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Nick-directed repair of palindromic loop mismatches in human cell extracts

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Summary

Palindromic sequences present in DNA may form secondary structures that block DNA replication and transcription causing adverse effects on genome stability. It has been suggested that hairpin structures containing mispaired bases could stimulate the repair systems in human cells. In this study, processing of variable length of palindromic loops in the presence or absence of single-base mismatches was investigated in human cell extracts. Our results showed that hairpin structures were efficiently processed through a nick-directed mechanism. In a similar sequence context, mismatch-containing hairpins have higher repair efficiencies. We also found that shorter hairpins are generally better repaired. A strand break located either 3′ or 5′ to the loop is sufficient to activate hairpin repair on the nicked strand. The reaction requires Mg^{2+}, the four dNTPs and hydrolysis of ATP for efficient repair on both palindromic loop insertions and deletions. Correction of each of these heteroduplexes was abolished by aphidicolin but was relatively insensitive to the presence of ddTTP, suggesting involvement of polymerase(s) α and/or δ. These findings are most consistent with the nick-directed loop repair pathway being responsible for processing hairpin heterologies in human cells.

Introduction

Palindromic DNA sequences in the human genome have a variety of important biological roles. For example, palindromes have regulatory roles in promoters (e.g. [1, 2]), including those transactivated by p53 [3, 4]. Palindromes also play a role in chromatin interactions with the nuclear matrix [5]. Inverted repeats in the palindromes can engage in intra- and intermolecular base pairing [6]. Such repeats have structural roles in tRNA and some ribonuclear proteins. They also contribute to genetic instability and gene amplification (for reviews, see [7]).

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Insertion mutations produce loop mismatches in heteroduplex DNA, and loops are also produced by DNA polymerase slippage [8]. Nonpalindromic insertions produce single-stranded loops, whereas palindromic insertions of sufficient length produce stem-loop structures. Palindromic sequences as short as 12-nt are predicted to form a stable stem loop structure [9]. Studies suggest that during DNA replication, a hairpin structure forms preferentially on the lagging strand template [10–12]. The ability to adopt hairpin and cruciform secondary structures is associated with frameshift mutations. These sequences can also be utilized by the polymerase allowing both intra- and interstrand switching events [13, 14]. Such mechanisms can involve imperfect inverted repeats and lead to

additional mutations. Hairpins and cruciforms may also act as a block to replication fork progression and obstruct transcription.

In addition, inverted repeats pose a special impediment to DNA replication fidelity. Several human genetic diseases illustrate inverted repeat mediated mutagenesis, such as hereditary angioneurotic edema [15], a subset of Duchenne muscular dystrophy [16], biotinase deficiency [17], and familial hypercholesterolemia [18].

Genetic studies in mammalian cells demonstrated that palindromic loops are processed via different mechanisms. Hairpin loops were efficiently repaired *in vivo* [9, 19–21]. The repair bias was dependent on loop length, shifting with increasing loop size from hairpin retention (12-nt) to hairpin loss (26- and 40-nt) [21]. However, when the loop was presented with a single-base mismatch, the bias was eliminated by nick-directed mismatch repair [9]. In contrast to mammals, palindromic loops longer than 12 bases are poorly repaired in yeast cells [22, 23].

The mismatch repair pathways mediated by the prokaryotic MutHLS system and their eukaryotic homologues are known to correct single base mismatches as well as small nucleotide insertions/deletions in a strand-specific manner [24, 25]. However these systems are limited to 7 unpaired nucleotides in *E. coli* [26] and about 8 unpaired nucleotides in human cells [27–30].

For processing large loops, mechanisms independent of the mismatch repair pathway have been observed in *E. coli*, yeast, and human. Studies have reported loops of 16, 27 and 216 bases can be repaired both *in vivo* and *in vitro* by mismatch repair-deficient yeast strains [31, 32]. Human mismatch repair-deficient cell extracts are known to repair 8-nt [28] to as high as 216-nt loops [33] in a nick-directed fashion. Recently, we also found a nick-directed loop repair pathway present in *E. coli* that can correct loops up to 429 nucleotides [34].

Hairpin specific removal of DNA secondary structures in *E. coli* is associated with the SbcCD protein [35]. In the presence of ATP, SbcCD possesses a double strand exonuclease activity. Without ATP, it can only cleave single-stranded DNA [36, 37]. Hairpin-loop structures resulting from replication can be excised by SbcCD *in vitro* [35], but double strand breaks consequently occur as a result of SbcCD cleavage *in vivo* [38] and can be repaired via recombination [39]. In mammalian cells, Rad50 and Mre11 form a large multiprotein complex [40] that shares sequence similarity to SbcCD proteins [35]. They also function in hairpin loop cleavage and facilitate the repair of DNA double-strand breaks *in vitro* [41] and *in vivo* [42].

