption of XRCC3. We hypothesized earlier that RAD54 might act past a point of commitment where alternative pathways cannot easily rescue the aborted recombination event. Extrapolating this model from the immunoglobulin locus to the genome as a whole, we hypothesized that, in contrast to RAD54, a disruption of RAD18 with XRCC3 might not be lethal if other pathways, such as translesion synthesis, could process frequent lesions (such as abasic sites) that might otherwise sometimes initiate recombination. This double mutant would also allow us to determine whether the aberrant events seen during immunoglobulin diversification were associated with the same normal recombination mechanisms responsible for gene conversion rather than being by-products of attempts by other pathways to process the abasic sites. To test this idea, we took advantage of a strategy in which XRCC3 expression can be conditionally inactivated after the tamoxifen-induced expression of Cre recombinase (16, 45, 47) (Fig. 4A). We disrupted RAD18 in *xrcc3* cells carrying a human XRCC3-IRES-GFP transgene and then induced Cre expression. Since the expression of the hXRCC3 transgene was linked to that of GFP, its loss can be monitored by cytometry. After the addition of tamoxifen, GFP was lost from practically all cells by 24 h (Fig. 4B) without loss of viability. Loss of the hXRCC3 transgene was confirmed by Western blotting (Fig. 4C), and the RAD18 disruption was confirmed by Southern blotting (data not shown). Viability was confirmed by subcloning and verification of the disruption of both genes (data not shown).

XRCC3 acts upstream of RAD18 in homologous recombination. SCE is a readily scorable outcome of homologous recombination in vertebrate cells. SCE represents resolution of recombination intermediates with crossover and has previously been shown to be dependent on XRCC3 (39). To examine whether disruption of XRCC3 was also able to suppress the

FIG. 1. Aberrant immunoglobulin diversification in *rad18* DT40. (A) Frequency of gene conversions (GC), point mutations (PM), deletions (del), and duplications (dup) in the rearranged and expressed immunoglobulin light-chain variable region (VL1) in wild-type, *rad18*, and *rad18* cells complemented with human RAD18 (*rad18:hRAD18*). The data set of gene conversions and point mutations in the wild type and the *rad18* mutant has been published previously (35) but is included here for reference. (B) Examples of gross rearrangements in the immunoglobulin light-chain genes of *rad18* cells. Southern blot of KpnI-digested genomic DNA probed with the expressed VL gene. The white arrowheads indicate a hybridizing fragment generated by the introduction of a KpnI site by gene conversion. The black arrowheads indicate clones with aberrant VL genes containing large-scale rearrangements. Although most of the hybridizing pseudogene bands will include signals from both alleles, the band with the asterisk is derived only from the rearranged allele (G4) (12) and has been lost in the clone in lane 8. (C) Suppression of the large-scale V gene rearrangements in *rad18* cells by expression of hRAD18. The bars indicate the

FIG. 2. Gene conversion tracts are shortened in *rad18* cells. Each point represents a unique gene conversion tract. The maximum gene conversion tract length was determined in each. The bar in each distribution represents the median length (the actual value being indicated to the right of the bar) of 116 unique tracts from the wild type, 86 unique tracts from *rad18* cells, 28 unique tracts from *rad18:hRAD18* cells, 54 unique tracts from *ube2v2* (*mms2*) cells, and 99 unique tracts from *rev1* cells. Tract lengths were calculated from previously published data (34, 35). Using the Kolmogorov-Smirnov test, the distribution of tract lengths in *rad18* cells was significantly different from wild-type ($P \leq 0.001$), *ube2v2* ($P \leq 0.001$), and *rev1* ($P \leq 0.012$) cells. There was no significant difference between wild-type, *ube2v2*, and *rev1* cells ($P = 0.411$ to $P = 0.830$). WT, wild type.

