Nick-dependent and -independent Processing of Large DNA Loops in Human Cells*

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DNA loop heterologies are products of normal DNA metabolism and can lead to severe genomic instability if unrepaired. To understand how human cells process DNA loop structures, a set of circular heteroduplexes containing a 30-nucleotide loop were constructed and tested for repair in vitro by human cell nuclear extracts. We demonstrate here that, in addition to the previously identified 5' nick-directed loop repair pathway (Littman, S. J., Fang, W. H., and Modrich, P. (1999) J. Biol. Chem. 274, 7474–7481), human cells can process large DNA loop heterologies in a loop-directed manner. The loop-directed repair specifically removes the loop structure and occurs only in the looped strand, and appears to require limited DNA synthesis. Like the nick-directed loop repair, the loop-directed repair is independent of many known DNA repair pathways, including DNA mismatch repair and nucleotide excision repair. In addition, our data also suggest that an aphidicolin-sensitive DNA polymerase is involved in the excision step of the nick-directed loop repair pathway.

DNA loop heterologies are unpaired, single-stranded DNA structures that can be generated during DNA metabolism. These structures reflect a form of genetic instability and are considered as an early phenotype in carcinogenesis (1). DNA loops can range in size from a single nucleotide to several thousand nucleotides (nt).1 Smaller loops (<20 nt) are generally formed during replication of repetitive DNA sequences (2–4), and larger loops can arise during recombination events between divergent sequences (reviewed in Ref. 5).

It has been demonstrated that eukaryotic cells are capable of processing DNA loops ranging from 1 to 5,600 nt in length (6–13). There is accumulating evidence, however, suggesting that eukaryotic cells possess multiple pathways to process these loops, including both the mismatch repair (MMR)-dependent and -independent pathways (6, 11, 12, 14–20). This redundancy of pathways for loop processing has been recently shown to apply to DNA loops less than 16 nt, but not to those larger than 16 nt. The former can be repaired by both the MMR-dependent and -independent pathway, and the latter can only be processed by the MMR-independent pathway(s) (20). The limit of MMR-dependent loop repair at 16 nt in length serves as a criterion to classify DNA loop heterologies into small loops (<16 nt) and large loops (>17 nt) (20). For small loops, the MMR-dependent pathway appears to utilize the same general mechanism to process these heterologies, i.e. excision is conducted by exonucleases from the pre-existing strand break to the heterology no matter whether the strand break is located 5’ or 3’ to the loop. However, small DNA loops are repaired differently by the MMR-independent pathway(s) (14–16, 20). Although the processing of small DNA loops with a strand break 5’ to the heterology occurs by a manner similar to that seen for MMR-dependent processing (i.e. involving exo-nucleases), the processing of looped heteroduplexes containing a 3’ strand break seems to involve endonuclease(s) that remove the loop directly, without excision occurring from the nick (20).

Very little is known about the requirements and mechanism of large loop (>17 nt) processing by the MMR-independent pathway in eukaryotes. Previous studies have indicated that the human MMR-independent pathway(s) can process large loops containing a strand break 5’, but not 3’ to the heterology (14). On the other hand, a very weak 3’-directed large loop repair activity has been identified in yeast (16). However, the mechanisms by which these large loop repair pathways operate are largely unknown. To understand large DNA loop repair in human cells, we tested the repair of a series of DNA loop heteroduplexes containing a 30-nt loop and a strand break either 5’ or 3’ to the heterology by human nuclear extracts. We demonstrate that there are at least two pathways for large loop repair in human cells, one of which has been previously demonstrated (14). In this work, we identify a human large loop repair activity that specifically removes the loop in a manner independent of a nick, which we designate as the loop-directed pathway. The loop-directed pathway is independent of several major DNA repair pathways, including MMR, nucleotide excision repair (NER), and the Werner syndrome protein WRN. We also show that the two large loop repair pathways have different underlying mechanisms.