To characterize hairpin processing activities in human cell extracts, we have constructed a set of hairpin-containing heteroduplexes. We demonstrate that *in vitro* processing of hairpins was nick-directed; and some features of repair activity suggest a large loop repair system in humans.

Materials and methods

Human cell lines and extracts preparation

HeLa S_3 cell extracts were prepared as described for human mismatch repair assays [43].

Construction of f1hp bacteriophage mutants

The phages used to create the hairpin substrates were constructed by insertion of a 50-base pair palindromic oligonucleotide into the *Hin*dIII cleavage site of f1P [44] disrupting the original *Hin*dIII site (Figure 1). Two nucleotide randomized positions residing in four overlapping restriction endonuclease recognition sites of the linker allowed for screening mutants by restriction analysis of replication form (RF) DNA. Mutant sequences were then confirmed by DNA sequencing. Mutant f1hp phages derived in this study are summarized in Table 1.

To construct hairpins of different lengths, f1hpT-A was digested with *Afl*II and re-ligated to produce f1hpS, a palindromic sequence of 20-nt. An elongated palindrome was also constructed by inserting a linker of 5′-CTAGCCATGGAATT-CCATGG-3′ into the*Spe*I site of f1hpT-A to generate f1hpL a 70-nt palindromic sequence (Figure 2b).

Hairpin containing heteroduplexes construction

E. coli strain NM522 was used to propagate RF f1 phage DNA and a *dam* strain RS5033 was used to propagate viral strand f1 phage DNA [26] for heteroduplex preparation.

Heteroduplex DNA substrates were prepared as described [34]. Phage f1hp mutant RF DNA

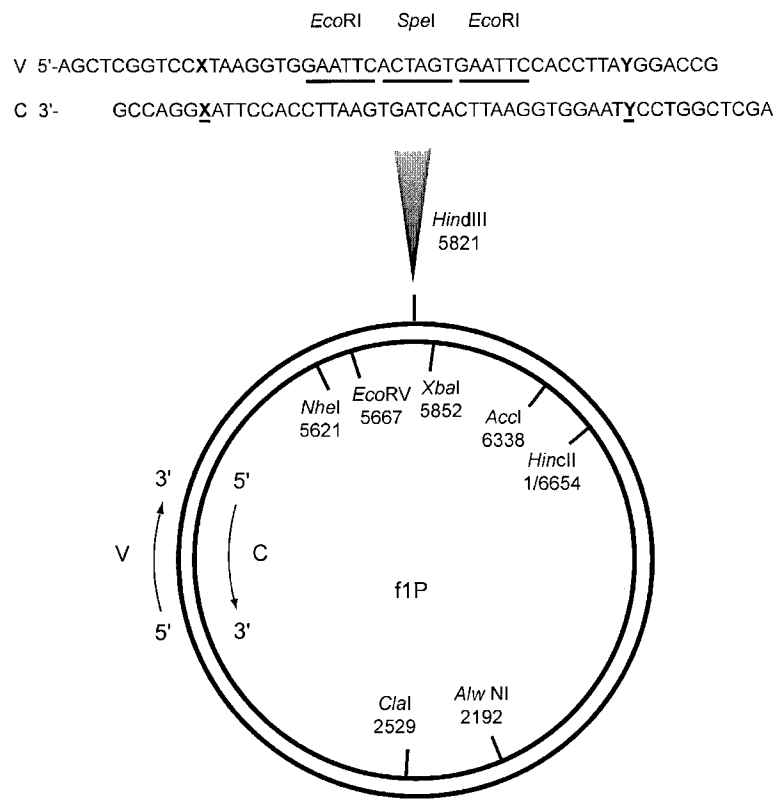

Figure 1. Phage f1P mutants containing palindromic sequences. Circular 6654-base pair map of the f1P shows restriction sites relevant to this study. Coordinates and the locations of the single-strand breaks tested are indicated by lines that contact one DNA strand only. A 50-bp palindromic oligonucleotide was inserted into *Hin*dIII site as shown. *X*,*X* and *Y*,*Y* are randomized pairs of sites that were designed to form base–base mismatches within the hairpin structure.

