Assessment of Anti-recombination and Double-strand Break-induced Gene Conversion in Human Cells by a Chromosomal Reporter

Received for publication, February 10, 2012, and in revised form, June 25, 2012. Published, JBC Papers in Press, July 7, 2012, DOI 10.1074/jbc.M112.352302

Keqian Xu, Xiling Wu, Joshua D. Tompkins, and Chengtao Her

From the School of Molecular Biosciences, Washington State University, Pullman, Washington 99164-7520

Background: DSB repair is frequently associated with gene conversion.

Results: Gene conversions at the site of a DSB and its surrounding regions are regulated differently.

Conclusion: hMSH2, hMLH1, and hMRE11 play different roles in proximal and distal gene conversions.

Significance: Delineating the mechanisms underlying DSB-induced gene conversion will help to decipher how mutations in DSB repair genes affect genome stability.

Gene conversion is one of the frequent end results of homologous recombination, and it often underlies the inactivation of tumor suppressor genes in cancer cells. Here, we have developed an integrated assay system that allows simultaneous examination of double-strand break (DSB)-induced gene conversion events at the site of a DSB (proximal region) and at a surrounding region ~1 kb away from the break (distal region). Utilizing this assay system, we find that gene conversion events at the proximal and distal regions are relatively independent of one another. The results also indicate that synthesis-dependent strand annealing (SDSA) plays a major role in DSB-induced gene conversion. In addition, our current study has demonstrated that hMLH1 plays an essential role in anti-recombination and gene conversion. Specifically, the anti-recombination activity of hMLH1 is partially dependent on its interaction with hMRE11. Our data suggests that the role of hMLH1 and hMRE11 in the process of gene conversion is complex, and these proteins play different roles in DSB-induced proximal and distal gene conversions. In particular, the involvement of hMLH1 and hMRE11 in the distal gene conversion requires both hMSH2 and heteroduplex formation.

DSBs are commonly viewed as the most deleterious lesions in DNA, and aberrant DSB repair represents an important risk factor for cancer development in humans (1). Despite this, active induction of DSBs in cancer cells is often seen and considered to be a major underlying mechanism for many genotoxic anti-cancer drugs. The HR pathway carries out high-fidelity DSB repair, during which it changes the sequence of recipient DNA to that of the homologous donor, leading to gene conversion. This happens not only at the site of DSB but also in surrounding regions due to the formation of heteroduplex DNA through single strand invasion, repair synthesis, or branch migration (2, 3). However, studies of such events in human cells are often limited by the intrinsic complexity of gene conversion processes.

As a direct consequence of DSB repair, gene conversion can be ascribed to two main mechanisms, namely synthesis-dependent strand annealing (SDSA) (4) and the classic double-Holliday junction repair (DSBR) model (5). SDSA, which is commonly accepted as a critical alternative pathway in homology-directed DSB repair (4), deviates from the classic DSBR model at several steps of the recombination process. Specifically, SDSA predicts that only one end of a DSB needs to invade the template DNA (i.e. donor copy), leading to subsequent DNA repair synthesis and branch migration. The invading strand with an extended single-strand 3’-end, later separated and released from the template strand, is able to bridge the gap by annealing to the opposite side of the DSB (4). In contrast, the DSBR model, proposed by Szostak and colleagues (5), predicts that both ends of a DSB need to invade the homologous template DNA at the beginning of the process. This leads to the formation of a double Holliday junction that undergoes branch migration and subsequent formation of non-crossover and crossover products.

Evidently, gene conversion at the site of a DSB requires gap repair if the formation of DSB causes nucleotide deletions. At regions surrounding a DSB, gene conversion generally occurs as a result of heteroduplex processing by the mismatch repair (MMR) pathway. A number of studies have demonstrated that gene conversion tracts can range from several hundred base pairs to 11.2 kb in mammalian cells (3, 6, 7). This underscores a necessity for a better understanding of how cells regulate gene conversions at various regions in reference to the locations of DSBs. In addition, gene conversion has been increasingly recognized as an important cause of many inherited human diseases (8), such as the inactivation of MMR genes in Lynch syndrome patients (9, 10). Although the detailed molecular mechanisms underlying the regulation of gene conversion in human cells remain to be revealed (11), it is known that the...
MMR pathway plays an essential role in restricting the formation of heteroduplexes in a mismatch-dependent fashion (12). Indeed, the effect of MMR-dependent suppression on recombination increases with sequence divergence, and MMR has little, if any, effect on recombination between two identical sequences (13–15). Recent evidence, however, has suggested that individual MMR proteins can also affect recombination frequency in a mismatch-independent manner. For example, it is reported that, although Msh2 deficiency has no significant effect on meiotic recombination (16), suppression of HR in mouse fibroblasts could be alleviated by the loss of Mlh1 (17). In addition, Turkier and co-workers (18) observed high-frequency recombination in mouse fibroblasts could be alleviated by the loss of Mlh1 (17). In addition, Turkier and co-workers (18) observed high-frequency recombination in mouse fibroblasts could be alleviated by the loss of Mlh1 (17).

Construction of pMMR-IR3 Gene Conversion Locus—The RFP and GFP ORFs, as well as the internal ribosomal entry site, were derived originally from plasmid vectors, pIRES2-EGFP and pDsRed2-N1 (Clontech, Palo Alto, CA). The 18-bp I-SceI restriction site was inserted between RFP codons 134 and 144, leading to a concomitant deletion of 26 nucleotides. The cre-recombination activity independent of MMR. Furthermore, this role of hMLH1 did not rely on its binding proteins, BLM and FANCJ, and was independent of ATM signaling (20). Clearly, the molecular mechanism underlying the role of hMLH1 in the regulation of recombination is hitherto elusive.

