Repair of Large Insertion/Deletion Heterologies in Human Nuclear Extracts Is Directed by a 5′ Single-strand Break and Is Independent of the Mismatch Repair System*

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The repair of 12-, 27-, 62-, and 216-nucleotide unpaired insertion/deletion heterologies has been demonstrated in nuclear extracts of human cells. When present in covalently closed circular heteroduplexes or heteroduplexes containing a single-strand break 5′ to the heterology, such structures are subject to a low level repair reaction that occurs with little strand bias. However, the presence of a single-strand break 5′ to the insertion/deletion heterology greatly increases the efficiency of rectification and directs repair to the incised DNA strand. Because nick direction of repair is independent of the strand in which a particular heterology is placed, the observed strand bias is not due to asymmetry imposed on the heteroduplex by the extrahelical DNA segment. Strand-specific repair by this system requires ATP and the four dNTPs and is inhibited by aphidicolin. Repair is independent of the mismatch repair proteins MSH2, MSH6, MLH1, and PMS2 and occurs by a mechanism that is distinct from that of the conventional mismatch repair system. Large heterology repair in nuclear extracts of human cells is also independent of the XPF gene product, and extracts of Chinese hamster ovary cells deficient in the ERCC1 and ERCC4 gene products also support the reaction.

Base pairing anomalies can occur within the DNA helix as a consequence of DNA biosynthetic errors or as a result of recombinational strand transfer between sequences that differ genetically (1–4). Such pairing errors may take the form of base-base mismatches or insertion/deletion (I/D) heterologies, in which one strand contains a segment of one or more unpaired nucleotides. Strand-specific correction of base-base and I/D mismatches produced during DNA biosynthesis plays an important role in mutation avoidance (2, 5, 6), and mismatch rectification within the recombination heteroduplex has been implicated in meiotic gene conversion in fungal systems (3, 4, 7, 8).

Base-base mispairs are subject to strand-specific correction by the mismatch repair systems of both prokaryotes and eukaryotes (5, 6, 9), but action of this system on I/D mismatches is limited to fairly small heterologies. The *Escherichia coli* mismatch repair pathway will correct I/D heterologies up to about 7 unpaired nucleotides, but larger heterologies are poorly processed by this system (10–13). A similar specificity is characteristic of the human mismatch repair system, which can correct I/D heterologies up to about 8 unpaired nucleotides (14–16).

There is evidence, in some cases contradictory, that both prokaryotes and eukaryotes can rectify I/D heterologies with larger unpaired segments by a pathway distinct from the mismatch repair system. Using transfection assay, Dohet *et al.* (10) demonstrated rectification of a bacteriophage λ heteroduplex containing an 800-nucleotide unpaired IS1 heterology by a pathway that was independent of *mutH*, *mutL*, and *mutS* gene function. In contrast, Carraway and Marinus (12) failed to detect repair of large heterologies upon transformation of covalently closed circular plasmid heteroduplexes into wild type *E. coli*. Although strand breaks are known to be required for efficient correction by conventional mismatch repair systems (6, 17), a potential activating role for strand discontinuities in large heterology repair in *E. coli* has not been reported.

Transformation of monkey cells with heteroduplex DNAs containing unpaired single-stranded loops has indicated that mammalian cells can rectify such structures (18–21). Heterology repair as scored by transformation assay is biased about 2:1 for heterology removal (19), and the presence of a single-strand break near the unpaired loop was found to alter repair outcomes to a significant but limited degree (21). *In vitro* experiments have also suggested that human cells possess a system distinct from the mismatch repair pathway for processing heteroduplexes with large I/D heterologies. For example, Umar *et al.* (15) found that incised heteroduplexes containing 8- and 16-nucleotide I/D mismatches were repaired in nick-directed fashion in extracts of a mismatch repair-defective *MLH1*–/– cell line. Similar results have been obtained by Genschel *et al.* (16), who demonstrated that a nicked heteroduplex containing an 8-nucleotide I/D heterology is processed in a strand-specific manner and to similar extents by the mismatch repair system and by a second pathway that is independent of MSH2 function. A 27-nucleotide I/D heterology was shown to be processed only by the latter system. Although the asymmetric rectification of the larger I/D heterologies in both of these studies was consistent with nick direction, definitive conclusions on this point have been precluded by the fact that individual heterologies were tested in only one strand orientation. Furthermore, the nicks responsible for putative strand direction in these studies were located 5′ to the I/D heterology; the effect of 3′ nick placement was not tested, nor was the depend-

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1 The abbreviations used are: I/D, insertion/deletion; V, viral; C, complementary; bp, base pair(s).

