Bi-directional Processing of DNA Loops by Mismatch Repair-dependent and -independent Pathways in Human Cells*

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Previous work has shown that small DNA loop heterologies are repaired not only through the mismatch repair (MMR) pathway but also via an MMR-independent pathway in human cells. However, how DNA loop repair is partitioned between these pathways and how the MMR-independent repair is processed are not clear. Using a novel construct that completely and specifically inhibits MMR in HeLa extracts, we demonstrate here that although MMR is capable of bi-directionally processing DNA loops of 2, 4, 5, 8, 10, or 12 nucleotides in length, the repair activity decreases with the increase of the loop size. Evidence is presented that the largest loop that the MMR system can process is 16 nucleotides. We also show that strand-specific MMR-independent loop repair occurs for all looped substrates tested and rigorously demonstrate a bi-directional Analysis of repair intermediates generated by the MMR-independent pathway revealed that although the processing of looped substrates with a strand break 5’ to the heterology occurred similarly to MMR (i.e. excision is conducted by exonucleases from the pre-existing strand break to the heterology), the processing of the heterology in substrates with a 3’ strand break is consistent with the involvement of endonucleases.

DNA insertion/deletion heterologies (also called DNA loops) are formed during normal DNA metabolism, particularly within simple repetitive sequences (1). These DNA loop structures are mutagenic if they are not corrected. It is known that the DNA mismatch repair (MMR) system in both prokaryotes and eukaryotes is capable of correcting small DNA loops (for reviews, see Refs. 2–7). The Escherichia coli MMR system can efficiently process DNA insertion/deletion heterologies up to 7 unpaired nucleotides (8–11). Gel-shift analyses using mismatch recognition complexes purified from both yeast and human cells have demonstrated that all these substrates can be processed by both MMR-proficient and MMR-deficient cell extracts. We demonstrate here that all these substrates can be processed by both MMR-dependent and -independent pathways in a strand-specific manner regardless of the nick orientations and that the MMR-independent repair of the heterologous with a strand break 3’ to the loop appears to involve endonuclease(s).

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† The abbreviations used are: MMR, mismatch repair; nt, nucleotide(s).

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The Human MMR Pathway Is Capable of Processing at Least 12-Nucleotide Loops—To determine the upper limits of loop size that MMR can process, MMR-proficient HeLa extracts were examined for repair of circular heteroduplexes with different loop sizes either in the complementary strand or in the viral strand. In addition to loops, these substrates also contain a strand break either 5′ (in the C strand) or 3′ (in the V strand) to the loop (Fig. 1). This set of substrates allowed us to test for bi-directional nick-directed repair of DNA loops. Repair of these substrates was scored either by the commonly used restriction enzyme assay (27) or by Southern blot analysis (20). As shown in Fig. 2, HeLa nuclear extracts were capable of correcting all loop substrates in a strand-specific manner with repair being targeted in each case to the nicked strand regardless of whether the nick was 5′ or 3′ to the heterology. The levels of repair were fairly constant between substrates containing from 5 to 12 unpaired nucleotides (nt), although there were a few variations (Fig. 2). The repair exhibited a -30-fold bias toward the nicked strand, as judged by the fact that the repair rate to the nicked strand was around 15% but only 0.5% to the continuous strand. These results suggest that the repair of 5–12 nt loops has nick-directed, bi-directional processing characteristics similar to that seen for mammalian base-base MMR (31).

To ascertain whether the loop heterologies were processed by the MMR system, an MMR-specific inhibitor (30) was used in our loop repair assay to block all MMR activity in HeLa extracts. This inhibitor was derived from hybridization of bacteriophages fd and M13 DNA and contains -290 mismatches per molecule. We have previously shown that this hybrid can completely inhibit in vitro MMR (30). If the loop repair observed was indeed conducted by MMR, addition of the fd:fdM13 hybrid in the reaction would completely block HeLa extracts from repairing looped heteroduplexes. These experiments are summarized in Fig. 3 and Table I. As expected, repair of a G/T mismatch was completely blocked by addition of fd:M13 heteroduplex DNA. This inhibition was not due to the presence of extra DNA in the reaction since addition of the same amount of fd:fd homoduplex DNA did not alter the amount of repair (Fig. 3). However, the repair of loop substrates of 2–12 nucleotides, although reduced, was not completely abolished under the same conditions regardless of whether the substrates contained a 5′ or a 3′ strand break (Fig. 3; Table I). These results suggest that the repair of loops of 2–12 nucleotides in HeLa extracts contains both MMR-dependent and -independent components. Although the amount of MMR-independent repair remained fairly constant (at about 10%, Fig. 3 and Table I) for all 2–12 nt substrates tested, the amount of MMR-dependent repair was gradually reduced as the loop size increased (from 33% for 2 nt down to ~3% for 12 nt, Table I).