To explore the molecular basis underlying the anti-recombination activity of hMLH1, as well as its correlation with gene conversion in human cells, we developed a reporter system that is capable of detecting DSB-induced proximal and distal gene conversions simultaneously in living cells. Studies performed with this system demonstrated that hMLH1 partially relied on its interaction with hMRE11 to exert an anti-recombination activity, whereas hMRE11 played a dual role in the regulation of recombination.

EXPERIMENTAL PROCEDURES

DNA Cloning and Sequencing—Genomic DNA was isolated from all relevant cell preparations using the Blood and Cell Culture DNA mini kit (Qiagen, Inc., Valencia, CA). The recipient and donor copies of the reporter sequence were amplified with primers specifically targeting unique sequences flanking the recipient and donor copies (supplemental Table S1), except that GFP ORFs from no-color and GFP+ cells were directly amplified from both recipient and donor copies. PCR products were cloned into vectors pcDNA6 or pGBKT7 (Clontech). Individual clones were sequenced to reveal alterations within RFP and GFP coding regions.

FACS Sorting and Analysis—Cells expressing different fluorescent proteins were separated and collected by the use of a fluorescence-activated cell sorter (FACS Vantage, Becton Dickinson, Franklin Lakes, NJ). Cultures were maintained in DMEM (Invitrogen) containing 10% FBS (Biomedia, Foster City, CA) and antibiotic-antimycotic (Invitrogen). The reporter construct pMMR-IR3, as well as pR-G-Rec2/IR, was transfected into 293TLα cells by the standard calcium phosphate procedure, and stable transfec-
encoding construct, pCMV(I-SceI)x3xNLS, cells were treated with 1% formaldehyde to crosslink protein to DNA. Chromatin fragmentation was carried out by sonication performed with a Branson Sonifier 250 (Branson Ultrasonics, Danbury, CT). ChIP analysis was conducted according to the manufacturer’s recommendations (EZ ChIP kit) with 5 μg each of hMRE11, hMLH1, and hMSH2 (Calbiochem, EMD, San Diego, CA). Controls for ChIP analysis were performed with rabbit IgG and acetyl histone H3 (Upstate). Immunoprecipitated DNA was used to perform PCR analysis in triplicates for quantifying protein enrichments on chromatin. Specifically, PCR reactions were performed with primers designed to amplify regions 303 to 57 (proximal), 57–338 (proximal), 438–697 (intermediate), and 814–1037 (distal) on pMMR-IR3 or pRG-Rec2/IR loci (supplemental Table S1).

RESULTS AND DISCUSSION

In Vivo DSB-induced Gene Conversion Assay System—To analyze in vivo gene conversion events, the pMMR-IR3 locus was stably integrated into 293TL (hMLH1 Tet-off) cells. PCR analysis of genomic DNA from this reporter cell line indicated that the relevant regions of the reporter locus were in its original configuration (data not shown). The rationale of this in vivo system is founded upon the principle that DSB repair is frequently associated with gene conversions at the site of DNA strand breaks and at distal regions that could form DSB-induced heteroduplex intermediates. The latter is resulted from the repair of mismatched nucleotides in the heteroduplex region by the MMR pathway (15). In comparison with the other fluorescent protein-based systems used in recombination analysis (11), the uniqueness of this reporter system stems from its ability to allow simultaneous detection of both proximal (short tract) and distal (long tract) gene conversion events triggered by a single DSB within the reporter locus in live cells.

For DSB induction in the genome of the reporter cell line, we have adapted the well established rare-cutting restriction endonuclease I-SceI system (22, 25, 26), which has been widely used in studies of DSB repair in vertebrate cells. The locus for analyzing DSB-induced gene conversion, designated as pMMR-IR3 hereafter, comprises a recipient and a donor copy (Fig. 1A). The recipient copy consists of RFP (I-SceI) and GFP (Stop 27), whereas the donor copy possesses RFP (439A>T) and GFP (Stop 42). On both copies, an internal ribosomal entry site (IRES) resides in between the mutant RFP and GFP ORFs (Fig. 1A). Although a functional CMV promoter precedes the recipient copy, no functional RFP or GFP proteins can be produced from this reporter. This is due to the replacement of a 26-bp segment of the RFP ORF by an 18-bp I-SceI recognition site, as well as the introduction of an internal stop codon within the GFP coding sequence. Specifically, a C3TT change was made at GFP codon 27 to introduce a stop codon, thus preventing GFP expression from the recipient copy (Fig. 1). Located 1973-bp downstream from the recipient copy is the promoterless donor sequence orientated as an inverted repeat (Fig. 1A). The recipient copy consists of RFP (I-SceI) and GFP (Stop 27), whereas the donor copy possesses RFP (439A→T) and GFP (Stop 42). On both copies, an internal ribosomal entry site (IRES) resides in between the mutant RFP and GFP ORFs (Fig. 1A). Although a functional CMV promoter precedes the recipient copy, no functional RFP or GFP proteins can be produced from this reporter. This is due to the replacement of a 26-bp segment of the RFP ORF by an 18-bp I-SceI recognition site, as well as the introduction of an internal stop codon within the GFP coding sequence. Specifically, a C→TT change was made at GFP codon 27 to introduce a stop codon, thus preventing GFP expression from the recipient copy (Fig. 1). Located 1973-bp downstream from the recipient copy is the promoterless donor sequence orientated as an inverted repeat (Fig. 1A). The recipient copy consists of RFP (I-SceI) and GFP (Stop 27), whereas the donor copy possesses RFP (439A→T) and GFP (Stop 42). On both copies, an internal ribosomal entry site (IRES) resides in between the mutant RFP and GFP ORFs (Fig. 1A). Although a functional CMV promoter precedes the recipient copy, no functional RFP or GFP proteins can be produced from this reporter. This is due to the replacement of a 26-bp segment of the RFP ORF by an 18-bp I-SceI recognition site, as well as the introduction of an internal stop codon within the GFP coding sequence. Specifically, a C→TT change was made at GFP codon 27 to introduce a stop codon, thus preventing GFP expression from the recipient copy (Fig. 1). Located 1973-bp downstream from the recipient copy is the promoterless donor sequence orientated as an inverted repeat (Fig. 1A).
which the Thr^{147} to Ser mutation causes significant reduction of RFP protein expression and dramatically decreases the fluorescence intensity of the mutant RFP protein (supplemental Fig. S1). The location of 439A→T alteration on the donor copy corresponds to the 11th nucleotide downstream of the I-SceI site on the recipient RFP copy (Fig. 1A). Therefore, the location of the original recipient and donor RFP copies after gene conversion can be readily traced by this mutation. In addition, a C→TT change was also made on the donor copy of GFP to create an internal TAA stop at GFP codon 42 (Fig. 1).