elevated SCE in the *rad18* mutant, we examined spontaneous and 4-nitroquinoline-1-oxide-induced SCE in the macrochromosomes. Consistent with previous reports (35, 43), we found *rad18* cells to have 7.6 exchanges per metaphase compared to 1.6 in the wild type. This was induced to 12.4 and 4.6, respectively, after treatment with 4NQO (Fig. 5A). Also consistent with previously published data, *xrcc3*-deficient cells exhibit low spontaneous levels of exchange that are not induced by DNA damage. In the context of the *rad18* mutant, this remains the case with the *xrcc3* disruption completely suppressing the spontaneous and damage-induced SCE of *rad18*. An elevated level of SCE was partially restored in the *rad18/xrcc3* mutant by expression of a human XRCC3 transgene (Fig. 5A). These data show that the *rad18* mutant is able to survive without a requirement for recombination with crossover.

xrcc3 mutant cells exhibit a defect in RAD51 focus forma-

percentage of sIg-negative cells selected from sIg-positive clones with an aberrant or missing rearranged VL1. The numbers of sIg-negative clones analyzed were as follows: wild type, 45; *rad18* mutant, 96; and *rad18:hRAD18*, 49. WT, wild type.

FIG. 3. A defect in double-strand break-induced gene conversion in *rad18* cells. (A) Assay for site-specific induction of gene conversion by a double-strand break. The DR-GFP construct (i) (26) contains a copy of GFP modified to incorporate the 17-bp recognition site for the homing endonuclease I-SceI. This site also introduces a stop codon into the GFP gene. After introduction of the I-SceI, the GFP gene is cut and can initiate gene conversion (ii) with a truncated GFP gene fragment, iGFP, resulting in restoration of a functional GFP (iii). (B) Each column represents the mean percentage of GFP-positive cells detected 48 h after introduction of the I-SceI endonuclease corrected for transfection efficiency. The mock-transfected wild-type cells in the first column indicate the background level of GFP-positive cells. The error bars represent one standard deviation from the mean. Each column is derived from between three and seven independent experiments. WT, wild type.

tion (7, 39), suggesting that XRCC3 is required in the homologous recombination reaction at a stage before the loading of RAD51 onto DNA. To examine whether the role of RAD18 in recombination was before or after this function of XRCC3, we compared the formation of RAD51 foci in the *rad18* and *rad18/xrcc3* double mutant to wild-type and *xrcc3* cells. We examined colocalization of RAD51 foci with γ H2AX, a marker of double-strand breaks, before and after irradiation with UV light. Although few foci were visible in the absence of damage, the number of foci of both RAD51 and γ H2AX was increased after exposure to UV light. The *rad18* mutation did not affect recruitment of RAD51 to γ H2AX foci, but both *xrcc3* and *rad18/xrcc3* cells exhibited a reduction in the number of

FIG. 4. Generation of *rad18/xrcc3* DT40. (A) Scheme for the generation of *rad18/xrcc3* DT40. The *xrcc3* cells carry a human XRCC3-GFP transgene flanked by loxP sites. They also carry a tamoxifen (TAM)-responsive Cre recombinase (47). After disruption of RAD18, Cre is induced to remove the hXRCC3 transgene, resulting in GFP-negative *rad18/xrcc3* cells. (B) Fluorescence-activated cell sorting analysis of GFP expression in *xrcc3*:hXRCC3-IRES-GFP cells before and 24 h after addition of tamoxifen. (C) The same populations monitored for expression of the hXRCC3 transgene by Western blotting. A non-specific band provides a loading control. The 38-kDa XRCC3 band is lost after 24 h of tamoxifen. WT, wild type.

γ H2AX foci containing RAD51. This suggests that disruption of RAD18 does not affect the early steps of the homologous recombination reaction, up to and including RAD51 loading.

