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Repair of single-stranded loops in heteroduplex DNA transfected into mammalian cells

URSULA WEISS AND JOHN H. WILSON

Verna and Marrs McLean Department of Biochemistry, Baylor College of Medicine, Houston, TX 77030

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ABSTRACT Repair of heteroduplex DNA, generated between two interacting DNA molecules during homologous recombination, has been implicated as a contributing factor in the process of gene conversion. To assess patterns of heteroduplex repair in mammalian cells, we constructed 13 different heteroduplexes from simian virus 40 wild-type and deletion mutant DNAs. Each heteroduplex contained one or multiple single-stranded loops in the intron of the gene for large tumor antigen, which is not essential during lytic infection. After transfection into cultured monkey cells, cellular repair was evaluated by restriction analysis of the amplified viral progeny from 1123 individual plaques, each representing the clonal expansion of a single repair event. Single-stranded loops were corrected prior to replication with an overall efficiency of 90%. At the position of a loop, one of the two heteroduplex strands served as a template for accurate repair 98% of the time. Repair of single-stranded loops was biased nearly 2 to 1 in favor of the strand without the loop. The efficiency, accuracy, and strand bias of repair were unaffected by loop size within the tested range, which was 25–247 nucleotides. The excision tract associated with repair of single-stranded loops rarely exceeds 200–400 nucleotides in length.

Formation of heteroduplex DNA is a critical intermediate step in homologous recombination since it mediates the union of the two duplexes undergoing strand exchange (1, 2). Mismatched bases and unpaired single strands can form in heteroduplexes between nonidentical parental DNAs; their repair is thought to contribute to phenomena such as gene conversion, marker effects, and high negative interference (3–6). Correction of mispaired DNA sequences can be studied conveniently by introducing heteroduplexes of viruses or plasmids into cells (7–12).

Two modes of repair have been identified in bacteria and mammalian cells. One is sensitive to the methylation status of parental strands; it may function in the correction of mismatches that arise from errors during replication (7, 13, 14). The other is methylation independent; it is probably more relevant to correction of mismatches that arise during recombination (15–17). In this report, we examined mammalian cell repair of heteroduplexes that contain single-stranded regions. Although single-stranded loops represent an energy barrier to formation of heteroduplexes, the participation of lengthy insertions and deletions in heteroduplex DNA of recombining molecules has been deduced from the outcome of genetic crosses in fungi and bacteria and has been demonstrated physically in phage λ crosses (18, 19, 34). In addition, long insertions and deletions can be incorporated into heteroduplexes *in vitro* in an ATP-dependent reaction promoted by the *Escherichia coli* recA protein (20).

The simian virus 40 (SV40) system offers several advantages for the analysis of heteroduplex repair in mammalian cells. (*i*) SV40 DNA can be introduced into cells by DEAE dextran-mediated transfection under conditions in which cells receive only one heteroduplex (21), thereby eliminating the potentially confounding effects of mixed infections and recombination between transfected molecules. (*ii*) Plaque formation by SV40 permits the ready isolation of clonal populations of virus that represent the amplified products of individual repair events. (*iii*) Since mismatches can be positioned within the large tumor antigen (T antigen) intron, repair products can be identified and isolated without selection. In combination, these advantages permit the analysis of a large number of individual repair events, including the entire spectrum of possible repair products.

MATERIALS AND METHODS

Cells and Viruses. The CV1 monkey kidney line was grown as described (22). The SV40 mutants dl883, dl884, dl890, and dl891 were derived from the wild-type strain 776 and have been described (23). The double mutants dl890–891, dl891–883, and dl890–883 were generated in the present study.

Formation of Heteroduplexes. Heteroduplexes were prepared from DNA samples that were grown in CV1 cells and were >90% supercoiled. Unless otherwise noted, all heteroduplexes were prepared by hybridizing heat-denatured single strands as described (24). One DNA of each heteroduplex was linearized with *Bam*HI; the other DNA was linearized with *Eco*RI. Circular heteroduplexes were separated from linear homoduplexes by agarose gel electrophoresis (24).

Four heteroduplex preparations were judged to contain at least 90% circles by either blot hybridization after agarose gel electrophoresis or electron microscopy. The integrity of single-stranded loops (57 and 247 bases long) was evaluated for two heteroduplex preparations by electrophoresis through alkaline agarose and blot hybridization. Only bands corresponding to linear full-length single strands were apparent in the autoradiogram.

Genotypic Analysis of Progeny Plaques. Heteroduplexes were transfected into CV1 cells at 0.01–0.05 ng per 60-mm plate with DEAE-dextran as described (22). Individual plaques were amplified in the presence of ^{32}P orthophosphate (25). Viral DNA was prepared according to Hirt (26), restricted with *Hin*dIII and *Taq* I, and subjected to electrophoresis on a 5% polyacrylamide gel.

RESULTS

Experimental Design. Heteroduplexes were prepared from SV40 mutants that contain deletions in the intron of the large T antigen gene (Fig. 1*A*). Since the intron is not essential for lytic infection, all of the mutant DNAs form plaques with an efficiency equal to wild-type DNA (unpublished data). The various arrangements of single-stranded loops that were studied are illustrated in Fig. 1*B*.