EXPERIMENTAL PROCEDURES

Cell Culture and Nuclear Extract Preparation—HeLa cells were grown in RPMI 1640 with 5% fetal bovine serum and 4 mM glutamine. HCT115, NALM6, and GM02345 cells were grown in RPMI 1640 with 10% fetal bovine serum. HCT116 and HEC-1A cells were grown in McCoy’s 5A with 10% fetal bovine serum. WS780 (obtained from Isabel Mellon, University of Kentucky) and AG08802 cells were grown in Dulbecco’s modified Eagle’s medium with 5% non-essential amino acids, 1× essential amino acids, and 1× vitamins (Invitrogen). GM02345 and AG08802 were purchased from Coriell Cell Repositories. Monolayer

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1 The abbreviations used are: nt, nucleotide; MMR, mismatch repair; NER, nucleotide excision repair; ccc, covalently closed circular; C, complementary; V, viral.
cultures (HCT116, HCT15, HEC-1A, and AG08802) were harvested from 20 to 30 roller bottles and suspension cells (HeLa, NALM6, GM02345) were harvested from 6-liter cultures in spinner flasks. Nuclear extracts were prepared as previously described (21, 22).

**Heteroduplex Substrate DNA Construction**—Bacterial phage f1MR0 was created by digestion of phage f1MR1 (23) double stranded DNA with XbaI and NheI, gel purification of the large fragment (6,423 bp), and ligation of the compatible ends. The resulting molecule contained a 30-bp deletion relative to phage fMR24 DNA (22). The sequence of f1MR0 was verified by DNA sequencing. Creation of heteroduplex substrates was performed as previously described (21, 23). Briefly, double stranded DNA from one phage (e.g. f1MR0) was linearized by Sau96I, and annealed with single stranded DNA from another phage (e.g. fMR24). The resulting 5'- nicked (in the complementary strand) heteroduplex was purified as described (23). To construct a looped substrate with a 3’ nick, its corresponding 5’- nicked substrate was incubated with DNA ligase in the presence of ethidium bromide to form a supercoiled covalently closed circular (ccc) substrate. The latter product was then incubated with glycopolypeptide II protein, an endonuclease that specifically nicks the viral strand at the site of the phage replication origin (24), to yield a nick 3’ to the loop (see Fig. 1). The nomenclature of substrates follows the format: (nick position) – loop size (loop strand). For example, 5’-30V describes a substrate with a 5’ nick and 30-nt loop in the viral (V) strand (see Fig. 1). 5’ nicks are located 115 bp away from the loop site on the complementary (C) strand. 3’ nicks are located 175 bp away from the loop site on the V strand.

**Loop Repair Assays**—Unless mentioned otherwise, large loop repair was assayed by Southern blot analysis as described (20). Briefly, 100 ng (24 fmol) of DNA substrate was incubated with 75 µg of nuclear extracts in 15-µl reactions containing 20 mM Tris-HCl (pH 7.6), 110 mM KCl, 5 mM MgCl2, 1.5 mM ATP, 1 mM glutathione, and 0.1 mM each of the four dNTPs. Reactions were incubated for 15 min at 37 °C and stopped by protease K digestion (30 µg/ml) for 15 min at 37 °C. DNA was isolated by phenol extraction and ethanol precipitation, and then was digested with SpI and BanII. The resulting products were separated on 6% denaturing polyacrylamide gel electrophoresis and detected by ethidium bromide staining. Reaction products were digested with SspI, separated on 6% denaturing polyacrylamide gels, and electrotransferred onto a nylon membrane as described (20). Where indicated, aphidicolin was added to a final concentration of 0.1 mM each of the four dideoxynucleotidetriphosphates (ddNTPs) was added to a final concentration of 0.1 mM and each of the four dideoxynucleotidetriphosphates (ddNTPs) was added to a final concentration of 0.1 mM and each of the four dideoxynucleotidetriphosphates (ddNTPs) was added to a final concentration of 0.1 mM and each of the four dideoxynucleotidetriphosphates (ddNTPs) was added to a final concentration of 0.1 mM and each of the four dideoxynucleotidetriphosphates (ddNTPs) was added to a final concentration of 0.1 mM and each of the four dideoxynucleotidetriphosphates (ddNTPs) was added to a final concentration of 0.1 mM and each of the four dideoxynucleotidetriphosphates (ddNTPs) was added to a final concentration of 0.1 mM and each of the four dideoxynucleotidetriphosphates (ddNTPs) was added to a final concentration of 0.1 mM and each of the four dideoxynucleotidetriphosphates (ddNTPs) was added to a final concentration of 0.1 mM and each of the four dideoxynucleotidetriphosphates (ddNTPs) was added to a final concentration of 0.1 mM and each of the four ddNTPs. Reactions were incubated for 15 min at 37 °C and stopped by DNA sequeencing of the strand size from a shorter strand to a longer strand for either the complementary strand or viral strand. A conversion of substrates containing a nick (in the C strand) 5’ to the loop, the nick in the V strand was created by glycoprotein II (gpII) and is 175 bp 3’ to the loop. The black circle in the SpI fragment and the whole substrate represents the loop structure. The loop sequence either in the C or V strands is shown in bold type at the top of the figure. The black and gray bars represent locations where oligonucleotide probes hybridize in the C and V strands, respectively. Numbers in parentheses indicate nucleotide positions of corresponding restriction enzymes in bacteriophage f1MR24.