Table 1. Bacteriophage f1hp derivatives for heteroduplex preparation.

f1hp mutants	Nucleotide displacement			
	X	Restriction marker	Y	Restriction marker
f1hpT–A	T	*Afl*II	A	*Afl*II
f1hpC–G	C	*Bsu*36I	G	*Bsu*36I
f1hpA–C	A	*Pfl*MI	C	*Cpo*I
f1hpT–G	T	*Afl*II	G	*Bsu*36I
f1hpG–A	G	*Cpo*I	A	*Afl*II
f1hpC–T	C	*Bsu*36I	T	*Pfl*MI
f1hpA–A	A	*Pfl*MI	A	*Afl*II
f1hpT–T	T	*Afl*II	T	*Pfl*MI
f1hpG–G	G	*Cpo*I	G	*Bsu*36I
f1hpC–C	C	*Bsu*36I	C	*Cpo*I

Bacteriophage f1hp derivatives contain a 50-bp palindromic synthetic linker:

V strand 5'-AGCTCGGTCC**X**TAAGGTGGAATTCACTAGTGAATTCCACCTTA**Y**GGACCG
C strand 3'- GCCAGG**X**ATTCCACCTTAAGTGATCACTTAAGGTGGAAT**Y**CCTGGCTCGA

X and **Y** are the randomized nucleotide sites in viral strand; **X** and **Y** are the pairing nucleotides in the complementary strand. The randomized positions were designed to reside in four overlapping restriction endonuclease recognition sites to determine mutant identities by restriction analysis of replication form DNA.

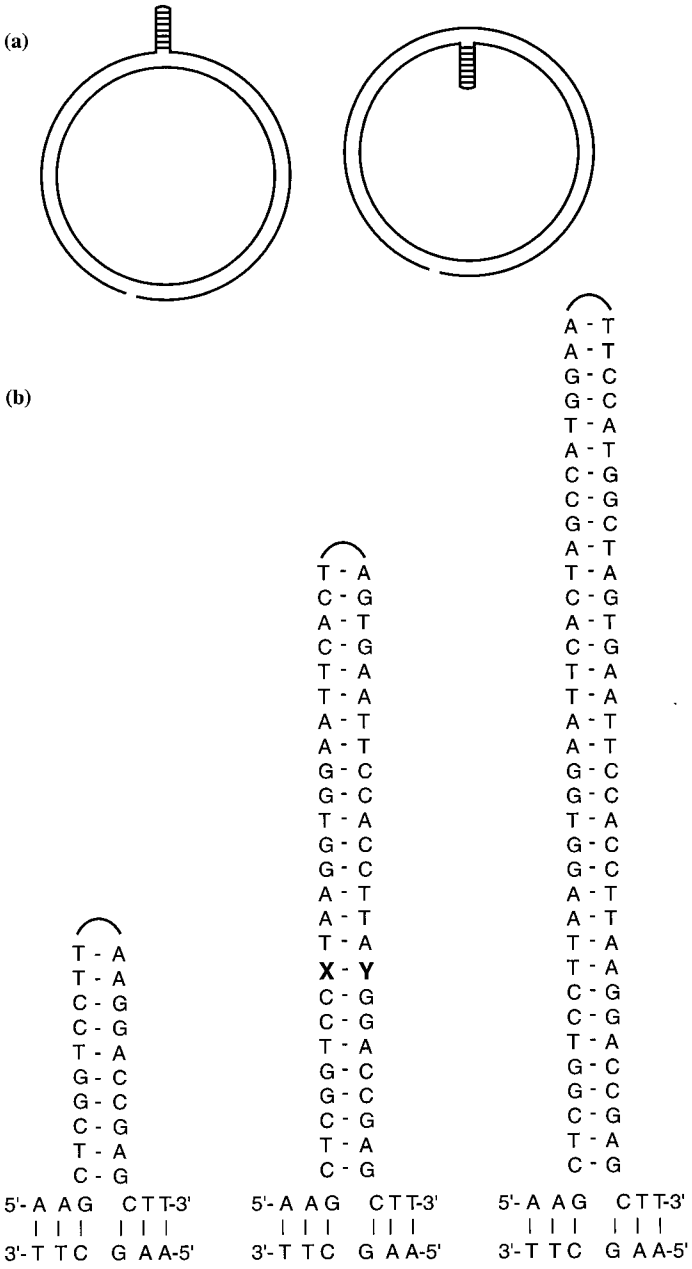

Figure 2. Schematic structures of the hairpin substrates. Heteroduplexes used in this study composed of variable length hairpins at the *Hin*dIII site (coordinate 5821) in the complementary (*Chp*-substrates) or viral (*Vhp*-substrates) strands. The number of hairpin substrates *hp20*, *hp50* and *hp70* indicate the amount of nucleotides within the hairpin structures. The X and Y in hp50 are the randomized sites that allow the making of base–base mismatches within the hairpin structure.

was linearized with *Cla*I and mixed with a 4-fold molar excess of viral DNA for alkaline denaturation and re-annealing. Heteroduplex DNA was sequentially purified by hydroxyapatite chromatography, ATP-dependent DNase digestion, Sephadex G-200 (Sigma) chromatography, and finally benzoylated naphthylated DEAE cellulose (Sigma) chromatography. All substrates have a nick in the *Cla*I site on the complementary strand unless otherwise stated. To make covalently closed circular substrates, nicked substrates were ligated in the presence of ethidium bromide (96 mmol dye/mol of nucleotide).