DSB induction by I-SceI expression initiates gene conversion at both the proximal and distal regions, which leads to the corresponding expression of RFP and/or GFP proteins (Fig. 2A). Gene conversion at the proximal region (RFP expression) has to be fulfilled by gap repair due to the deletion of 26 nucleotides in the first copy of RFP (Fig. 1A). It is likely that the distal gene conversion would require the formation of heteroduplex intermediates from both copies of GFP ORFs and the subsequent heteroduplex repair (Fig. 1B). In this design, at either end of the DSB, the terminal non-homologous sequence at the 3′-end of
the invading strand has to be removed before DNA synthesis can occur. Based on both SDSA and DSBR models (4, 5), the proximal DSB repair can lead to the formation of T/T mismatch at RFP codon 147. However, it is known that the T/T mismatch is poorly recognized and repaired by MMR (28, 29). Conversely, the GFP heteroduplex region harbors insertion/deletion loops of one extra-helical nucleotide adjacent to either a G/T or a C/A mismatch at GFP codons 27 and 42 (Fig. 1B). These insertion/deletion loops are preferred MMR substrates (30–32). Evidently, I-SceI expression in this reporter cell line could lead to the generation of RFP + and/or GFP + cells, reflecting proximal and distal gene conversion events, respectively (Fig. 2A). Intriguingly, the expression of GFP and GFP proteins following I-SceI-induced DSB was largely discordant, with only a small fraction of cells expressing both GFP and RFP (Fig. 2).

**hMLH1 Differentially Regulates Proximal and Distal Gene Conversions**—To investigate whether proximal and distal gene conversions were under differential regulation, we first addressed the effects of hMLH1 on DSB-induced gene conversion. The expression of hMLH1 in 293TLα/pMMR-IR3 cells was suppressed by doxycycline to a level that was below the immunoblotting detection limit (supplemental Fig. S2). To initiate DSB-induced gene conversion, I-SceI was transiently expressed in 293TLα/pMMR-IR3 cells to introduce a defined DSB on the recipient copy (Fig. 1A). DSB-induced proximal and distal gene conversion events were monitored by the appearance of RFP + and/or GFP + cells through FACS analysis (Fig. 2A).

As shown in Fig. 2B, hMLH1 deficiency led to a large increase in the numbers of I-SceI-induced RFP + cells and a moderate increase of GFP + cells. This observation suggests that hMLH1 has a strong inhibitory effect on proximal gene conversion in comparison to distal gene conversion. The different effects of hMLH1 deficiency on DSB-induced proximal and distal gene conversions can be measured by the ratio of GFP + to RFP + cells, which changed from 0.83 to 0.44 when hMLH1 was silenced (Fig. 2C). The occurrence of distal gene conversion in the absence of hMLH1 could suggest the existence of hMLH1-independent heteroduplex repair activity. Consistent with this view, *in vitro* MMR reconstitution assays demonstrated that only 3′- to 5′-directed MMR reactions required hMLH1 (33, 34).

**hMLH1 Partially Relies on hMRE11 to Exert Its Anti-recombination Activity**—It is known that hMLH1 acts together with hMRE11 in anti-recombination, an important role for hMRE11 in non-homologous end-joining (NHEJ) (23). Although to a lesser extent than hMLH1 deficient cell, expression of hMRE11 452–634 led to a significant increase in RFP + cells (Fig. 2B), and a reduction of the GFP/RFP ratio to a similar level elicited by hMLH1 deficiency (Fig. 2C). Similar results were also obtained with hMRE11 silencing (Fig. 2B). These observations suggest that hMLH1 partially relies on its interaction with hMRE11 to exert anti-recombination activity.

The reduction of the GFP/RFP ratio is indicative of a compromised heteroduplex repair at the distal region. Because the hMLH1-hMRE11 interaction is involved in 3′- to 5′- MMR and hMLH1 is only required for 3′- to 5′ but not 5′- to 3′ MMR (23, 24, 33, 43), the ~40% reduction of GFP/RFP ratio is compatible with the idea that MMR could act in the process of heteroduplex repair at the distal region. Consistent with the essential role of hMRE11 in the early steps of DSB repair (44), RNAi-mediated hMRE11 knockdown on the hMLH1-deficient background partially diminished the promoting effects of hMLH1 deficiency on recombination without significantly changing the GFP/RFP ratio (Fig. 2C).