**RESULTS**

**Human Cells Possess Both Nick-dependent and -independent Large Loop Repair Pathways**—To study the repair of large DNA loops in human cells, we constructed a set of DNA substrates containing a 30-nt loop and a strand break, where the loop was placed either on the nicked strand or on the continu-}

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**FIG. 1. DNA substrates containing a 30-nt loop.** DNA substrates containing a 30-nt loop and a strand break were constructed from bacteriophage f1MR24 and f1MR0 as described under “Experimental Procedures.” The loop was either in the complementary (C) or viral (V) strands. The nick in the C strand was created by Sau96I and is 115 bp 5’ to the loop; the nick in the V strand was created by glycoprotein II (gpII) and is 175 bp 3’ to the loop. The black circle in the SpI fragment and the whole substrate represents the loop structure. The loop sequence either in the C or V strands is shown in bold type at the top of the figure. The black and gray bars represent locations where oligonucleotide probes hybridize in the C and V strands, respectively. Numbers in parentheses indicate nucleotide positions of corresponding restriction enzymes in bacteriophage f1MR24.

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**C strand loop**

| Size | ATGCACAGCCTGCGACCAACACGCAGATC |
|------|---------------------------------|
| 3’   | -CTTAAAGATC TTTAAGCCAG... C   |
| 5’   | -GATTTGCTAG -AAATCCGCCAT... V |

**V strand loop**

| Size | CGACGATCCGCGCTGTGTCGTCAG |
|------|--------------------------|
| 3’   | -CTTAAAGATC TTTAAGCCAG... C   |
| 5’   | -GATTTGCTAG -AAATCCGCCAT... V |

**SpI fragment**

| Size | (5213) |
|------|--------|
| C    | SspI (5967) |
| V    | C      |

**Sau96I fragment**

| Size | (5767) |
|------|--------|
| C    | Sau96I |
| V    | C      |

**BanII fragment**

| Size | (5824) |
|------|--------|
| C    | BanII |
| V    | C      |

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**Large DNA Loop Repair in Human Cells**
substrates were performed using 75/5 H9262/H11032 substrates with a 3'-5' nick. The membrane was hybridized with 32P-end-labeled oligonucleotide probes as described (20). Repair in the C and V strands was detected by a C-strand probe (V5216) with BanII and SspI prior to electrophoresis through a 6% denaturing polyacrylamide gel. DNA was then transferred onto a nylon membrane and

- strand probe (V5216) showed repair of a new but shorter fragment (443 nt) in the reaction containing the looped 3'-30V substrate 5'-30V. As shown in Fig. 2A, conversion of the original longer fragment (473-nt) to a shorter fragment occurred in the looped 30V substrate as indicated by the fact that there was a conversion of the original longer fragment (473-nt) to a new but shorter fragment (443 nt) in the reaction containing the active HeLa extract (Fig. 2A, vii, HL). No repair of the nicked strand of the 3'–30C substrate was seen, however (Fig. 2A, viii, HL).