Following exonuclease III (Pharmacia) treatment to remove residual nicked DNA, covalently closed circular substrates were purified by Sephadex G-200, and benzoylated naphthylated DEAE cellulose chromatography.

Heteroduplexes of base–base mismatch 3′-AA, and random large loop 5′-C32, and 5′-V32 were as described [44].

Heteroduplex repair assays

In vitro heteroduplex repair was described previously [45]. Reactions (10 µl) contained 20 mM Tris–HCl (pH 7.6), 5 mM $MgCl_2$, 110 mM KCl, 50 µg/ml BSA, 1 mM ATP, and 0.1 mM each of dATP, dGTP, dTTP, and dCTP; and 0.1 µg (22 fmol) of heteroduplex DNA. The optimum amount of human extracts was 9.0 mg of protein/ml. Incubation was at 37 °C for 30 min. The reaction was terminated by adding EDTA to 20 mM and SDS to 0.5% followed by proteinase K treatment at 37 °C for 30 min. DNA was purified by phenol extraction and ethanol precipitation. Repair was scored by restriction endonuclease digestion and agarose gel electrophoresis (Figure 3). The DNA products were quantified after ethidium bromide staining using a gel documentation CCD camera (UVP Ltd.) [26].

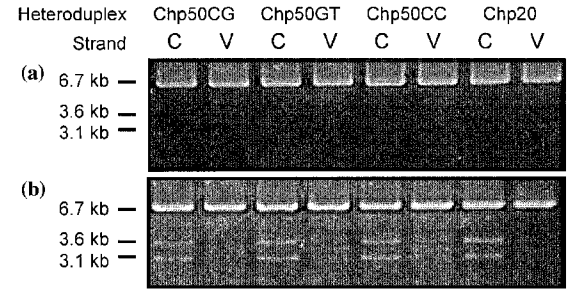

Figure 3. Repair of hairpin substrates in human cell extracts. Heteroduplex repair with human extracts was performed as described in the 'Materials and methods'. All the substrates contained a strand break at the *Cla*I site at the C strand. DNA products were digested with *Alw*NI and the appropriate diagnostic restriction endonuclease and then subjected to agarose gel electrophoresis to score heteroduplex correction on each DNA strand. C represents the scoring of reactions on the C strand and *V* represents the reactions on the V strand. Panel (a) untreated heteroduplex substrates digested with diagnostic restriction endonucleases. Panel (b) diagnostic restriction endonuclease scoring of the HeLa extracts repair reaction. The *bar* pointing to the 6.7 kb fragment represents unrepaired substrate, while *bars* 3.1 and 3.6 kb fragments indicate repaired products.

Results

Construction of heteroduplexes with hairpin structures

We constructed a set of heteroduplexes with a mismatch-containing hairpin structure by pairing a set of phage f1P [44] and f1hp mutant DNA (Figures 1 and 2, Tables 1 and 2). Each of these heteroduplexes contains a 50-nt hairpin structure at the *Hin*dIII site in either complementary or viral DNA strand. A site-specific strand break at variable positions in the heteroduplexes was designed to evaluate the potential activating role of the nicks. Covalently closed, circular heteroduplexes were also prepared for comparison. The covalently closed circular form heteroduplex substrates we prepared were in supercoiled form, and after purification 3–12% of the DNA remain nicked as judged by agarose gel electrophoresis. The proposed secondary structure generated by extra-helical palindromic sequences was tested by an *Eco*RI digestion; all of the covalently closed circular form Chp50 substrates in Table 2 can be relaxed to open circular form upon treatment with *Eco*RI (data not shown), implying the formation of a properly folded hairpin structure.

The design of the substrates permitted independent evaluation of correction on either DNA strand. Digestion of the unprocessed heteroduplex DNA with *Alw*NI and the indicator restriction endonuclease will yield 6.7-kb fragment only (Figure 3a). A similar digestion of DNA after a repair reaction with human extracts will yield 3.6- and 3.1-kb fragments (Figure 3b). All substrates used for this study were tested to ensure they were refractory to digestion by the indicator restriction endonucleases prior to repair or in the presence of EDTA-inactivated cell extracts (Figures 3a and 4, 0 min point; and data not shown).

A time course showing the amount of repaired products of a Chp50GT heteroduplex of both nicked and closed strands when incubated with HeLa extracts is shown in Figure 4. The extent of repair exceeded 95% and reached plateau by 30 min; this was chosen as the standard reaction time. A similar efficiency was observed with several other hairpin substrates (data not shown) indicating a similar repair rate profile.

Table 2. Hairpin heteroduplexes.