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ence of the efficiency of rectification on the presence of a strand break.

In order to further define the nature of the reaction responsible for repair of large extrahelical segments, we have constructed a set of heteroduplexes containing unpaired I/D loops of 12–993 nucleotides, evaluated the dependence of repair on presence and placement of a single-strand break, and tested the possibility that strand placement of a genetic heterology may confer asymmetry on the rectification process.

EXPERIMENTAL PROCEDURES

Bacteriophages and Heteroduplex Construction—A set of f1MR bacteriophages for construction of heteroduplex DNAs has been previously described (14, 23–26). Bacteriophage f1MR1 was derived from f1A by elimination of the CiaI site at position 6040 by oligonucleotide mutagenesis, followed by insertion of a 27-base pair synthetic duplex into the EcoRI site at position 5617, destroying the latter site (23, 24). The intermediate phage in this protocol, which retains the EcoRI site, is designated f1MR11 (Fig. 1). Bacteriophages f1MR9, f1MR24, and f1MR28 have been described previously (14, 24). Phage f1MR32 was constructed by insertion of 5′-AGTCGCCAAGCAGCTGCTGTG- GTGCC-3′ annealed with 3′-CGTCGCGTGACGCAACACCGCATC-5′ into f1MR1 (23, 24) that had been digested with HindIII and XhoI. Phage f1MR30 was constructed by insertion of a HindIII-XhoI restriction fragment derived from plasmid pBR322 (coordinates 29–229) into HindIII, XhoI-cleaved f1MR1, whereas f1MR31 was constructed by insertion of a HindIII-XhoI fragment from plasmid pQE-30 (Qiagen, coordinates 187–1164) into f1MR1 digested in a similar manner.

Heteroduplex DNAs were constructed by hybridization of restriction endonuclease-cleaved replicative form DNA to single strands isolated from f1MR virions (25, 27). Substrates containing one or a few unpaired nucleotides were prepared using phage DNAs described previously (14, 16, 26). Other heteroduplexes used in this study are summarized in Fig. 1, with the unpaired heterology placed either in the viral (V) or complementory (C) DNA strand as specified. For preparation of the heteroduplex containing a 62-nucleotide insertion in the C strand, the oligonucleotide d(CTAGAC(TG)73CA) (14) was added to the hybridization reaction. The presence of the oligonucleotide prevented the formation of branched complexes in which a phage viral strand hybridized to the 62-nucleotide unpaired segment within the heteroduplex. As judged by resistance to NheI (Fig. 1), isolated preparations of this heteroduplex were free of significant levels of the oligonucleotide. A similar problem, which could not be circumvented in this manner, was encountered in attempts to prepare heteroduplexes with larger insertions in the C strand. For this reason, unpaired 216- or 993-nucleotide heterologies were placed in the viral strand only.

Heteroduplexes were prepared in covalently closed circular form or contained a site-specific, strand-specific nick as indicated. In the latter case, DNAs contained a strand break in the C strand at the ScaI or HindIII or HincII site, or in the V strand at the cleavage site for f1 gpII protein (25, 27). Nicks placed in this manner were 114 bp 5′, 797 bp 5′, or 170 bp 3′ to the I/D mismatch, respectively, as measured along the shorter path joining the two DNA sites in the circular substrates (Fig. 1).

Nuclear Extracts and Repair Reactions—Human cell lines HeLa S3, M1T1, H6, SO, LoVo, and HEC-1-A were grown as described (14, 26–29). Chinese hamster ovary cell lines UV20 and UV41, which were provided by Dr. Aziz Sancar (University of North Carolina, Chapel Hill, NC), were grown as described previously (30). The human GM08437 cell line, which was obtained from the NIGMS/Coriell repository, was cultured in supplements (C) DNA strand as specified. For preparation of the heteroduplex containing an unpaired 27-nucleotide loop in the complementary strand, the number of unpaired nucleotides in the heterology, whereas 3′-V216 refers to the heteroduplex with a 216-nucleotide unpaired segment in the viral strand with the nick located 3′ to the heterology. With all of the substrates used, heterology rectification and any associated strand specificity can be monitored by restriction endonuclease assay (Fig. 1).