The disparity between the results of the 3'-30C and 3'-30V substrates suggested to us that the repair observed for the 3'-30V substrate may not be nick-directed. Indeed, a previous study has indicated the presence of a non-nick directed, but loop-stimulated pathway for loop repair in yeast cell extracts (15). To test our hypothesis of the presence of a loop-stimulated repair pathway in human extracts that is not nick-directed, we first examined the repair that occurred on the continuous strands of 5'-30V and 3'-30C substrates. If the loop itself can provoke repair, loop removal should occur on the V strand of substrate 5'-30V and the C strand of 3'-30C. Results indicate that loop-specific removal indeed occurred in these substrates (Fig. 2A, iii, for 3'-30C, and vi, for 5'-30V). Little (if any) repair was observed in the continuous strand containing no loops (see Fig. 2A, iv and v). It is worth mentioning that a residual 473-nt band was detected in substrate 3'-30V by the C strand probe (Fig. 2A, iv, HL), which seemed to be a loop addition (12) in the continuous strand. However, based on our experience in the preparation of 30 substrates, which were derived from ligation of their corresponding 5' substrates before nicking by glycoprotein II in the viral strand (see "Experimental Procedures" for 30 substrate preparations), there is a very small fraction of unligated substrates, which still contain a 5' nick. Therefore, the residual 473-nt is most likely derived from a background 5' nick-directed repair of the unligated substrates.

To confirm the presence of a nick-independent repair pathway in human cells, we tested for loop removal of two ccc substrates that contain a 30-nt loop either in the C (ccc-30C) or V strands (ccc-30V). As shown in Fig. 2B, conversion of the longer fragment to a shorter fragment occurred in the looped strand for both substrates. As expected, little repair was detected in the non-looped strand for both ccc substrates (data not shown). The presence of a non-loop-directed repair pathway was observed in all cell lines examined. The same results, together with the data described above, suggest that a large loop structure in human cells can be removed in a manner independent of a strand break. Therefore, human cells appear to possess at least two large loop repair pathways: one that is directed by a 5' strand break, whereas the other is loop-directed loop removal and is nick-independent. In general, nicked-directed repair appears to be more efficient than loop-directed repair as the amount of the former is always higher than the latter (see Fig. 2C).

The Loop-directed Repair Pathway Is Independent of MMR, NER, and WRN Proteins—To determine whether several major DNA repair pathways are involved in large DNA loop repair, we assayed cell lines deficient in various DNA repair pathways for their ability to process the 5'-30V substrate, which allows for detection of both nick-dependent and -independent pathways in a single reaction. The cell lines used in this analysis are deficient in MMR (HCT116, HCT15, and NALM6), NER (GM02345 and AG08802), or Werner syndrome protein WRN (WS780). The WRN protein has a 3' → 5' helicase activity as well as a 3' → 5' nuclelease activity, and is believed to participate...
in recombination repair and double strand break repair (25, 26). Repair activities were compared with the levels found in HeLa extract, which is wild type for MMR (21, 27), NER (28), and WRN activity (29). Our results indicate that all cell lines tested, regardless of deficiency in MMR, NER, or WRN, showed some degree of activity for both nick-directed and nick-independent pathways (Table I, Figs. 2 and 3). For the nick-directed pathway, nuclear extracts derived from MLH1-deficient HCT116 (Table I), MSH2-deficient HCT15 (Table I), MSH6-deficient NALM6 (Table I, Figs. 2 and 3), and WRN-deficient WS780 (Table I) had levels of repair comparable with those of HeLa cells. Cells with mutations in NER genes XPA (GM02345) or XPG (AG08802) were capable of processing large DNA loops in a nick-directed manner, but the repair level was significantly lower than that in other cells (Fig. 3 and Table I).

To determine whether these cells are partially defective in this repair pathway, nuclear extracts derived from MLH1-deficient HCT116 (Table I), MSH2-deficient HCT15 (Table I), MSH6-deficient NALM6 (Table I, Figs. 2 and 3), and WRN-deficient WS780 (Table I) had levels of repair comparable with those of HeLa cells. Cells with mutations in NER genes XPA (GM02345) or XPG (AG08802) were capable of processing large DNA loops in a nick-directed manner, but the repair level was significantly lower than that in other cells (Fig. 3 and Table I).