Together, our data suggest that hMRE11 plays a dual role in the process of proximal gene conversion, exerting both a promoting and a suppressing effect. Specifically, the promoting effect is reflected by the reduction of gene conversion when hMRE11 was silenced on the hMLH1-deficient background (Fig. 2B). The suppressing effect of hMRE11 on gene conversion is inferred from the observation that disruption of hMLH1-hMRE11 interaction or silencing of hMRE11 could lead to a significant increase of gene conversion at both proximal and distal regions (Fig. 2B). Intuitively, the proximal and distal gene conversion events are coordinated by the initiation of DSB processing. However, the present data suggests that the proximal and distal gene conversions are regulated differently. It is of note that both hMLH1 and hMRE11 were reported previously to promote deletional NHEJ, and deficiency of either gene lessened NHEJ activities (42, 45). Therefore, additional work is needed to test whether deficiency of hMLH1-hMRE11 could also play an indirect role in shifting the balance between HR and NHEJ.

**Characteristics of DSB-induced Gene Conversion**—To further investigate the molecular nature of these gene conversion events, DNA sequencing analysis of pMMR-IR3 genomic locus from I-SceI-transfected 293TLα/pMMR-IR3 cells was performed to validate corresponding repair events. As illustrated in Fig. 1A, each of the four RFP or GFP ORFs contains a unique sequence alteration. Thus, the locations of these four sequence alterations could be identified after DSB-induced gene conversion events. Specifically, recipient and donor copies of the reporter locus in cells expressing RFP-and/or GFP (i.e. RFP +, GFP +, or RFP +/GFP +), as well as cells expressing no fluorescent proteins (no-color) were PCR-amplified with primers that could distinguish between recipient and donor copies (supplemental Table S1). Because the “functional” GFP protein can only be produced from the wild type GFP sequence located on the recipient copy, the GFP coding sequences on both the recipient and the donor copies were simultaneously amplified and analyzed in no-color and GFP + cells. Amplified PCR fragments were cloned, and the portions containing RFP and GFP ORFs were sequenced.
The results of 106 DNA sequencing reactions were provided in Table 1, in which the numbers represent the number of times that a given sequence variation was identified on either recipient or donor copies. Because the number of sequencing reactions for each cell population is relatively small, thereby only qualitative analysis was performed on the sequencing data included in Table 1. For instance, in RFP+ cells, RFP ORF on the recipient copy was sequenced from four different clones, which represented two RFP (I-SceI) and two wild type RFP sequences. On the other hand, the GFP ORF was sequenced from five different clones, which were all GFP (Stop 27) (Table 1). In addition, on the donor copy of RFP+ cells, seven GFP and four GFP ORF clones were sequenced, and they represented seven GFP (439A→T) and four GFP (Stop 42) sequences (Table 1). As a whole, the information summarized in Table 1 indicates that the RFP expression was indeed due to recombinational repair of I-SceI-induced DSB using the donor RFP ORF as a template. The GFP expression was attributable to elimination of the internal stop codon within the GFP ORF on the recipient copy, implicating MMR actions on heteroduplex intermediates. It is of note that, in no-color cells, neither recipient nor donor RFP ORFs were altered (Table 1), reflecting the lack of I-SceI cleavage or repair by precise NHEJ of I-SceI-induced DSB on the recipient copy. Although sequence analysis confirmed that the two GFP ORFs in no-color cells contained corresponding internal stop codons 27 and 42 (Table 1), there was one wild type GFP ORF and one with both stop codons among a total of 14 sequenced GFP ORFs (Table 1). As illustrated in Fig. 1A, GFP can only be expressed when the wild type GFP ORF resides on the recipient copy. Therefore, if no-color cells harbor a wild type GFP sequence, it has to be on the donor copy.

Sequence analysis of pMMR-IR3 genomic loci from partially purified RFP+, GFP+, and RFP+/GFP+ cells indicated that the expression of RFP was due to the generation of wild type RFP ORF by DSB-induced proximal gene conversion, and the appearance of GFP+ cells resulted from distal gene conversion. In particular, wild type RFP ORFs were only identified from the recipient copy at the pMMR-IR3 locus in RFP+ cells. As for GFP+ cells, besides wild type GFP ORFs, sequencing analysis has revealed that the recipient GFP ORFs contained either the 439A→T alteration or a deletion (Table 1 and supplemental Fig. S4). This deletion, identified only in a fraction of GFP+ cells, extended for 256-bp from the site of I-SceI cleavage to the last RFP codon on the recipient copy (Table 1 and supplemental Fig. S4). Examination of the sequence of the deletion products provided no evidence for the presence of microhomology at the joint. Consistent with a previous report (46), this observation indicated that deletional NHEJ-mediated DSB repair at the proximal region could be associated with distal gene conversion. In other words, NHEJ at proximal region could associate with HR at the distal region. In addition, as expected, the recipient copy at the pMMR-IR3 locus from RFP+/GFP+ cells contained both wild type RFP and wild type GFP ORFs (Table 1).