The *xrcc3* disruption also allowed us to confirm that the increased deletions and duplications seen in the immunoglobulin light chain locus of *rad18* cells (Fig. 1C) are associated with gene conversion rather than with other modes of processing AID-induced damage, such as translesion synthesis or excision repair. We created and diversified multiple sIg-positive subclones of the *xrcc3* and *rad18/xrcc3* mutants. We found that, after 4 weeks in culture, the frequency of sIg-negative loss variants, which provides a very approximate measure of immunoglobulin diversification, was two- to fourfold higher than for the wild type in both *xrcc3* and *rad18/xrcc3* cells (data not shown), as previously noted after disruption of XRCC3 (31). To assess the pattern of diversification, we sequenced the re-

FIG. 5. (A) Suppression of elevated SCE in *xrcc3/rad18* cells. The histograms represent the percentage of metaphases (y axis) containing a given number of SCE (x axis). The mean number of SCE is indicated. At least 50 metaphases were counted for each graph. (B) Formation of γ H2AX and RAD51 foci before and after UV irradiation. Left-hand panels show untreated cells; right-hand panels show cells at 2 h after exposure to 2 J/m² UV. Colors: γ H2AX, green; RAD51, red. Areas where the two colocalize appear yellow in the merge panel. (C) Numbers of γ H2AX foci that colocalize with RAD51 foci. For each line, γ H2AX foci were counted from more than 30 nuclei, with investigators blind to the genotype of the cells. wt, wild type.

arranged V gene from sIg-negative cells and examined the density of point mutations and gene conversion events in mutated sequences as previously described (34). Both the *xrcc3* and *rad18/xrcc3* cells exhibited a shift in their pattern of immunoglobulin diversification from predominantly gene conversion to point mutation (Fig. 5A and B), as previously observed for the *xrcc3* mutant (31). The analysis of point mutations in all sequences suggests that the loss of *rad18* reduces the frequency of the point mutation (Fig. 6A). However, there remains substantial RAD18-independent translesion synthesis of abasic sites in the immunoglobulin locus, and the level of mutations in mutated sequences is similar to that seen in wild-type cells (Fig. 6B). It is, however, not possible to put an accurate figure on the scale of any reduction because of the manner in which this experiment is configured. The pattern of mutation in the *rad18/xrcc3* mutant (Fig. 6C) is comparable to that of the *xrcc3* single mutant (31).

The *xrcc3* disruption also suppressed the large-scale aberrant rearrangements seen in *rad18* cells. Southern blotting of the immunoglobulin locus, performed as described above, reduced the frequency of large-scale rearrangements in the *rad18/xrcc3* mutant to a level comparable to that for wild-type cells (Fig. 6D). Together, these data therefore suggest that RAD18 plays a role in immunoglobulin gene conversion that is downstream of XRCC3 and RAD51 loading.

Toxicity of RAD51-dependent recombination in the absence of RAD18. The viability of the *rad18/xrcc3* mutant is surprising given the inviability of the *rad18/rad54* mutant. We therefore asked what effect the *xrcc3* disruption had on the sensitivity of *rad18* cells to genotoxic stress. Unlike *rad18* cells, the *xrcc3* mutant was not sensitive to UV light (Fig. 7A), suggesting that recombination is not normally used to repair UV lesions. Surprisingly, the double mutant exhibited sensitivity intermediate to that of *rad18* and *xrcc3*, suggesting that, in part, the hyper-