MMR-independent Repair of 2–12 nt Loops Is Bi-directional—A previous study has shown that MMR-independent repair of a DNA loop that is 27 nucleotides in length or larger in human cells can only be directed by a 5′ nick (24). Our results, however, suggest that both 5′ and 3′ nicks can direct...
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Fig. 2. Strand-specific, nick-directed loop repair in HeLa nuclear extracts. Loop repair assays were performed using 75 μg of HeLa cell nuclear extract and 100 ng of substrate DNA. Repair to either strand was scored using the restriction enzyme sensitivity assay for all substrates except 3'-8V and 3'-12V, for which the Southern blot assay was used (see “Experimental Procedures”). Values are the average determined from at least three separate assays, and error bars represent the standard error of the mean. A G/T mismatch is repaired at ~50% efficiency (data not shown). Stippled bars represent repair of the nicked strand, and black bars represent repair of the continuous strand.

Fig. 3. Analysis of the MMR-dependent and -independent contributions to loop repair. Loop repair assays were performed in reactions containing 250 ng of substrate DNA and 75 μg of HeLa nuclear extract in the presence of no additional DNA (black bars), 75 μg of fd homoduplex DNA (white bars), or 75 μg of fd:M13 heteroduplex DNA (cross-hatched bars). The extent of nick-directed repair was monitored using the restriction enzyme sensitivity assay. Values are determined from at least three separate assays as described previously.

| Substrate | Total repair + fd:fd | MMR-independent repair + fd:M13 | MMR-dependent repair | Inhibition by fd:M13 |
|-----------|----------------------|-------------------------------|----------------------|---------------------|
| 5'-G/T    | % 52.2               | 0 %                          | 52.2 %               | 100 %               |
| 5'-2V     | % 41.2               | 8.2 %                        | 33.0 %               | 80 %                |
| 3'-4C     | % 40.2               | 9.1 %                        | 31.1 %               | 77 %                |
| 5'-5C     | % 20.3               | 10.2 %                       | 10.1 %               | 51 %                |
| 5'-8V     | % 17.4               | 10.2 %                       | 7.2 %                | 42 %                |
| 3'-8C     | % 21.0               | 12.8 %                       | 8.2 %                | 39 %                |
| 5'-12V    | % 14.5               | 11.3 %                       | 3.2 %                | 22 %                |
| 3'-12C    | % 10.1               | 7.4 %                        | 2.7 %                | 27 %                |

TABLE I

Relative loop repair by MMR-dependent and -independent pathways

Experiments were performed as described in Fig. 3. MMR-dependent repair was obtained by subtracting the MMR-independent repair (in the presence of fd:M13) from the total repair (in the presence of fd:fd). Percent of repair inhibited by fd:M13 was calculated by dividing the MMR-dependent repair with the total repair (+ fd:fd).

loop repair using an MMR-independent mechanism. To confirm that MMR-independent loop repair of 2–12 nt heterologies is bi-directional, we performed loop repair in nuclear extracts derived from several well characterized MMR-defective cell lines: MLH1-deficient HCT116, PMS2/MSH6-deficient HEC-1A, and MSH6-deficient HCT15. The results (shown in Fig. 4) indicate that, as seen for HeLa extracts (Fig. 3 and Table I), all MMR-deficient extracts efficiently repaired all looped heterologies tested with no significant differences in the repair rate between the 5' and 3' substrates. Repair in all cases was only detected in the nicked strand but not in the continuous strand (data not shown), indicating that the MMR-independent processing of the looped substrates is dependent on a nick and can occur in both 5'→3' and 3'→5' orientations. A subset of these substrates were also tested for repair by extracts derived from an MSH2-deficient lymphoblastoid cell line, NALM-6 (33). Again, nick-directed and bi-directional loop repair was observed in this MSH2 mutant cell line (data not shown). It is worth mentioning that the repair rate in these MMR-deficient cells is comparable with that observed in HeLa extracts supplemented with fd:M13 (compare Fig. 4 with Fig. 3). In sum, we demonstrate using either MMR-deficient or MMR-inhibited nuclear extracts that 5' and 3' nicks direct nearly equivalent amounts of repair for several of our loop substrates. These results strongly suggest that MMR-independent repair of 2–12 nt looped substrates occurs bi-directionally.