Analysis of Repair Intermediates Produced under Conditions of Limited Repair-DNA Synthesis—Trapping of intermediates resulting from excision of I/D heterologies was accomplished by omission of exogenous dNTPs from reactions and thereby restricting repair DNA synthesis (25). Excision tract end points were mapped relative to an indicated restriction endonuclease cleavage site by indirect end labeling (31) after electrophoresis through 1.5% alkaline agarose (0.03 N NaOH, 2 m EDTA) or 10% denaturing polyacrylamide gels (50 m Tris borate, pH 8.3, 1 m EDTA, 8.3 m urea). After transfer and UV cross-linking to an ICN Biotrans nylon membrane, that portion of a heteroduplex strand of interest was visualized by probing with a 5′-32P-labeled oligonucleotide that hybridized near the strand terminus produced by restriction cleavage (25). Oligonucleotides V2531 (d(ATGGTTCATTTGATACAGTT), corresponding to f1MR11 viral strand nucleotides 2531–2550) and V3216 (d(ATGGTTCATTTGATACAGTT), corresponding to viral strand nucleotides 5216–5235) were used as probes to end label complementary strand products produced by Bsp106 or SpfI cleavage (Fig. 1).

RESULTS

Strand-specific Repair of Large I/D Heterologies Is Directed by a 5′ Strand Break—Although the human mismatch repair system is able to repair I/D mismatches in a nick-directed, strand-specific manner (14, 15, 26), the activity of this system on such structures appears to be restricted to heterologies containing less than about eight unpaired nucleotides (15, 16). Incised heteroduplexes containing 12-, 16-, and 27-nucleotide I/D heterologies are also processed by human nuclear extracts, but in these cases, rectification is independent of MSH2 and MLH1 mismatch repair proteins (15, 16). Although repair of such structures has been found to occur on the incised DNA strand, the role of the nick in directing the reaction has been uncertain due to the fact that the tested substrates contained the heterology in only one of the two possible unpaired configurations. Hence, it has been unclear whether the observed repair asymmetry is due to presence of a strand break or to asymmetry imposed on the DNA by the unpaired heterology itself.

In order to clarify the nature of this reaction, we have constructed a set of circular heteroduplexes containing unpaired heterologies ranging in size from 12 to 993 nucleotides (Fig. 1), in several cases in both possible configurations, and have evaluated the role of strand-specific single-strand breaks in the processing of such structures. We designate these heteroduplexes according to the DNA strand containing the unpaired segment, the number of unpaired nucleotides in the heterology, and placement of the strand break 5′ or 3′ to the heterology as viewed along the shorter path joining the two DNA sites in the circular DNA. In this convention, 5′-C27 indicates a heteroduplex containing an unpaired 27-nucleotide loop in the complementary DNA strand with the strand break located 5′ to the heterology, whereas 3′-V216 refers to the heteroduplex with a 216-nucleotide unpaired segment in the viral strand with the nick located 3′ to the heterology. With all of the substrates used, heterology rectification and any associated strand specificity can be monitored by restriction endonuclease assay (Fig. 1).

As summarized in Table I, unpaired heterologies of 27 or 216 nucleotides were subject to limited processing in HeLa nuclear extracts when present in a covalently closed circular DNA. However, heterologies in this size range were efficiently rectified in open circular DNAs containing a single-strand break 114 or 797 bp 5′ to the unpaired region in the 6.4-kilobase pair circular heteroduplexes (Fig. 2 and Table I). Repair of these...
large heterologies was 30–70% of that observed for a G-T mismatch or T, A, or GT I/D mispairs, all of which are processed by the conventional mismatch repair system (14, 16, 26, 27, 32, 33). Significant repair with some strand bias was also observed for the 5′-heteroduplex containing a 993-nucleotide unpaired region, but this substrate was processed less well than those containing the smaller nonhomologous segments.