It is conceivable that DSB-induced gene conversion at the proximal and distal regions can be carried out by either SDSA or DSBR pathway (Fig. 3). In SDSA, right-end invasion directly repairs the DSB, leading to a high likelihood of expression of RFP, whereas the production of both RFP and GFP by means of heteroduplex DNA repair can be readily achieved by left-end invasion (Fig. 3). A hallmark of SDSA in this case is that the donor copy will be in its original configuration after gene conversion, e.g. the presence of both 439A→T (RFP) and Stop 42 (GFP) sequence markers on the donor copy. In contrast, DSBR could contribute to both proximal and distal gene conversions in a more complicated fashion. It is generally initiated by strand invasions from both ends of the concerned DSB. It is then followed by repair synthesis, branch migration, heteroduplex repair, and Holliday junction resolution (Fig. 3). Therefore, DSBR is expected to lead to a frequent dissociation of 439A→T (RFP) and Stop 42 (GFP) sequence markers on the donor copy (Fig. 3). To this end, our results of DNA sequencing and FACS analyses support a scenario by which SDSA played a predominant role in controlling gene conversion events (Table 1 and Fig. 3). The key supporting evidence for this view is derived from the observation that the vast majority of the donor copies in RFP+, GFP+, RFP+/GFP+, and no-color cells comprise both RFP (439A→T) and GFP (Stop 42) (Table 1). The relatively abundant appearance of RFP+ cells in comparison with GFP+ cells as well as the low quantity of cells positive for both RFP and GFP are also compatible with SDSA being a major mechanism. This is because SDSA always associates with a higher level of DSB-induced RFP expression during right-end invasion despite

### TABLE 1

**Sequence analysis of RFP and GFP coding regions on recipient and donor copies**

Sequence analysis of RFP and GFP coding regions resided on recipient and donor copies from no-color, RFP-positive, GFP-positive cells, as well as cells positive for both RFP and GFP. Coding regions of RFP and GFP from different population of cells were amplified with specific PCR primers (see supplemental Table S1) and were cloned accordingly. DNA sequencing reactions were performed on selected clones. The numbers in this table represent the number of times that a given sequence marker was identified by DNA sequencing.

|        | RFP (I-SceI) | RFP (439A→T) | GFP (stop 27) | GFP (stop 42) | RFP (WT) | RFP (deletion) | GFP (WT) | GFP (two stops) |
|--------|-------------|--------------|---------------|---------------|----------|----------------|----------|-----------------|
| No-color |             |              |               |               |          |                |          |                 |
| Recipient | 8           | 5            | 5             | 7             | 1        |                |          |                 |
| Donor    | 6           | 6            | 3             | 4             | 2        |                |          |                 |
| RFP      |             |              |               |               |          |                |          |                 |
| Recipient | 2           | 5            | 7             | 4             | 2        |                |          |                 |
| Donor    | 7           | 7            | 9             | 9             | 4        |                |          |                 |
| GFP      |             |              |               |               |          |                |          |                 |
| Recipient | 7           | 4            | 8             | 4             | 2        |                |          |                 |
| Donor    | 7           | 5            | 9             | 9             | 5        |                |          |                 |
| RFP and GFP |           |              |               |               |          |                |          |                 |
| Recipient | 2           | 5            | 4             | 4             | 2        |                |          |                 |
| Donor    | 5           | 5            | 5             | 5             | 5        |                |          |                 |
the status of 3′-to-5′ MMR (Fig. 3). In the case of left-end invasion, 3′-to-5′ MMR will likely compromise RFP and promote GFP expression. Conversely, inactivation of 3′-to-5′ MMR likely promotes RFP and decreases GFP expression (Fig. 3). Moreover, the aforementioned RFP deletion in GFP cells can also be explained by an excessive processing of the recipient strand and looping out a portion of the invading strand in the annealed intermediate (left-end invasion in SDSA). Although the involvement of DSBR could not be excluded, PCR analysis of pMMR-IR3 loci in RFP, GFP, RFP/GFP, and no-color cells yielded no evidence for the occurrence of crossover recombination, which would only occur in the process of DSBR (Fig. 1A; data not shown).

FIGURE 3. Schematic illustration of I-SceI induced DSB formation and the subsequent generation of RFP+ and GFP+ cells via proximal and distal gene conversions. I-SceI is used to generate a unique DSB on the recipient copy of the RFP coding sequence, whereas the donor copy encodes a mutant RFP sequence, RFP (439A→T). The two major recombinational DSB repair pathways (SDSA and DSBR) and the corresponding consequences of DSB repair are schematically presented.

Expression of RFP from the recipient copy is dependent on DNA replication and subsequent segregation. 3′-to-5′ MMR is expected to have a negative effect on the expression of RFP, while GFP expression requires the repair of heteroduplex at GFP Stop 42 (preferred MMR substrate) by 3′-to-5′ MMR. GFP expression can also occur when DNA repair synthesis is terminated in between the two GFP stop codons. No separation of RFP A439T and GFP Stop 42 on the donor copy.

DSB-induced hMLH1 and hMRE11 Protein Loading at Proximal and Distal Regions is Differently Regulated by hMSH2—To directly analyze the roles of hMLH1 and hMRE11 in gene conversion, ChIP analysis was utilized to investigate DSB-induced loading of these proteins at the proximal and distal regions on the pMMR-IR3 locus (Fig. 4A). The protein loading patterns at the two proximal regions located on either side of the DSB were very much alike (Fig. 4, B and C), except that hMSH2 was slightly more enriched at the proximal region 57–338. Although comparable levels of hMLH1 and hMRE11 were found at proximal and distal regions, hMSH2 loading at the distal region was much greater than that at the proximal region (Fig. 4C). To validate that the loading of hMLH1-hMRE11 at
the distal region was associated with hMSH2-mediated heteroduplex binding, the effect of hMSH2 on DSB-triggered hMLH1-hMRE11 loading was examined. RNAi-mediated hMSH2 knockdown (supplemental Fig. S2) resulted in ∼50% reduction of hMLH1 and a lesser reduction on hMRE11 loading at the proximal region, but hMSH2 knockdown caused a significant reduction of both hMLH1 and hMRE11 proteins at the distal region (Fig. 4C), suggesting that DSB-induced loading of hMLH1-hMRE11 at proximal and distal regions is differently regulated by hMSH2. However, the effect of hMSH2 at the proximal region is complex, as silencing of hMSH2 led to a ∼50% reduction of gene conversion events at both proximal and distal regions (data not shown). It is likely that, at the proximal region, hMSH2 is also required for efficient removal of single-stranded nonhomologous 3′-ends before strand invasion (47, 48). Furthermore, DSB-triggered chromatin recruitment of hMSH2, hMLH1, and hMRE11 was relatively specific for the proximal and distal regions, as only low levels of protein loading were detected at a region located between the proximal and distal regions (Fig. 4C). Collectively, these results demonstrate that, at the distal region, hMLH1-hMRE11 mainly functions with hMSH2 in the process of heteroduplex repair. Conversely, at the proximal region, only a fraction of DSB-induced loading of hMLH1 and hMRE11 is dependent on hMSH2.