5'- and 3'-Nicked Loop Substrates Are Processed by Different Mechanisms—To understand how DNA loops are removed by the MMR-independent pathway, loop repair was carried out using both MMR-proficient and MMR-deficient extracts under conditions of limited DNA synthesis, e.g. in the absence of dNTPs. By blocking repair DNA synthesis, repair events prior
to DNA resynthesis are trapped, and the reaction intermediates can be visualized by Southern blot analysis (24, 31). Fig. 5 shows the repair intermediates of four looped substrates (5'-8C, 3'-8C, 5'-12V, 3'-12V) by HeLa or HCT116 nuclear extracts in the absence of dNTPs. For the 5'-nick substrates, intermediates accumulated over a broad range (from the nick to loop to several points beyond the loop) in both MMR-proficient and -deficient extracts (Fig. 5A), a phenomenon observed for MMR intermediates (31). This finding suggests that the removal of the heterology in 5'-looped substrates occurs through exonucleolytic excision starting from the pre-existing nick and proceeding toward the loop. However, for the 3'-nick substrates, the patterns of repair intermediates in HeLa and HCT116 cells are significantly different. As shown in Fig. 5B, repair intermediates in HCT116 cells were confined to an area immediately surrounding the loop site (lanes 6 and 8), and this occurs regardless of whether the loop is on the nicked (lane 8) or continuous (lane 6) strand. In comparison with the results shown in Fig. 5A, it appears that the MMR-independent pathway uses different mechanisms to process 3'- and 5'-nick substrates. Interestingly, the bands corresponding to repair intermediates that immediately flank the loop site were less intense as compared with those made from the 3'-looped heterology, as seen previously for MMR-dependent repair assay that loop repair intermediates were recovered between the loop and the probe (Table I). Thus, reduced processing of the larger loop by the MMR pathway decreases as the loop size increases (Table I). Thus, reduced processing of the larger loop by the MMR pathway appears to lead to more repair of the substrate by the MMR-independent pathway.

Removal of Heterology in 3'-Nicked Substrates Does Not Originate at the Nick—The pattern of repair intermediates produced by the MMR-deficient HCT116 extracts when processing 3' substrates suggest that removal of 3'-looped heterology by the MMR-independent pathway is a short patch repair and may involve endonucleases. To further determine the nature of excision on the 3'-nick substrates, repair intermediates generated under conditions of limited DNA resynthesis were double-digested with Sau96I and SspI and subjected to Southern hybridization using a 32P-labeled probe located between the nick and the loop site (Fig. 6). If excision occurs from the nick to the loop, the probe would lose its corresponding binding sequence, and Sau96I will not be able to digest the repair intermediates since the region containing its recognition sequence (located between the nick and the loop) will be single-stranded. The results, shown in Fig. 6, indicate that this is not the case. Fragments with end points that flank the loop site are again evident in both the 3'-8C and 3'-12V substrates (Fig. 6), supporting the idea that incisions were made around the loop site. These fragments were only detected on the nicked strand, regardless of which strand the loop is on. It is worth noting that reactions utilizing HeLa extracts (Fig. 6, lanes 1 and 3) again show reduced intensity of bands as compared with those utilizing HCT116 extracts (Fig. 6, lanes 2 and 4). Clearly, the
processing of these substrates by the MMR pathway (from the strand break to the heterology) in HeLa cells left less substrate available for repair via the MMR-independent pathway.

**DISCUSSION**

In this study, we examined MMR-proficient and MMR-deficient cells for their ability to process heteroduplexes containing a loop of 2, 4, 5, 8, 10, or 12 nucleotides, which are believed to be substrates for both MMR-dependent and -independent pathways. We demonstrate that both pathways can repair these substrates in a strand-specific manner and rigorously show that DNA loop repair by MMR-independent pathway occurs bi-directionally. Mechanistic studies revealed that the MMR-independent pathway processes looped heteroduplexes with a 3’ strand break in a way distinct from that of MMR.