Substrates	C strand	Diagnostic enzyme	V strand	Diagnostic enzyme
Chp50TA	f1hpT–A	*Eco*RI	f1P	*Hin*dIII
Chp50CG	f1hpC–G	*Eco*RI	f1P	*Hin*dIII
Vhp50CG	f1P	*Hin*dIII	f1hpC–G	*Eco*RI
Chp50GT	f1hpA–C	*Eco*RI	f1P	*Hin*dIII
Chp50CA	f1hpT–G	*Eco*RI	f1P	*Hin*dIII
Chp50TC	f1hpG–A	*Eco*RI	f1P	*Hin*dIII
Chp50AG	f1hpC–T	*Eco*RI	f1P	*Hin*dIII
Chp50TT	f1hpA–A	*Eco*RI	f1P	*Hin*dIII
Chp50CC	f1hpG–G	*Eco*RI	f1P	*Hin*dIII
Chp50AA	f1hpT–T	*Eco*RI	f1P	*Hin*dIII
Chp50GG	f1hpG–G	*Eco*RI	f1P	*Hin*dIII
Vhp50GG	f1P	*Hin*dIII	f1hpG–G	*Eco*RI
Chp20	f1hpS	*Eco*RI	f1P	*Hin*dIII
Vhp20	f1P	*Hin*dIII	f1hpS	*Eco*RI
Chp70	f1hpL	*Eco*RI	f1P	*Hin*dIII
Vhp70	f1P	*Hin*dIII	f1hpL	*Eco*RI

Circular heteroduplexes containing a set of hairpin loop were prepared using the phage DNAs shown. Heteroduplexes with hairpin loops are depicted by Chp or Vhp, the complementary or viral strand where hairpin structures are located, followed by the number of nucleotides, and matched or mismatched base-pair formed within the hairpin. Diagnostic enzyme indicates the restriction endonucleases for scoring the repair.

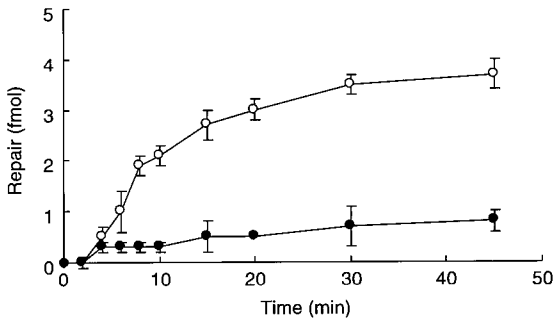

Figure 4. Time course of hairpin repair of nicked and closed strands in HeLa extracts.Heteroduplex repair with HeLa extracts was determined as described in 'Materials and methods'. Heteroduplexes of Chp50GT contained a single-strand break on the C strand at the *Cla*I site, 3293-bp away from the heterology was tested. Repair occurred in both nicked C strand (open circle) and closed V strand (closed circle) were assayed. The *error bars* represent one S.D. from three determinations. The input substrate in each reaction was 22 fmol.

Hairpin structures are processed in a nick-directed fashion in human extracts

To determine whether hairpin structures were corrected via the nick-directed mismatch repair pathway *in vitro*, heteroduplexes containing hairpin structures with a pre-existing nick were tested for repair in mismatch repair-proficient HeLa cell extracts. If repair occurred on the complementary strand, then the viral strand was used as the template for resynthesis and the product could be digested by the indicator restriction endonuclease present on the viral strand. This type of repair is designated as C in this study. When repair occurred on the viral strand using complementary strand as template is designated as V (Table 3 and Figure 3b). Figure 3 illustrates the behavior of several better-repaired examples in the restriction assay.

When subjected to HeLa cell extracts treatment, as shown in Table 3, all substrates displayed limited processing with little strand bias when present in covalently closed circular form (*ccc* entries). However, when heteroduplexes contained a pre-existed nick, all hairpin substrates showed bias on the open C strand (*Nicked* entries). In nick-directed reactions, repair efficiencies of removing hairpin structures containing mismatches (*Chp* substrate entries), were higher (range 2.5–4.6 fmol) than those of perfect-matched hairpins (range 1.9–2.2 fmol). Different mismatches varied in efficiency of repair as much as 1.8-fold.

Table 3. Hairpin repair in human cell extracts.