FIG. 6. Immunoglobulin diversification in *rad18/xrcc3* cells. (A) The pie charts represent the proportions of sequences with the indicated number of changes. The total number of sequences analyzed is indicated in the center of the pies. The PCR error for 30 cycles of PCR was estimated to result in one to two mutations per 100 VL sequences, assuming an error rate for *Pfu* Turbo of 1.3×10^{-6} per bp per cycle. (B) The bars represent the number of unique gene conversions and unique point mutations in sorted sIg-negative cells of the indicated mutants. These experiments were performed alongside those published in reference 35, and the wild-type and *rad18* data sets are therefore presented here again for comparison. The data are derived from 115 mutated sequences from seven wild-type subclones, 103 mutated sequences from ten *rad18* subclones, 59 mutated sequences from two *xrcc3* subclones, and 62 mutated sequences from four *rad18/xrcc3* subclones. (C) Pattern of mutation in VL of *rad18/xrcc3* cells. The frequency of each substitution is expressed as a percentage of the total number of unique mutations ($n = 43$), which was derived from four cellular subclones. (D) Suppression of the large-scale V gene rearrangements in *rad18* cells by disruption of XRCC3. The bars indicate the percentage of sIg-negative cells selected from sIg-positive clones with an aberrant or missing rearranged VL1. The wild-type and *rad18* data are reproduced from Fig. 1 for comparison. Numbers of sIg-negative clones analyzed: *xrcc3* clones, 25; and *rad18/xrcc3* clones, 51. WT, wild type.

sensitivity of the *rad18* mutant to UV light is dependent on functional XRCC3. Methyl methanesulfonate (MMS) is a monofunctional DNA alkylating agent that creates replication-stalling base lesions. *xrcc3* also exhibited little sensitivity to MMS, whereas *rad18* was much more sensitive. Again, the sensitivity of *rad18* was suppressed in the double mutant (Fig. 7B). Cisplatin results in the formation of DNA adducts and interstrand cross-links whose repair is more complex, requiring multiple overlapping pathways. Thus, as expected, both RAD18 and XRCC3 contributed significantly to the tolerance

of this agent. Nevertheless, there was some suppression of the sensitivity of *rad18* after disruption of XRCC3. Expression of YFP-hRAD18 completely suppressed the sensitivity of the *rad18* mutant to MMS (Fig. 7D) and UV (33), whereas expression of hRAD18 in the *rad18/xrcc3* mutant had little effect on MMS sensitivity, the residual sensitivity being similar to that of the *xrcc3* single mutant (Fig. 7D). Together, these data suggest that the presence of functional recombination contributes to the sensitivity of *rad18* cells to a range of genotoxic stresses, a point considered further in the discussion below.

FIG. 7. Suppression of DNA damage sensitivity in the *xrc3/rad18* double mutant. Wild type (□), *xrc3* (○), *rad18* (◇), and *rad18/xrc3* (△) cells. (A) Treatment with 265-nm UV light. (B) Treatment with MMS (ppm = parts per million). (C) Treatment with cisplatin. (D) Complementation of MMS sensitivity of *rad18* (◆) and *rad18/xrc3* (▲) cells by human RAD18 (single experiment). WT, wild type.

DISCUSSION

Evidence for a recombination function for RAD18. Here we provide two lines of evidence that, in a vertebrate model, RAD18 plays a role in XRCC3-dependent (and we therefore believe RAD51-dependent) recombination leading to gene conversion. First, *rad18* cells exhibit diminished levels of successful gene conversion in response to an endonuclease-induced double-strand break in a GFP-based reporter system, a form of recombination known to be dependent on XRCC3 (26). Second, *rad18* cells exhibit an increased number of aberrant rearrangements in their immunoglobulin loci, which arise in association with abasic site-induced gene conversion, a process also known to be dependent on XRCC3 (31).

Notably, the effect RAD18 disruption has on recombination is relatively modest compared to the substantial reduction in gene conversion in both the DR-GFP reporter system and the immunoglobulin loci seen in cells mutated for core recombination factors, such as the RAD51 paralogues, BRCA2 and RAD54 (4, 15, 18, 23, 26, 31). Further, although the average gene conversion tract length is reduced, the *rad18* mutant is still capable of creating relatively long tracts. This suggests that the loss of RAD18 increases the chance of gene conversion tract synthesis ending early. Speculatively, the association of decreased tract length and increased duplications and dele-