A single-strand break located either 3′ or 5′ to a mispair is sufficient to provide strand specificity for mismatch repair MutSc- , MutSβ-, and MutLo-dependent human mismatch repair system (Ref. 25 and the last two entries of Table I). As described above, a single-strand break located 114 or 797 bp 5′ to the heterology supports strand-specific repair of 27-, 62-, and 216-nucleotide heterologies. However, a nick located 170 bp 3′ to the heterology was ineffective in this respect. As can be seen in Table I and Fig. 3, repair of 3′-C27, 3′-V27, and 3′-V216 heteroduplexes was not significantly different from that observed with the corresponding covalently closed, circular substrates, and no strand specificity was evident in the low level repair values obtained with any of these 3′-heteroduplexes.

It is important to note that whereas all of the 5′-heteroduplexes used in this study contained the strand break in the complementary strand, tested substrates included several with the unpaired nonhomology present in either the complementary or viral strand (Table I). When the nonhomologous segment was present in the C strand, processing of the 5′-heteroduplex produced the deletion repair product, whereas presence of the nonhomology in the V strand yielded the insertion repair product. Consequently, the strand-specific asymmetry observed for repair of 5′-heteroduplexes cannot be attributed to the simple presence of an unpaired segment in a particular DNA strand. Rather, this effect must be due to the 5′ strand break. This conclusion is also consistent with the finding that unpaired heterologies of 27–216 nucleotides are processed only at a basal level and without evident strand bias when present in closed circular DNAs or in heteroduplexes containing a nick 3′ to the heterology (Table I and Fig. 3). The observation that the efficiency of repair directed by a 5′ strand break decreases with increasing distance between the heterology and the nick also supports this view. Thus, although basal processing of unpaired heterologies does occur, the presence of a 5′ strand break substantially increases the efficiency of the reaction and confers strand specificity on the process.

The low level of strand-independent repair that we observe with covalently closed circular heteroduplexes or 3′-substrates (Table I) could be the consequence of events directed by a 5′ strand break produced by endonucleases present in the extract (27). However, it is also possible that I/D heterologies are directly recognized and processed in a nick-independent fashion by other activities present in the nuclear extract. Some evidence supporting the latter view is described below.

**Requirements for Large Heterology Repair in Human Nuclear Extracts**—The only exogenous cofactors required for *in vitro* repair of large I/D heterologies by HeLa nuclear extracts are ATP, Mg2+, and the four dNTPs. Omission of any of these components resulted in substantial decrease in repair of the several 5′-heteroduplexes tested (Table II). The reaction was also inhibited by 90 μM aphidicolin but not by 0.5 mM ddTTP. Because aphidicolin is a specific inhibitor of DNA polymerases α, δ, and ε (34), whereas dideoxynucleotides are potent inhibitors of the β DNA polymerase (35, 36), these observations indicate involvement of polymerase α, δ, and/or ε in repair of large I/D mismatches. DNA polymerase δ has been previously implicated in the conventional mismatch repair system (37).

**Repair of Large Insertion/Deletion Heterologies Yields a Covalently Continuous DNA Product**—Repair by the bacterial...
mismatch correction pathway has been shown to culminate with ligation of the repaired DNA strand (38). As shown in Fig. 4, we have used an indirect end labeling method (25, 31) to determine whether large heterology repair is associated with ligation of the repaired DNA strand. As judged by production of the full-length (~6400 nucleotides) linear form after cleavage with Bsp106, the repaired C strand was recovered in covalently continuous form in 80–95% of the molecules isolated after incubation with HeLa nuclear extracts (Fig. 4, odd lanes 1–9). The small amounts of incised C strand evident in the Bsp106 hydrolysates is most likely derived from unligated heteroduplex. This point is most easily seen with the end-labeled C strand product produced by Bsp106 cleavage of the V216(H) heteroduplex that contained a single-strand break in the HindII site. As shown in Fig. 1, the distance between the Bsp106 site, which is the position of indirect end labeling, and the HindII site is 3884 nucleotides, a value that corresponds well to the 3900-nucleotide species observed after Bsp106 cleavage of DNA products obtained after incubation with HeLa nuclear extract.