The Loop-directed Repair Pathway Requires Little DNA Synthesis—All known DNA repair pathways include a DNA resynthesis step that can be inhibited by a DNA polymerase inhibitor. To assess the impact of DNA synthesis in large loop DNA repair, repair reactions were performed either in the absence of exogenous dNTPs or in the presence of the DNA synthesis inhibitor aphidicolin (an inhibitor for pol α, pol δ, and pol ε), or ddNTPs (chain elongation terminators). We first analyzed the requirement for DNA synthesis for the nick-dependent pathway of large DNA loop repair using substrates 5'-30V and 5'-30C. As shown in Fig. 4A, the nick-directed repair was completely blocked by any inhibitory factor regardless of whether the loop was on the nicked strand (substrate 5'-30C) or on the continuous strand (substrate 5'-30V), suggesting that like most DNA repair pathways, nick-directed large loop repair requires active DNA synthesis that may involve pol α, pol δ, and/or pol ε. This is consistent with a previous report implicating pol δ in MMR (30). Similar analysis was performed to determine the impact of DNA synthesis on the nick-independent pathway using substrates 5'-30V and 3'-30V. Surprisingly, the loop removal for both substrates was not completely inhibited in all three conditions of limited DNA synthesis, although the amount of repair was reduced, with an inhibition of 17% by the omission exogenous dNTPs, 58% by aphidicolin,
and 67% by ddNTPs (Fig. 4B). These results indicate that nick-independent loop removal requires a greatly reduced amount of DNA synthesis compared with the nick-dependent system.

Because DNA synthesis in DNA repair is preceded by strand excision, the limited inhibition of the loop-directed repair by DNA synthesis inhibitors also suggests a limited repair excision during this reaction. To explore this possibility, repair intermediates were monitored by Southern blot analysis as previously described (20), under the conditions of limited DNA synthesis. Fig. 5, A and B, shows the analysis of nick-directed repair for both 5'-30V and 5'-30C substrates using a probe that binds to the 3' end of the C strand of a fragment produced by digestion with SspI (also see Fig. 1, SspI fragment). As expected, under the normal repair conditions (lanes 1 and 5), only a band with the full size SspI fragment (the top band, 724 nt in length for lane 1, 754 nt in length for lane 5) and a band (554 nt in length for lane 1, and 554 nt in length for lane 5) corresponding to the originally nicked DNA fragment were detected. The top band in these cases represents both complete repair (excision of the loop, resynthesis, and ligation) and direct ligation of the nick prior to repair, whereas the lower band indicates the original substrate that was neither ligated, nor otherwise processed. Under the conditions of limited DNA synthesis (lanes 2–4 and 6–8), in addition to the whole SspI fragment (724 nt in length for lane 5A and 754 nt in Fig. 5B) and the fragment containing the original nick (524 nt in Fig. 5A and 554 nt in Fig. 5B), smaller bands between 524 and ~400 nt were also observed, which represent the excision intermediates. Although a slightly different pattern of intermediate tracts between the nick and the loop was evident between these two substrates, prominent DNA fragments with ends that correspond very close to the loop site were clearly detected in both cases (between 400- and 420-nt markers), particularly in reactions containing ddNTPs (lanes 4 and 8) and omitting exogenous dNTPs (lanes 2 and 6). Interestingly, at least 50% less repair intermediates were seen in the aphidicolin-containing reactions (lanes 3 and 7, discussed below). These results indicate that an extensive repair excision is associated with nick-directed loop repair and that the bulk of excision occurs between the nick and loop site. Similar experiments were performed to determine excision intermediates for the nick-independent loop repair pathway by using substrates 5'-30V and 3'-30V. The results from these experiments demonstrated that repair intermediates were difficult to detect. Even after prolonged exposure of the film, we were able to detect only very light bands centered on the loop, which had a similar intensity as many nonspecific repair bands above the loop site (Fig. 5C). Similar results were observed using the 3'-30V substrate (not shown). Although less effective repair by the loop-directed pathway may partially contribute to detectable excision intermediates, the data shown in Fig. 4 suggest to us that loop-directed loop removal is less sensitive to conditions of limited DNA synthesis, which would also explain the less detectable excision intermediates observed in Fig. 5.