tions suggests a model in which loss of RAD18 leads to less processive D-loop extension, which may lead to abortion of the recombination reaction and the formation of a new double-strand break. This double-strand break may subsequently become a substrate for further recombinational repair. Such events may be reflected in our recent demonstration that the majority of the elevated SCEs seen in *rad18* DT40 are not dependent on K164 of PCNA, suggesting that RAD18 plays a role independent of PCNA ubiquitination in suppressing SCE (33). In addition, the breaks may be captured by nonhomologous end joining. Supporting this latter conjecture, disruption of Ku70 in the *rad18* mutant suppresses the accumulation of Southern blotting detectable rearrangements of the immunoglobulin light chain loci of DT40 (data not shown).

The exact mechanism by which RAD18 mediates its role in recombination is a subject of current investigation. It seems likely that it will depend on the ubiquitin ligase activity of the protein since a catalytically dead mutant of RAD18, *rad18C28F*, is phenotypically identical to *rad18* (33). However, our preliminary data suggest that gene conversion in a *pcnaK164R* mutant of DT40 is essentially normal and is not associated with aberrant rearrangements. Together with the nonoverlap of SCE phenotypes of the *rad18* and *pcnaK164R* mutants, this suggests that RAD18 may have ubiquitination targets other than PCNA.

Relationship between the role of RAD18 in PRR and HR. To date, interpretation of the phenotype of vertebrate *rad18* mutants has focused on the impact of loss of RAD18 on postreplication repair, with the effects on recombination and the synthetic lethality of *rad18* and *rad54* mutations being explained in terms of channeling of lesions from one pathway to the other (41–43). However, we show clearly that the profound inhibition of RAD51-dependent recombination caused by *xrc3* disruption, far from causing lethality in *rad18* cells, actually reduces their sensitivity to DNA damage. Coupled with our demonstration of a role for RAD18 within the recombination reaction itself, this observation suggests that the absence of RAD18 renders recombination toxic. Does this replace the idea that lesions are channeled to recombination from disabled postreplication repair in the *rad18* mutant? No, it does not, at least not entirely. As discussed above, *pcnaK164R* cells show more damage-inducible SCEs than the wild type but less than the *rad18* mutant (33), suggesting that the abolition of PCNA ubiquitination does result in some increased pressure on recombination pathways. Therefore, we believe that elevated SCE and damage sensitivity of the *rad18* mutant arise from two sources: first from disabled postreplication repair and second from the the channeling of lesions to homologous recombination, which has itself also been compromised by the disruption of RAD18. We suggest that when XRCC3 is disrupted, the early block to RAD51-dependent recombination alleviates the recombination-dependent component of the sensitivity of *rad18* by preventing the reaction occurring. We suggest that the inviability of the *rad18/rad54* double mutant may be explained by the inability of other pathways to rescue recombination in the *rad54* mutant, exacerbating the recombination and PRR defect of the *rad18* mutant to the point that the cells are inviable.

A key question remaining is which pathways are used in the absence of both RAD18 and XRCC3. Existing data already

point to two possibilities. In the case of double-strand breaks, RAD52-dependent single-strand annealing may be an alternative (36, 38): RAD52 is able to partially substitute for XRCC3, since although vertebrate *rad52* mutants display almost no phenotype, a concurrent mutation of XRCC3 and RAD52 in DT40 is inviable (16). For single-stranded lesions, such as the abasic sites that initiate immunoglobulin gene conversion, REV1-dependent translesion synthesis is able to substitute for XRCC3-dependent recombination (31, 34). It therefore seems likely that for overall cellular survival of the *rad18* mutant in response to complex DNA damage, a combination of backup pathways is used.

ACKNOWLEDGMENTS

We thank Shunichi Takeda for providing the *rad18* targeting constructs and communicating data prior to publication, Javier di Noia for the DTDR17 cells, Kevin Hiom for the *brca1*-DTDR17 cells, and members of the lab for discussion and comments on the manuscript. D.S. was funded by the Leukemia Research Fund.

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