When the products of heteroduplex incubation with HeLa nuclear extract were hydrolyzed with Bsp106 and the appropriate restriction enzyme diagnostic for strand-specific rectification, a fraction of the full-length 6400-nucleotide C strand was converted to a smaller species with a mobility similar to that of the 3100-nucleotide marker (Fig. 4, even lanes 2–10), which is the expected size of repair products (Fig. 1). However, with some 5‘-heteroduplexes containing a C strand nick at the Sau96I site, this repair product was evident only as an increase in signal over a background DNA species of similar size that is evident in samples treated with Bsp106 alone (e.g. see Fig. 4, lanes 3 and 4 or lanes 9 and 10). As discussed above for the V216(H) heteroduplex with a HindII nick, we think it likely that this background signal is due to persistence of some molecules with a discontinuity in the C strand at or near the Sau96I site. Due to the fact that the Sau96I strand break in these heteroduplexes is separated from the heterology by only 114 bp, this species is poorly resolved from the C strand fragment that is diagnostic for repair.

In view of this background problem, production of the covalently continuous repair product was confirmed by physical isolation of covalently closed, circular duplex DNA produced upon incubation of C27 and C62 heteroduplexes with HeLa nuclear extract. As shown in Fig. 4 (lanes 11–14), repaired

**TABLE I**

| Heterology | C strand nick (5’) | V strand nick (3’) | CC circle |
|------------|--------------------|--------------------|----------|
|            | C                  | V                  | C        | V      |
| C27        | 3.1 ± 0.72         | 0.17 ± 0.11        | 0.75 ± 0.73 | 0.62 ± 0.76 | 0.55 ± 0.07 | 0.76 ± 0.99 |
| V27        | 4.6 ± 1.0          | 1.2 ± 0.44         | 0.74 ± 0.42 | 1.1 ± 0.80 | 0.70 ± 0.54 | 0.41 ± 0.18 |
| C62        | 3.9 ± 0.56         | 1.1 ± 0.56         | 1.0 ± 0.08 | 1.5 ± 0.63 | 0.72 ± 0.55 | 0.94 ± 0.48 | 0.65 ± 0.23 |
| V62        | 3.9 ± 0.84         | 1.0 ± 0.08         | 0.46 ± 0.42 | 1.5 ± 0.63 | 0.72 ± 0.55 | 0.94 ± 0.48 | 0.65 ± 0.23 |
| V216       | 6.5 ± 1.3          | 0.46 ± 0.42        | 1.5 ± 0.63 | 0.72 ± 0.55 | 0.94 ± 0.48 | 0.65 ± 0.23 |
| V216(H)    | 4.5 ± 1.3          | 0.89 ± 0.24        | 0.82 ± 0.28 | 2.3 ± 0.80 | 0.84 ± 0.18 | 0.64 ± 0.23 |
| V993       | 1.9 ± 0.24         | 0.82 ± 0.28        | 2.3 ± 0.80 | 0.84 ± 0.18 | 0.64 ± 0.23 |
| C1         | 10 ± 1.7           | 2.3 ± 0.80         | 0.84 ± 0.18 | 0.64 ± 0.23 |
| V1         | 10 ± 2.1           | 2.9 ± 1.3          | 0.84 ± 0.18 | 0.64 ± 0.23 |
| V2         | 7.9 ± 2.3          | 1.7 ± 0.40         | 1.0 ± 0.20 | 9.5 ± 1.3 | 0.80 ± 0.70 | 1.5 ± 0.60 |
| G-T        | 9.2 ± 3.2          | 1.3 ± 1.1          | 1.8 ± 0.20 | 11 ± 1.2 |

**FIG. 2. Strand-specific repair of large I/D heterologies.** Repair in HeLa nuclear extracts (75 μg) was scored as described under "Experimental Procedures." Heteroduplexes contained a single-strand break in the complementary DNA strand at the Sau96I site (114 bp 5’ to the heterology) or in the case of the V216(H) heteroduplex at the HindII site (797 bp 5’ to the heterology). 3‘-Heteroduplexes contained an incision in the V strand at the gpII cleavage site (170 bp from the heterology). Repair shown for each DNA strand is the average of at least three determinations ± 1 S.D. C1, V1, and V2 heteroduplexes contained a T insertion on the V strand. CC circle, covalently closed circular heteroduplex DNA.
molecules were present in the closed circular population produced with both heteroduplexes.