Substrate	Repair (fmol)			
	Nicked		ccc	
	C	V	C	V
Chp50TA	2.2 ± 0.06	0.4 ± 0.19	1.2 ± 0.16	0.5 ± 0.14
Chp50CG	1.9 ± 0.12	0.2 ± 0.10	0.6 ± 0.03	0.6 ± 0.17
Vhp50CG	3.7 ± 0.22	0.3 ± 0.06	0.3 ± 0.03	0.3 ± 0.06
Chp50GT	2.5 ± 0.24	0.7 ± 0.04	1.1 ± 0.26	0.4 ± 0.27
Chp50CA	3.8 ± 0.17	1.2 ± 0.20	1.0 ± 0.08	0.4 ± 0.18
Chp50TC	4.2 ± 0.31	0.5 ± 0.03	1.6 ± 0.07	0.4 ± 0.13
Chp50AG	3.1 ± 0.39	0.6 ± 0.14	0.7 ± 0.16	0.5 ± 0.18
Chp50TT	4.6 ± 0.04	0.9 ± 0.24	1.0 ± 0.25	0.4 ± 0.16
Chp50CC	4.5 ± 0.63	0.8 ± 0.26		
Chp50AA	3.0 ± 0.64	0.7 ± 0.12	1.2 ± 0.17	0.7 ± 0.21
Chp50GG	3.9 ± 0.10	0.8 ± 0.07	2.0 ± 0.28	0.4 ± 0.10
Vhp50GG	3.5 ± 0.25	0.8 ± 0.17	0.6 ± 0.15	0.5 ± 0.03

Repair in HeLa extracts (90 μg/100 ng substrate) were determined as described under 'Materials and methods'. Nicked form, substrates containing a *Cla*I nick in the complementary strand; ccc form, covalently closed, circular heteroduplexes. C, repair occurring on the complementary strand; V, repair occurring on closed viral strand. Repair results are averages of three separate experiments ± one S.D. Complete repair would correspond to 22 fmol.

The presence and location of the nick affects repair efficiency

Since the repair was biased to the nicked strand, we constructed covalently closed circular (ccc) forms of most substrates. The repair extent on the C strand of the ccc form substrates were at least 50% lower than that of the nicked substrates (Table 3). In order to assess dependence of the reaction on location of the nick, we tested five other HpTT heteroduplexes with differently placed nicks in the complementary strand. This set (Figure 5) includes two substrates with breaks 3′ to the hairpin, at the *Eco*RV (154 bp) or *Nhe*I (200 bp) sites, as viewed along the shorter path joining the two sites in the circular molecule. Three substrates had nicks 5′ to the hairpin with nicks at the *Xba*I, *Acc*I, or *Hin*cII sites: 31 bp, 517 bp, and 833 bp from the hairpin, respectively.

As summarized in Figure 5, in a covalently closed circular DNA (*ccc* entry) hairpin loop with a T–T mismatch was subject to low level processing with our human extracts and displayed little strand bias. The presence of a strand break substantially increases the efficiency of the reaction and confers strand specificity to the process. The efficiency of hairpin DNA processing is

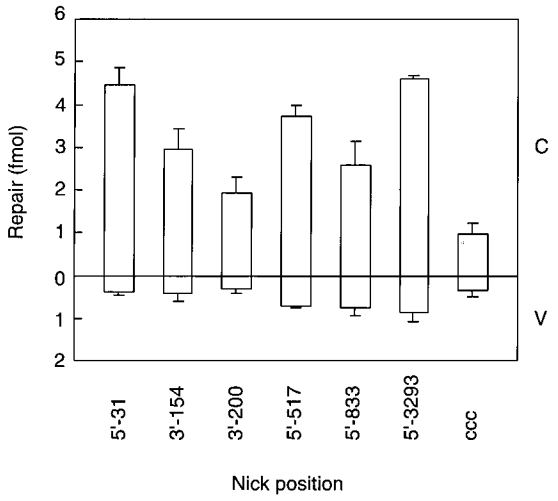

Figure 5. Nick positions and hairpin repair efficiency. Heteroduplex repair with mismatch repair proficient human cell extracts was determined as described in 'Materials and methods'. Heteroduplexes of Chp50TT contained a single-strand break on the C strand at the *Xba*I site 31-bp 5′ to the heterology (*5′-31*), at the *Eco*RV site 154-bp 3′ to the heterology (*3′-154*), at the NheI site 200-bp 3′ to the heterology (*3′-200*), at the *Acc*I site 517-bp 5′ to the heterology (*5′-517*), at the *Hin*cII site 833-bp 5′ to the heterology (*5′-833*), or at *Cla*I site 3293-bp away from the heterology (*5′-3293*). Covalently closed circular (*ccc*) heteroduplexes were also tested. Repair that occurred in both C and V strands was measured. The *error bars* represent one S.D. from three determinations. Complete repair would correspond to 22 fmol.

independent of the distance separating the heterologies and strand break since the substrates with a nick at *Alw*NI site (5′-3223) exhibited a similar level of repair to a nick closer to the heterologies (5′-31). This observation is similar to our recently reported bacterial nick-directed loop repair pathway [34], both 3′ and 5′ nicks can activate repair to the nicked strands, and the repair efficiency is independent of distances between the nicks and heterologies.