**DSB-induced Protein Recruitment at Distal Region Is Dependent on Heteroduplex Formation**—To further validate that the role of hMSH2 on DSB-induced hMLH1 and hMRE11 loading at the distal region is dependent on the formation of heteroduplex DNA, ChIP analysis was performed with a different substrate, pRG-Rec2/IR, that could not form a DSB-triggered distal heteroduplex. The configuration of pRG-Rec2/IR locus is identical to pMMR-IR3 except that both recipient and donor copies of pRG-Rec2/IR contain wild type GFP ORFs, and the RFP (439A→T) on the donor copy is also replaced by the wild type RFP sequence.
Although the patterns of DSB-induced hMSH2, hMLH1, and hMRE11 loading to the proximal regions (57–338) on pMMR-IR3 and pRG-Rec2/IR loci were very similar, the results of ChIP assay indicated that their loadings to the distal region (814–1037) were very different (Fig. 5). As shown in Fig. 5A, DSB induction triggered significant recruitments of hMSH2, hMLH1, and hMRE11 to the distal region (814–1037) on the pMMR-IR3 locus. However, in the absence of GFP (Stop 27) and GFP (Stop 42), hMSH2, hMLH1, and hMRE11 were not significantly recruited to the distal region 814–1037 after DSB induction (Fig. 5B). These findings are consistent with the view that DSB-triggered hMLH1 and hMRE11 recruitments to the distal region on the pMMR-IR3 locus require both hMSH2 and the heteroduplex formation, thereby supporting a role for hMLH1-hMRE11 in the process of distal gene conversion.

The slightly elevated levels of hMSH2 and hMLH1 loading at the proximal region on the pMMR-IR3 locus (in comparison to that of pRG-Rec2/IR) could be due to the formation of the T/T (RFP 439A→T) heteroduplex (Fig. 5). Together, the results of our study are consistent with a regulatory role of hMLH1 in the process of recombination. The observed hMLH1 loading at the proximal region of DSB (at both pMMR-IR3 and pRG-Rec2/IR loci) likely underlies its anti-recombination property. Because the hMRE11 complex is among the first to arrive at the site of DSBs (49, 50), it is conceivable that the loading of hMLH1 to chromatin is at least partially regulated through the interaction with hMRE11.

In summary, the generation of this versatile DSB-induced gene conversion system has allowed us to study in vivo proximal and distal gene conversion at a defined chromosomal locus in human cells. Our analysis indicated that DSB-induced gene conversions at both the proximal and distal regions are mainly mediated by SDSA. Specifically, we demonstrate that hMLH1 exerts an hMRE11-dependent anti-recombination activity. The observed coupling of proximal deletional NHEJ with distal gene conversion on the same DSB substrate suggests that NHEJ and HR can function cooperatively in the process of DSB repair. This observation is consistent with a previous study showing that DSB repair event could be initiated by HR and completed by NHEJ (46). Our results and the reported role of hMRE11 in deletional NHEJ (42) warrant future studies to determine the precise function of hMLH1-hMRE11 in the regulation of NHEJ and HR. Furthermore, the pMMR-IR3 locus may provide a powerful means to elucidate the effects of chromatin remodeling on in vivo MMR. The potential existence of a functional interaction between nucleosomes and MMR proteins during the process of mismatch removal has been recently suggested (51, 52). Finally, it is likely that some Lynch syndrome-associated hMLH1 mutations may disrupt the function of hMLH1, e.g. through disruption of the hMLH1-
Anti-recombination and Gene Conversion

hMRE11 interaction, in anti-recombination and gene conversion, thereby increasing genomic instability to a level that promotes cancer development.

Acknowledgments—We thank Fengxue Zhu for technical assistance in generating the reporter construct and Caron Glotzbach and Lisa Schaefer for assistance in the fluorescence in situ hybridization analysis.