Repair of Large I/D Heterologies Is Independent of MSH2, MSH6, MLH1, PMS2, and XPF Gene Products—In human cells, the conventional, nick-directed mismatch repair pathway is capable of processing the eight base-base mismatches as well as I/D heterologies of 1 to about 8 unpaired nucleotides (14–16, 25–27, 32). Previous experiments have shown that a 5’-heteroduplex containing a 16-nucleotide I/D heterology is subject to repair in MLH1–/– HCT116 cells (15) and that 12- and 27-nucleotide heterologies are repaired in MSH2–/– LoVo cells (16). Fig. 5 confirms and extends these observations. As expected, a 5’-heteroduplex containing a CA dinucleotide insertion in the complementary DNA strand is not significantly repaired in MSH2–/– LoVo cells (28), MLH1–/– H6 cells (39), or PMS2–/– MSH6–/– HEC-1-A cells (40) and is subject to limited correction in MSH6–/– MT1 cells (26, 41). Addition of purified MutSa restored a normal level of repair on the CA I/D heteroduplex to LoVo and MT1 extracts, and MutLa restored repair to the H6 extract. However, 12-, 27-, and 62-nucleotide heterologies were repaired in all extracts, and the addition of MutSa or MutLa had no significant effect on the degree to which they were processed. Repair of these larger heterologies can therefore occur by a pathway that is independent of MSH2, MSH6, MLH1, and PMS2.

Kirkpatrick and Petes (42) have found that rectification of an unpaired 26-nucleotide heterology during meiotic segregation in S. cerevisiae depends on the MSH2 and RAD1 gene products. As noted above, we have found the repair of large heterologies in human nuclear extracts to be independent of human MSH2.

In addition, we have tested this reaction in nuclear extracts derived from Chinese hamster ovary UV41 and UV20 cells deficient in ERCC4 or ERCC1 gene products respectively, dominant homologs of S. cerevisiae RAD1 and RAD10 proteins. Both extracts displayed repaired a 5’-C62 I/D insertion/deletion heterology normally, and similar results were obtained with the GM08437 human cell line deficient in the XPF gene product, the human homolog of RAD1 (not shown). Therefore, large heterology repair in extracts of mitotic mammalian cells apparently occurs by a pathway distinct from that responsible for heterology rectification in meiotic yeast cells.

**Analysis of Repair Intermediates Implies That Repair of Large I/D Heterologies Occurs by a Mechanism Different from Mismatch Repair—Restriction of repair DNA synthesis by omission of dNTPs or inclusion of aphidicolin has permitted visualization of excision intermediates that are produced during mismatch correction (25). We have used a similar approach in an attempt to trap excision intermediates produced during nick-directed repair of large heterologies. Fig. 6 shows the results of such an analysis with the V216 and C27 heteroduplexes containing a C strand nick at the Sau96I site 114 bp 5’ to the heterology junction (Fig. 1), as well as a single nucleotide T I/D heteroduplex with a nick at the same position. The complementary strand of each of these substrates was largely converted to a covalently closed species (6.4-kilobase pair band) under complete repair conditions. As observed previously for excision tracts generated by the mismatch repair system (25), excision intermediates were evident with the T I/D heteroduplex when dNTPs were omitted from the reaction. Under these conditions, about half of the molecules retained a discontinuity in the C strand, with the shortened 5’-termini mapping from

### Table II

**Reaction requirements**

Exptected for omissions or additions indicated, repair assays containing 75 µg of HeLa nuclear extract were performed as described under “Experimental Procedures.” All heteroduplexes contained a nick in the C DNA strand at the Sau96I site 114 bp 5’ to the heterology. 100% repair values were 4.7 fmol for V27, 3.1 fmol for C27, and 6.5 fmol for V216.