The low levels of products derived from covalently closed strands could be due to endogenous endonucleases present in the extracts.

Repair efficiency and hairpin size

To determine the repair efficiencies for hairpin structures of different sizes, we have constructed two substrates, one with a 20-nt and the other with a 70-nt perfect-matched hairpin at the *Hin*dIII site (Figure 2) and a strand break at the *Cla*I site. This design minimizes the effects of sequence context on hairpin repair efficiency. As shown in Figure 6, the repair efficiency varies with hairpin length. The substrate with the shorter hairpin structure (hp20; 20-nt) of both insertion and deletion configurations exhibited the higher repair efficiency when compared to the longest hairpins (hp70).

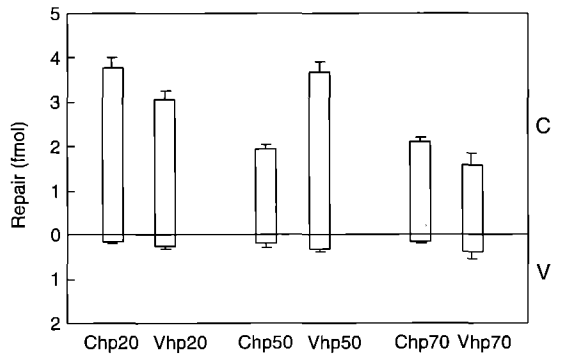

Figure 6. Repair efficiency and Hairpin length. Heteroduplex repair with HeLa cell extracts was determined as described in 'Materials and methods'. All the substrates tested contained a nick at *Cla*I site. Heteroduplexes containing 20-nt hairpin in the C strand (Chp20) and in the V strand (Vhp20); 50-nt hairpin in the C strand (Chp50) and in the V strand (Vhp50); 70-nt hairpin in the C strand (Chp70) and in the V strand (Chp70) were subjected to repair reaction. Repair (*white bar*) in both C and *V* strands were assayed. The *error bars* represent one S.D. from three determinations.

Requirements for hairpin repair are the same as nick-directed loop repair in human cells

Both mismatch repair and large loop repair by human cell extracts requires the addition of $MgCl_2$, ATP and the four dNTPs [33, 43]. In order to find out whether any of these components are essential for hairpin repair, we systematically omitted the cofactors in separate reactions. The repair efficiency of the reaction with all the components present was normalized to 100%. As shown in Table 4 and similar to large loop repair, Mg^{2+}, ATP and the four dNTPs are required for *in vitro* hairpin repair with human cell extracts. The repair was abolished when any of these components were omitted.

Exogenous ATP was also required: both loop and hairpin repair were significantly decreased by a non-hydrolysable ATP analog. The hairpin reaction was inhibited by ddTTP; and abolished by aphidicolin, similar to the loop repair in human (Table 4 and [28, 33]).

Discussion

In this study, we examined human cell extracts for their ability to process substrates with a hairpin structure. We demonstrated that hairpin loops can be efficiently repaired and the repair is biased to the nicked strand. We also used covalently closed circular (*ccc*) substrates and found that the relative repair of the C strand of the ccc form is significantly lower, suggesting that processing of hairpin heteroduplexes is nick-directed.

The results presented in this report confirm the earlier *in vivo* studies suggesting that a palindromic loop can be repaired in human cells [9, 21]. However, our results of repair specificity conflict with some of the genetic data [21]. In that study the repair bias of palindromic loops in mammalian cells was dependent only on loop length, with a bias shifting from loop retention to loop loss with increasing loop size. They employed a single-strand annealing model in an *in vivo* recombination scheme. In their design insertion mutations were equidistant from predicted nicks on both top and bottom strands. They hypothesized that such design eliminates potential bias caused by nick-directed repair [21]. In contrast, in our substrates only one DNA strand is nicked and the loop

Table 4. Reaction requirements of loop repair and hairpin repair.

Reaction conditions	3′-A–A	5′-C32	5′-V32	Chp20	Vhp20
Complete	100	100	100	100	100
Without Mg	< 1	< 1	< 1	< 1	< 1
Without dNTPs	13	< 1	< 1	< 1	< 1
Without ATP	13	< 1	< 1	< 1	< 1
Add ATP-γ-S	12	< 1	< 1	< 1	< 1
Add ATP/ATP-γ-S (1:1)	19	< 1	< 1	< 1	< 1
Add Aphidicolin (0.09 mM)	15	< 1	< 1	< 1	< 1
Add ddTTP (0.5 mM)	61	62	90	59	39