REFERENCES

1. O’Driscoll, M., and Jeggo, P. A. (2006) The role of double-strand break repair: Insights from human genetics. Nat. Rev. Genet. 7, 45–54
2. Kirkpatrick, D. T., Dominski, M., and Petes, T. D. (1998) Conversion-type and restoration-type repair of DNA mismatches formed during meiotic recombination in Saccharomyces cerevisiae. Genetics 149, 1693–1705
3. Papadaki, M. N., and Patrinos, G. P. (1999) Contribution of gene conversion in the evolution of the β-like globin gene family. Hum. Genet. 104, 117–125
4. Helleday, T., Jo, L., van Gent, D. C., and Engelward, B. P. (2007) DNA double-strand break repair: From mechanistic understanding to cancer treatment. DNA Repair 6, 923–935
5. Szostak, J. W., Orr-Weaver, T. L., Rothstein, R. J., and Stahl, F. W. (1983) The double-strand-break repair model for recombination. Cell 33, 25–35
6. Neuwirth, E. A., Honma, M., and Grosovsky, A. J. (2007) Interchromosomal crossover in human cells is associated with long gene conversion tracts. Mol. Cell. Biol. 27, 5261–5274
7. Bosch, E., Hurles, M. E., Navarro, A., and Jobling, M. A. (2004) Dynamics of a human interparalog gene conversion hotspot. Genome Res. 14, 835–844
8. Chen, J. M., Cooper, D. N., Chuzhanova, N., Férec, C., and Patrinos, G. P. (2007) Gene conversion: Mechanisms, evolution, and human disease. Nat. Rev. Genet. 8, 762–775
9. Zhang, J., Lindroos, A., Ollila, S., Russell, A., Marra, G., Mueller, H., Peltonaki, P., Plaslová, M., and Heinimann, K. (2006) Gene conversion is a frequent mechanism of inactivation of the wild-type allele in cancers from MLH1/MSH2 deletion carriers. Cancer Res. 66, 659–664
10. Auclair, J., Desseigne, F., Lasset, C., Saurin, J. C., Joly, M. O., Pinson, S., Xu, X. L., Monntgain, G., Ruano, E., Navarro, C., Puiseux, A., and Wang, Q. (2007) Novel biallelic mutations in MSH6 and PMS2 genes: Gene conversion as a likely cause of PMS2 gene inactivation. Hum. Mutat. 28, 1084–1090
11. Moynahan, M. E., and Jasin, M. (2010) Mitotic homologous recombination maintains genomic stability and suppresses tumorigenesis. Nat. Rev. Mol. Cell Biol. 11, 196–207
12. Surtees, J. A., Argueso, J. L., and Alani, E. (2004) Mismatch repair proteins: Key regulators of genetic recombination. Cytogenet Genome Res. 107, 146–159
13. Chen, W., and Links-Robertson, S. (1999) The role of the mismatch repair machinery in regulating mitotic and meiotic recombination between diverged sequences in yeast. Genetics 151, 1299–1313
14. Datta, A., Hendrix, M., Lipsitch, M., and Links-Robertson, S. (1997) Dual roles for DNA sequence identity and the mismatch repair system in the regulation of mitotic crossing-over in yeast. Proc. Natl. Acad. Sci. U.S.A. 94, 9757–9762
15. Harfe, B. D., and Links-Robertson, S. (2000) DNA mismatch repair and genetic instability. Annu. Rev. Genet. 34, 359–399
16. Qin, J., Baker, S., Te Riele, H., Liskay, R. M., and Arneheim, N. (2002) Evidence for the lack of mismatch repair-directed antirecombination during mouse meiosis. J. Hered. 93, 201–205
17. Chao, C., Deng, L., Chen, Y., Kucherlapati, R., Stambrook, P. J., and Tischfield, J. A. (2004) Mlh1 mediates tissue-specific regulation of mitotic recombination. Oncogene 23, 9017–9024
18. Wang, Q., Pononmavaara, O. N., Lasarev, M., and Turker, M. S. (2006) High frequency induction of mitotic recombination by ionizing radiation in Mlh1 null mouse cells. Mutat. Res. 594, 189–198
19. Yan, T., Seo, Y., and Kinsella, T. J. (2009) Differential cellular responses to prolonged LDR-IR in MLH1-proficient and MLH1-deficient colorectal cancer HCT116 cells. Clin. Cancer Res. 15, 6912–6920
20. Siehler, S. Y., Schrauder, M., Gerischer, U., Cantor, S., Marra, G., and Wiesmüller, L. (2009) Human MutL complexes monitor homologous recombination independently of mismatch repair. DNA Repair 8, 242–252
21. Czep, P., Stojic, L., Mojas, N., Russell, A. M., Heinimann, K., Canna, M. D., di Pietro, M., Marra, G., and Jiricny, J. (2003) Methylation-induced G/M arrest requires a full complement of the mismatch repair protein hMLH1. EMBO J. 22, 2245–2254
22. Tagrion, D. G., and Nickoloff, J. A. (1997) Chromosomal double-strand breaks induce gene conversion at high frequency in mammalian cells. Mol. Cell. Biol. 17, 6386–6393
23. Zhao, N., Zhu, F., Yuan, F., Haack, A. K., Fujishige, S., Gu, L., and Her, C. (2008) The interplay between hMLH1 and hMRE11: Role in MMR and the effect of hMLH1 mutations. Biochem. Biophys. Res. Commun. 370, 338–343
24. Vo, A. T., Zhu, F., Wu, X., Yuan, F., Gao, Y., Gu, L., Li, G. M., Lee, T. H., and Her, C. (2005) hMRE11 deficiency leads to microsatellite instability and defective DNA mismatch repair. EMBO Rep. 6, 438–444
25. Rogut, P., Sif, M., and Jasin, M. (1994) Expression of a site-specific endonuclease stimulates homologous recombination in mammalian cells. Proc. Natl. Acad. Sci. U.S.A. 91, 6064–6068