Previous studies in human cells have suggested that both MMR-dependent and MMR-independent pathways are capable of repairing DNA loop structures with small insertion/deletion DNA loops being processed by MMR and large loops being processed by an MMR-independent system (20–22, 24). However, how large a DNA loop can be processed by MMR was not clear. In E. coli, it has been documented that MMR can correct unpaired heteroduplexes containing no more than 7 nucleotides (8, 10, 11, 34, 35). In yeast, the upper limit of the loop size increases to 13 nucleotides (18, 19). Given the overlapping activities of DNA loop repair in human cells, it is difficult to distinguish loop repair activity conducted by MMR from that conducted by an MMR-independent pathway. Using fd:M13 hybrid as an MMR inhibitor, we successfully separated DNA loop repair activities in human cells. Interestingly, when the loop size increases, the ability of MMR to process looped heteroduplexes decreases proportionally (Table I and Fig. 7). Our data show that the MMR pathway in human cells can process DNA loops containing at least 12 unpaired nucleotides. Although we did not test loop substrates containing more than 12 nucleotides in this study, the data in Table I suggest that the MMR system is capable of processing DNA loops more than 12 nucleotides long. When the percent of repair inhibited by fd: M13 (i.e., the percent of repair contributed by the MMR-dependent pathway, Table I) was plotted versus loop size, we can extrapolate that the loop repair activity by MMR will drop to zero when the loop size reaches 17 nucleotides (Fig. 7). Therefore, the largest loop size that MMR can process is likely to be 16 nucleotides with larger loops being processed by entirely MMR-independent pathways. Our data are consistent with a previous observation by Wilson et al. (17) that although human MutSβ can recognize DNA loops up to 24 nt, only those with ≤16 nt can activate the ATPase activity of MutSβ.

Another important finding in this study is the identification and characterization of 3’-nick-directed loop repair by an MMR-independent pathway. Previous studies have shown that MMR-independent loop repair in human cells can be certainly directed by a 5’ strand break (21, 22, 24), but whether or not a 3’ nick can be used for loop repair was less clear. Whereas repair of heteroduplexes containing up to 5 unpaired nucleotides can be directed by a 3’ nick (21), large looped heteroduplexes with a strand break 3’ to the heterology are processed poorly with little strand bias (24). In this study, we clearly show that both MMR-depleted HeLa extracts and MMR-deficient extracts can efficiently process looped heteroduplexes containing 5–12 unpaired nucleotides regardless of whether the strand break is 5’ or 3’ to the heterology (Figs. 3 and 4, Table I). It is clear that there are at least two types of MMR-independent loop repair pathways in human cells: one that is specific for large loops (possibly 17 nucleotides or more) and occurs only through a 5’ to 3’ orientation and the other that is responsible for repairing small loops (16 nucleotides or less) and can work bi-directionally.

Our analysis of repair intermediates under the condition in which DNA synthesis is blocked revealed that the MMR-independent repair pathway processes 3’- and 5’-nicked substrates differently. In MMR, strand-specific excision of the mispaired base is oriented from the strand break toward the mismatch, regardless of whether the strand break is 3’ or 5’ to the mismatch (31, 36). Thus, intermediates recovered from DNA synthesis-blocked reactions span a broad range, normally from the strand break to a point ~200 nucleotides beyond the heterology (31, 36). The intermediates detected in 5’-looped substrates processed by either MMR or the MMR-independent pathway are similar to those observed in mismatched substrates (Fig. 5A). However, in the case of 3’-nicked substrates, repair intermediates were recovered in an area immediately flanking ei-
ther side of the loop (Fig. 5B), suggesting that the removal of the heterology in this type of substrate involves endonucleases. This hypothesis is supported by the fact that excision from the nick to the loop did not occur since the intermediates produced in DNA synthesis-inhibited reactions were sensitive to Sau96I (~50 nucleotides 5′ to the nick site, Fig. 1) and since probe C5746–5765 (located between the nick and the loop) could hybridize to the nicked strand and detect repair intermediates (Fig. 6). These results imply that some portion of DNA loop repair may be conducted by nucleotide excision repair or a pathway similar to nucleotide excision repair in which damaged DNA is removed through double endonucleolytic cleavage (37). However, our preliminary experiments using nuclear extracts from cell lines mutated in XPA, XPB, or XPG ruled out the involvement of nucleotide excision repair in loop repair.2 Therefore, the repair of DNA loops with a 3′ nick is likely conducted by an activity independent of not only MMR, but also nucleotide excision repair. However, how the heterology in the 3′ substrate is removed remains to be elucidated.

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