| Reaction conditions | V27 | C27 | V216 |
|---------------------|-----|-----|------|
| Complete            | (100) | (100) | (100) |
| -MgCl2              | <1 | <1 | <1 |
| -ATP                | <1 | 35 | <1 |
| -dNTPs              | 23 | <1 | <1 |
| +0.5 mM ddTTP       | 144 | 118 | 124 |
| +Aphidicolin (90 µM)| 16 | 22 | 1 |

**Fig. 4.** 5’-Heteroduplexes containing large I/D heterologies are repaired to covalently closed-circular products. Repair reactions (see under “Experimental Procedures”) were scaled up to 20 µl (lanes 1–10) or 50 µl (lanes 11–14) and contained 7.5 mg/ml HeLa nuclear extract and 10 µg/ml of the indicated 5’-heteroduplex. With the exception of the V216(H) heteroduplex, which contained a nick in the C strand at the HincII site, all DNAs contained a nick in the C strand at the Sau96I cleavage site (Fig. 1). For lanes 1–10, DNA isolated from reactions was divided into two parts. Half was linearized with Bsp106 (–), and the other half was digested with Bsp106 and the restriction endonuclease (EcoRI for C27 and C62, NheI for V27, V216, and V216(H)) diagnostic for repair (+). After electrophoresis through alkaline agarose and transfer to nylon membranes, the complementary DNA strand was visualized by indirect end labeling by hybridization to 5’-32P-labeled dATGTTTTCATTGGTACGGT (see under “Experimental Procedures” and Ref. 25). This synthetic oligonucleotide, which corresponds to V strand nucleotides 2531–2550, hybridizes to the 3’-end of the linear form of the C strand produced by cleavage with Bsp106 (Fig. 1). For the samples shown in lanes 11–14, DNA isolated from repair reactions was subjected to native agarose gel electrophoresis in the presence of 0.5 µg/ml ethidium bromide (see under “Experimental Procedures”) and covalently closed relaxed DNA recovered from the gel. Relaxed DNA samples were then analyzed as described above for lanes 1–10. DNA standards were run on each gel (not shown).
the nick to about 200 nucleotides beyond the original location of the mispair. Different results were obtained with C27 and V216 heteroduplexes. Under complete reaction conditions, about 1% of the recovered molecules contained discrete discontinuities in the vicinity of the original location of nick and the heterology, and these species increased dramatically upon omission of dNTPs (Fig. 6).

In view of the proximity of the heterology and the strand break in these heteroduplexes (separation distance of only 114 bp), these putative excision intermediates were examined at higher resolution by mapping relative to the 3'-end in the C strand by hydrolysis with SspI, which cleaves 400 nucleotides from the position of the I/D heterology (Fig. 1). As shown in Fig. 7, omission of dNTPs resulted in a substantial reduction in production of repair products, an effect most easily seen with the V216 heteroduplex, where the 933-nucleotide repair fragment is well resolved from the 717-nucleotide fragment in the unrepaird DNA (lanes 2 and 4). Omission of dNTPs also resulted in accumulation of major C strand species with termini mapping to a region just 5' of the position of the heterologies for both C27 and V216 heteroduplexes (Fig. 7, lanes 3 and 4). A second species of about 400 nucleotides corresponding to a discontinuity in the C strand at the location of the heterology was also evident with both heteroduplexes, but as can be seen, this species was also produced from the covalently closed circular form of the C27 substrate and was observed with the incised C27 heteroduplex in the presence of dNTPs. Hence, the latter species may be unrelated to the strand-specific repair reaction described here, although it could be implicated in the low level of strand-independent repair that we have observed (Table I). Because neither of these species was observed with the incised A-T homoduplex control (Fig. 7, lane 7), their production is clearly dependent on presence of an I/D heterology. Discontinuities corresponding to the location of the Sau96I strand break were observed in a small fraction of V216 and C27 heteroduplexes after incubation with HeLa extract in the presence of dNTPs (Fig. 7, lanes 2 and 3, 515- and 542-nucleotide species). Similar discontinuities were evident in C strand products obtained in the absence of dNTPs, but in this case, the yield was elevated, and species of reduced chain length were present (Fig. 7, lanes 4 and 5), indicative of excision from the strand break. Similar degradation products were not observed with the A-T homoduplex control DNA (Fig. 7, lane 7). Although these observations do not suffice to establish the mechanism for nick-directed repair of large I/D heterologies, they clearly show that this reaction occurs by a mechanism that is distinct from that of mismatch repair as deduced using similar methods (25).