26. Donoho, G., Jasin, M., and Berg, P. (1998) Analysis of gene targeting and intrachromosomal homologous recombination stimulated by genomic double-strand breaks in mouse embryonic stem cells. Mol. Cell. Biol. 18, 4070–4078
27. Deleted in proof
28. Fang, W. H., and Modrich, P. (1993) Human strand-specific mismatch repair occurs by a bidirectional mechanism similar to that of the bacterial reaction. J. Biol. Chem. 268, 11838–11844
29. David, P., Efrati, E., Tocco, G., Krauss, S. W., and Goodman, M. F. (1997) DNA replication and postreplication mismatch repair in cell-free extracts from cultured human neuroblastoma and fibroblast cells. J. Neurosci. 17, 8711–8720
30. Owen, B. A., H. Lang, W., and McMurray, C. T. (2009) The nucleotide binding dynamics of human MSH2-MSH3 are lesion-dependent. Nat. Struct. Mol. Biol. 16, 550–557
31. Hughes, M. I., and Jiricny, J. (1992) The purification of a human mismatch-binding protein and identification of its associated ATPase and helicase activities. J. Biol. Chem. 267, 23876–23882
32. Palombo, F., Iaccarino, I., Nakajima, E., Ikejima, M., Shimada, T., and Jiricny, J. (1996) hMutSβ, a heterodimer of hMSH2 and hMSH3, binds to insertion/deletion loops in DNA. Curr. Biol. 6, 1181–1184
33. Constantin, N., Dzantiev, L., Kadyrov, F. A., and Modrich, P. (2005) Human mismatch repair: Reconstitution of a nick-directed bidirectional reaction. J. Biol. Chem. 280, 39752–39761
34. Kadyrov, F. A., Dzantiev, L., Constantin, N., and Modrich, P. (2006) Endonucleolytic function of MutLα in human mismatch repair. Cell 126, 297–308
35. Wu, X., Xu, Y., Chai, W., and Her, C. (2011) Causal link between microsatellite instability and hMRE11 dysfunction in human cancers. Mol. Cancer Res. 9, 1443–1448
36. Her, C., Vo, A. T., and Wu, X. (2002) Evidence for a direct association of hMRE11 with the human mismatch repair protein hMLH1. DNA Repair 1, 719–729
37. Stracker, T. H., and Petri, J. H. (2011) The MRE11 complex: Starting from the ends. Nat. Rev. Mol. Cell Biol. 12, 90–103
38. Wang, Y., Cortez, D., Yazdi, P., Neff, N., Elledge, S. J., and Qin, J. (2000) BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. Genes Dev. 14, 927–939
39. Dinkelmann, M., Spehalski, E., Stoneham, T., Buis, I., Wu, Y., Sekiguchi, J. M., and Ferguson, D. O. (2009) Multiple functions of MRN in end-joining pathways during isotype class switching. Nat. Struct. Mol. Biol. 16, 808–813
40. Xie, A., Kwok, A., and Scully, R. (2009) Role of mammalian Mre11 in classical and alternative nonhomologous end joining. Nat. Struct. Mol.
41. Rass, E., Grabarz, A., Plo, I., Gautier, J., Bertrand, P., and Lopez, B. S. (2009) Role of Mre11 in chromosomal nonhomologous end joining in mammalian cells. Nat. Struct. Mol. Biol. 16, 819–824
42. Zhuang, J., Jiang, G., Willers, H., and Xia, F. (2009) Exonuclease function of human Mre11 promotes deletional nonhomologous end joining. J. Biol. Chem. 284, 30565–30573
43. Zhang, Y., Yuan, F., Presnell, S. R., Tian, K., Gao, Y., Tomkinson, A. E., Gu, L., and Li, G. M. (2005) Reconstitution of 5′-directed human mismatch repair in a purified system. Cell 122, 693–705
44. Sartori, A. A., Lukas, C., Coates, J., Mistrik, M., Fu, S., Bartek, J., Baer, R., Lukas, J., and Jackson, S. P. (2007) Human CtIP promotes DNA end resection. Nature 450, 509–514
45. Bannister, L. A., Waldman, B. C., and Waldman, A. S. (2004) Modulation of error-prone double-strand break repair in mammalian chromosomes by DNA mismatch repair protein Mlh1. DNA Repair 3, 465–474
46. Richardson, C., and Jasin, M. (2000) Coupled homologous and nonhomologous repair of a double-strand break preserves genomic integrity in mammalian cells. Mol. Cell. Biol. 20, 9068–9075
47. Saparbaev, M., Prakash, L., and Prakash, S. (1996) Requirement of mismatch repair genes MSH2 and MSH3 in the RAD1-RAD10 pathway of mitotic recombination in Saccharomyces cerevisiae. Genetics 142, 727–736
48. Sugawara, N., Pâques, F., Colaiácovo, M., and Haber, J. E. (1997) Role of Saccharomyces cerevisiae Msh2 and Msh3 repair proteins in double-strand break-induced recombination. Proc. Natl. Acad. Sci. U.S.A. 94, 9214–9219
49. D’Amours, D., and Jackson, S. P. (2002) The Mre11 complex: At the crossroads of DNA repair and checkpoint signaling. Nat. Rev. Mol. Cell Biol. 3, 317–327
50. Uziel, T., Lerenthal, Y., Moyal, L., Andegeko, Y., Mittelman, L., and Shiloh, Y. (2003) Requirement of the MRN complex for ATM activation by DNA damage. EMBO J. 22, 5612–5621
51. Li, F., Tian, L., Gu, L., and Li, G. M. (2009) Evidence that nucleosomes inhibit mismatch repair in eukaryotic cells. J. Biol. Chem. 284, 33056–33061
52. Javaid, S., Manohar, M., Punja, N., Mooney, A., Ottesen, J. J., Poirier, M. G., and Fishel, R. (2009) Nucleosome remodeling by hMSH2-hMSH6. Mol. Cell 36, 1086–1094