Repair assays containing 90 μg of HeLa cell extract was performed as described under 'Materials and methods'. Repair on the C strand was scored. Heteroduplex 3′-A–A is substrate containing a nick 3′ to the A–A mismatch; 5′-C32 and 5′-V32 are loop substrates containing a nick 5′ to the 32 bp loop on the C and V strand, respectively [44]. Repair values of 100% were 7.8 fmol for 3′-A–A, 4.6 fmol for 5′-C32, 1.7 fmol for 5′-V32, 3.8 fmol for Chp40, and 3.1 fmol for Vhp40.

retention or loss is exclusively dependent on the presence of this nick. Our results clearly demonstrate that a nick can activate and direct repair to the nicked strand regardless of the orientation of the nick (Figure 5). However, the mechanisms and the specific role of nick activation in different orientations are yet to be defined. Whether the predicted nicks equidistant from the heterologies would provoke a similar degree of repair remains to be determined. The nature and role of recombination intermediates in loop repair remains to be studied.

Previous studies in mammalian cells have shown that Mre11 alone exhibits endonuclease activity on hairpin structures [41]. When palindromic loops were on the nicked strand (C strand of nicked Chps substrates in Table 3), it is difficult to distinguish between nick-directed correction and Mre11-typed hairpin degradation. Both reactions occur on the same strand. Notably, in our reactions the removal of the hairpin loop on the C strand was greatly reduced upon covalent closure of the strand break (Table 3, reaction on C strand of ccc substrates). Furthermore, we only observed nick-directed hairpin fill-in and background levels of hairpin removal when the hairpins were on the closed strand opposite of the nick (nicked Vhps substrates in Table 3). These observations clearly rule out the involvement of the Mre11 pathway in our nick-directed reactions. However, our results can not exclude a hairpin-specific nicking activity in human cells. Our assay uses hairpin-containing circular DNAs and detects the restoration of interrupted restriction sites after hairpin heterologies are fully repaired. It would miss any intermediates that were the result of hairpin specific nicking activities.

Experiments in eukaryotic cells indicate that loop repair could be directed by a nick. This preference is shown in both human cell extracts [33] and yeast extracts [31]. Similar preference has been demonstrated in *E.coli* cells [34]. Several lines of evidence suggested that the correction of hairpin loops involves the same mechanism as the previously reported large loop repair [28, 33]. We have shown that *in vitro* DNA loop repair requires exogenous cofactors, namely $MgCl_2$, ATP and the four dNTPs. Our analysis of hairpin processing has shown that a strand break is required and enhanced repair efficiency is biased to the nicked strand. All of these features are very similar to nick-directed human loop repair [28, 33].

Our study showed that smaller hairpin loops were repaired better than larger ones, e.g. the shortest hairpin structure (20-nt) seems to provoke the highest repair activity. In addition, substrates with mismatches within the hairpin structure were repaired more efficiently than perfect hairpins. This may be due to the limitation of twist and writhe within hairpin structures. Longer palindromic sequences are more likely to form 'perfect-matched' double strand structure that may escape the detection by loop repair pathway, while shorter hairpins might retain some non-structured signature. By the same token, hairpin structures

containing mismatches may be similar to non-structured loops. Loops and mismatches have much shorter persistence lengths than native B-form DNA [46] and hence are inherently more flexible, a possible requirement for recognition or function of this repair system. Assuming a persistence length of approximately 150 bp consistent with native B-form DNA, hairpins would have to exceed several persistence lengths in order to be inherently flexible [47]. Consistent with this notion, even the longer homoduplex hairpins used here are not long enough to create flexible targets. Therefore repair that uses palindromic extrusions as a substrate could require unstructured DNA and or the flexibility that is associated with it as a preferred target. Thus proteins of the loop repair system would be able to recognize such extrusions more easily, and they would be more efficiently repaired. In yeast, loop repair appears distinct from that of hairpins. However, consistent with what we have observed here, it appears that hairpins in yeast are less efficiently repaired than random loops of the same size range [32].

The mechanism of this nick-directed palindromic and random loop repair as well as the enzymology of hairpin-loop repair in the human system remains to be determined. It is likely that some of the same proteins involved in replication or other repair systems are also involved in hairpin repair such as polymerases, DNA binding proteins and helicases. If this repair system has a similar function in preventing fixation of replication errors like the mismatch repair system, then replication and repair may well be coupled. Then the strand breaks or chain termini on the newly synthesized strand are recognized and used specifically as strand discrimination signals before they are ligated. An initial effort toward identifying the requirements of hairpin repair would use DNA polymerase inhibitors and withhold the exogenous co-factors. The blocking of repair by aphidicolin but only partial inhibition by ddTTP implicates the replicative polymerase α, δ, and/or ε as serving a role in hairpin repair. By identifying the polymerase(s) involved in repair, any known accessory proteins for that polymerase become logical candidates for hairpin repair. The *in vitro* system described here should provide an assay for purifying the components that catalyze this reaction and make it possible to elucidate the mechanism of hairpin repair in human cells.

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