**DISCUSSION**

As judged by dependence of repair on MSH2 and MLH1 products, the processing of I/D mismatches of 1–4 nucleotides in human cell extracts occurs almost exclusively by mismatch repair (14, 15, 26, 33). Based on the rates of repair observed in nuclear extracts of MSH2+/− or MLH1−/− cells as compared with those observed when such extracts are supplemented with the deficient activities, we have found that a 5-nucleotide I/D mismatch is rectified primarily by the mismatch repair system, although limited MSH2 and MLH1-independent repair does occur (Ref. 16 and this study). Analogous experiments with 8-
and 12-nucleotide I/D heteroduplexes have indicated that the former substrate is processed to a similar degree by the MSH2- and MLH1-dependent and -independent pathways, with rectification of the latter DNA being largely independent of the mismatch repair system. We show here that 27-, 62-, and 12-nucleotide I/D heterologies are efficiently processed in human nuclear extracts by a reaction that is independent of MSH2, MSH6, MLH1, and PMS2, clearly distinct from the conventional mismatch repair system.

Although large heterologies were subject to limited processing when present in covalently closed circular heteroduplexes or heteroduplexes containing a single-strand break 3' to the heterology, repair was enhanced substantially by a single-strand break placed 5' to the I/D heterology, and in this case, rectification was highly biased to the incised strand. This dependence on nick heterology orientation also distinguishes the reaction from mismatch repair, which can be directed to a particular strand by an incision located either 3' or 5' to the mispair (25). The two pathways are also dissimilar, as judged by the nature of excision intermediates that accumulate upon restriction of repair DNA synthesis (Fig. 7 and Ref. 25).

Transfection of monkey cells with SV40 heteroduplexes by Weiss and Wilson (19, 21) has previously indicated that large I/D heterologies are efficiently rectified in mammalian cells. Although presence of a strand break 71 or 125 nucleotides from the unpaired loop was found to bias rectification to a significant degree in this system, the primary determinant of repair was found to be the heterology itself (21). In contrast, we have observed only a limited degree of nick-independent processing of large I/D heteroduplexes in nuclear extracts of human cells, although we have detected a possible product of endonuclease activity was observed in extracts of yeast MSH2. Although a 60% reduction in repair in yeast extracts of mitotic S. cerevisiae analyzed by Weiss and Wilson (21) were prepared by denaturation and hybridization of two duplex DNAs and thus contained a mixture of the two combinations of the parental strands. As they note (21), use of such a mixture would artificially depress the effect of a strand break on repair in their transfection assay if the nick-directed reaction were to depend on a particular chemical polarity as we have found to be the case in extracts of human cells.

Genetic analysis has indicated that large I/D heterologies are repaired during meiosis in S. cerevisiae in a reaction that depends on the yeast MSH2 and RAD1 gene products (42). The nick-directed reaction that occurs in extracts of mitotic human cells described here apparently occurs by a distinct pathway because it is independent of MSH2 and the mammalian homologs of yeast RAD1 and RAD10. A study by Lahue and co-workers (43) describes a system for study of large heterology repair in extracts of mitotic S. cerevisiae. As in the case of the cell-free human system described here, repair in yeast extracts is independent of yeast MSH2. Although a 60% reduction in activity was observed in extracts of rad1 mutant cells, the addition of purified RAD1 protein or the RAD1/RAD10 complex failed to restore normal levels of repair to rad1 cell-free extracts.

The demonstration of strand-specific repair of large I/D heterologies in human cell extracts raises questions concerning the functions of this reaction. Although such a system may function in the processing of recombination heteroduplexes, the strand specificity of the reaction suggests a role in correction of...
I/D heterologies that arise by DNA misalignment events during chromosome replication (22). If a potential role in replication fidelity is assumed, the obligate polarity imposed by the requirement for a 5′ strand terminus would presumably restrict action of the system to the lagging DNA strand, where 5′ terminal discontinuities occur. Isolation of the activities involved in the pathway and the identification of the corresponding structural genes should serve to clarify the roles of this system.

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