**An Aphidicolin-sensitive DNA Polymerase Is Involved in Stimulating Repair Excision in the Nick-dependent Pathway**—Our data show that an aphidicolin-sensitive DNA polymerase is apparently involved in nick-directed large loop repair, as judged by the fact that no repair products were observed in reactions containing aphidicolin (Fig. 4A). Surprisingly, much less excision intermediates were observed in aphidicolin-containing reactions (Fig. 5, lanes 3 and 7) compared with other reactions that inhibit DNA synthesis. These results suggest that an aphidicolin-sensitive DNA polymerase may be required for loop repair-associated excision. To test this possibility, repair products from reactions with limited DNA synthesis were digested with BseRI and BanII. Whereas BseRI consistently linearizes the DNA substrate (see Fig. 1), digestion of the DNA substrate by BanII depends on the availability of its recognition sequence, because the BanII recognition sequence is located in between the nick and the loop (see SspI fragment in Fig. 1) and is subjected to excision during the repair reaction. Digestion by BanII would occur if there is no excision, or excision followed by resynthesis. If excision occurs without resynthesis in the presence of DNA synthesis inhibitors, a single strand DNA gap that spans the nick and the loop along the shorter distance would be generated, which prevents BanII...
from being able to cut. If both enzymes are able to digest the DNA under these conditions, fragments of 3.7 and 2.7 kb will be evident, indicating that the region of DNA in between the loop and pre-existing nick is double stranded and that repair excision does not occur in this region.

As an example, a 5' G-T heteroduplex was processed by HeLa nuclear extracts under various conditions of limited DNA synthesis. As expected, an increased prominence of the 6.4-kb band was observed regardless of how DNA synthesis was inhibited (Fig. 6, lanes 6–8), indicative of the presence of a single stranded DNA gap around the BanII sequences as a result of the mismatch-provoked excision and inhibited DNA synthesis. Similar assays were performed using the 5'-30C and 5'-30V substrates. As shown in Fig. 6, there were significant amounts (26%) of BanII-resistant species (6.4 kb in size) in reactions in the absence of dNTPs for both the 5'-30V (lane 11) and 5'-30C (lane 16) substrates, indicating production of a single stranded DNA gap within the BanII recognition sequence. However, the amount of the 6.4-kb species was significantly reduced in reactions containing aphidicolin (Fig. 6, lanes 13 and 18). These results suggest that the repair reaction was either completed or inhibited prior to excision. The former possibility is contradictory to the repair data shown in Fig. 4. Therefore, aphidicolin appears to be able to inhibit nick-directed excision as well as synthesis. In other words, an aphidicolin-sensitive DNA polymerase (pol α, δ, or ε) is potentially required for the excision step of nick-directed repair loop. The excision mechanism for the nick-directed DNA repair pathway is apparently different from that for MMR. For example, regardless of the kinds of DNA synthesis inhibitors, at least a 20-fold increase in the amount of the 6.4-kb band was observed during the repair of a G/T mismatch (see lanes 5–8). For looped substrates, there is an ~10-fold increase when omitting exogenous dNTPs (lanes 11 and 16), but an increase less than 3-fold in reactions containing aphidicolin. Control reactions using homoduplex DNA showed little BanII-resistant 6.4-kb species (Fig. 6, lanes 1–4), implying that the excision is provoked by a loop or a mismatch.

**DISCUSSION**

The results presented here demonstrate that there are at least two distinct repair pathways for large DNA loops in human cells: one that is directed by a strand break (nick-directed repair) and the other that is directed by the loop itself (loop-directed repair). The loop-directed repair is a novel loop repair pathway in human cells. Using Southern blot analysis, we show that the repair pathway specifically removes the loop structure from DNA regardless of whether the loop is in the continuous strand or the nicked strand (see Fig. 2A and B). We further show that the loop-directed pathway requires limited DNA synthesis because the repair reaction is marginally sensitive to aphidicolin, ddNTPs, or the absence of exogenous dNTPs (see Fig. 4B), all of which strongly block DNA synthesis. The data in this study demonstrate that the nick-directed DNA repair pathway relies on a nick 5' (but not 3') to the loop for repair, and the repair only occurs in the nicked strand, which is in agreement with a previous study in human cell extracts (14).

In addition, our data suggest that an aphidicolin-sensitive DNA polymerase is somehow involved in the excision step of the nick-directed pathway (see Fig. 6).

DNA large loop repair has been shown in yeast, and previous data suggest that this pathway requires components from both MMR and NER pathways. Kirkpatrick and Petes (10) have demonstrated that the processing of a 26-nt loop requires the gene products of MSH2 and RAD1, MMR and NER components, respectively. Additionally, the purified RAD1-RAD10 complex is capable of enhancing repair in vitro of a 27-nt loop (15). Our results suggest, however, that loop repair pathways in human cells may occur through a mechanism that is not homologous to that seen in yeast. We demonstrate that both the nick-directed and the loop-directed pathways in human cells seem to be independent of the MMR, NER, and WRN pathways, as judged by the fact that cells defective in MSH2, MLH1, XPA, XPG, or WRN are competent in the repair of large looped heteroduplexes for both nick- and loop-directed mechanisms (see Table I and Fig. 3). Although we did not test the involvement of the XPF gene product (the homolog of yeast RAD1) in loop repair, a recent study has indicated that XPF is not required for nick-directed large loop repair (14). Therefore, it is likely that different mechanisms are used to process large DNA loops in human and yeast cells.

The analysis of excision intermediates and repair products under conditions of limited DNA synthesis indicates that MMR, the nick-directed loop repair, and the loop-directed loop repair pathways utilize distinct mechanisms for repair excision. Like MMR and small loop repair, the excision of the nick-directed large loop repair appears to initiate at the pre-existing nick and proceeds toward the loop site (see Refs. 14, 15, and 20; and Fig. 5). However, unlike MMR, where the excision step can occur in the absence of a DNA polymerase, the nick-directed excision appears to depend on an aphidicolin-sensitive DNA polymerase (Figs. 5 and 6). For the MMR reaction, none of the three conditions causing limited DNA synthesis had an effect on gap formation between the nick and the mismatch, as a single stranded region that is refractory to BanII digestion is created under all conditions (Fig. 6, lanes 6–8). In the case of nick-directed large loop repair, aphidicolin decreases excision (Fig. 6, lanes 13 and 18), whereas lack of exogenous dNTPs (Fig. 6, lanes 11 and 16) or the presence of ddNTPs (Fig. 6, lanes 12 and 17) only slightly inhibits repair excision. This finding suggests that an aphidicolin-sensitive DNA polymerase is required for the excision step of nick-directed large loop repair. However, how such a polymerase may play a role in repair excision is a puzzle, as none of the known mammalian DNA polymerases contain a 5' → 3' exonuclease activity (31). It is possible that a preformed complex of a po-
lymerase and a nuclease is recruited to the nick, with DNA synthesis occurring immediately on the heels of digestion.

Additionally, the nick-independent loop repair pathway appears to operate by a much different mechanism. The most obvious difference is that the loop-directed repair requires little DNA synthesis because all conditions of limited DNA synthesis only slightly reduce the repair (Fig. 4). This is consistent with the fact that only limited excision intermediates and a very small patch of excision can be detected in these reactions (Fig. 5C). Therefore, it seems likely that the loop in the loop-directed pathway is removed by incisions (rather than excision) from activities similar to XPG or XPF/ERCC1, but these activities would need to make incisions either to the immediate 5' side of a loop, or on both sides of the loop, creating a strand break or a very small (several nucleotide) gap. The former case would create a flap DNA structure that could be processed by flap endonuclease 1 or a similar activity. In either case, only the looped DNA sequence is removed and there would be no need for extensive DNA synthesis. Clearly, much more work is needed to fully elucidate the mechanisms of the pathways.

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