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Abbreviations: SV40, simian virus 40; T antigen, large tumor antigen.

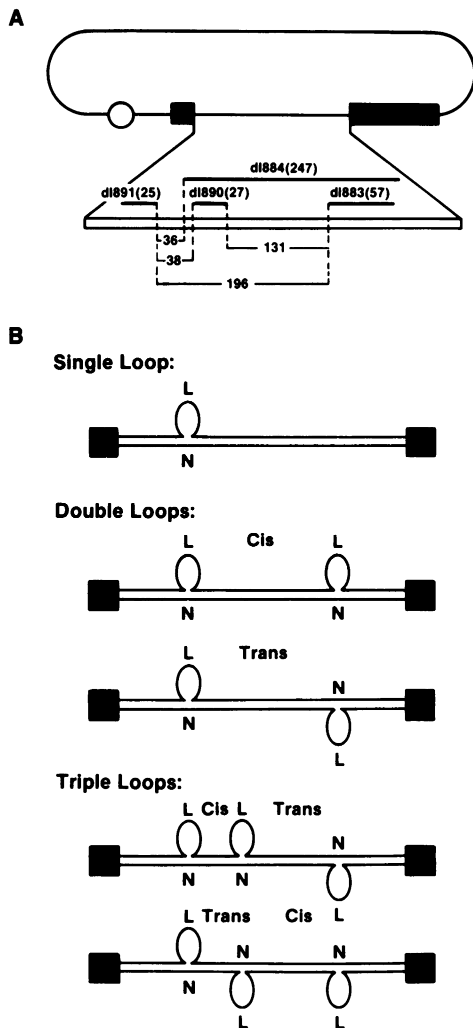

FIG. 1. (A) The SV40 genome. The circular SV40 genome is shown. Shaded boxes represent the exons of the large T-antigen gene. Circle represents the origin of replication. The intron of this gene is shown enlarged below. The positions of the SV40 deletion mutations are shown by heavy black lines. The number of base pairs deleted in each mutant is indicated in brackets next to the mutant designation. (B) Schematic diagram of heteroduplex types. The looped and nonlooped strands at each site are expressed as L and N, respectively. We use L and N as a shorthand designation for genotype. For example, in cis-loop heteroduplexes one parent is LL and the other is NN.

Purified heteroduplexes were transfected into CV1 cells at very low DNA concentrations to ensure that individual cells were infected by only a single DNA molecule. Progeny genomes were analyzed by digestion with restriction enzymes followed by electrophoresis on polyacrylamide gels. The T antigen intron is contained within a 1.1-kilobase *Hin*dIII fragment, which is split into an upper and a lower band by digestion with *Taq* I. Assignment of genotype is straightforward since each mutation produces a diagnostic band of characteristic mobility: dl884 lacks the unique *Taq* I site; dl883 displays altered mobility of the upper *Hin*dIII/*Taq* I band; and deletions 891 and 890 show different migration of the lower *Hin*dIII/*Taq* I band. Furthermore, although dl891 differs in length from dl890 by only 2 base pairs, these mutants can be readily distinguished from one another, as shown in the representative gel depicted in Fig. 2.

The sensitivity of this electrophoretic assay of genotypes has been noted in previous studies (27) and is supported by two additional observations in this study. (*i*) Twenty-one plaques with a novel diagnostic fragment were shown by sequence analysis to harbor a nonparental mutant genome (5 of the 21 contained a single base substitution). (*ii*) Twenty-six plaques with apparently normal diagnostic fragments were shown by sequence analysis to contain the parental mutations.

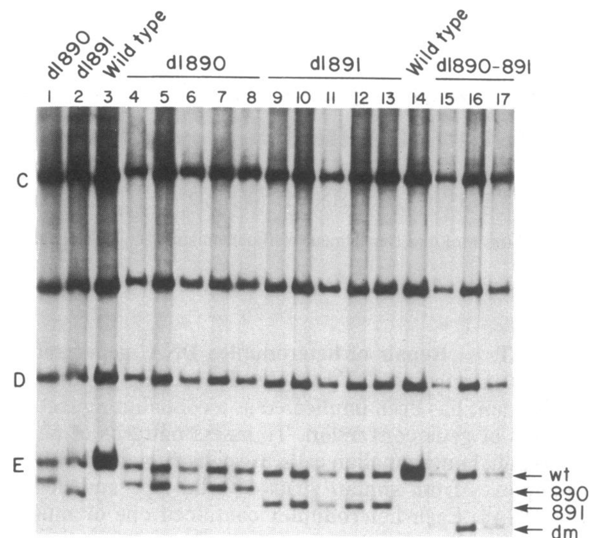

FIG. 2. Restriction analysis of DNA from individual plaques arising after transfection of heteroduplex H (dl891/dl890). Digestion of SV40 with *Hin*dIII and *Taq* I produces seven fragments. Only five fragments are shown in the autoradiogram. Fragments C, D, and E are constant for all SV40 mutants in this study. The other fragments encompass the intron of the T antigen gene. The diagnostic fragments for plaques from heteroduplex dl891/dl890 are marked with arrows. Lanes 1–3 contain DNA from dl890, dl891, and wild-type 776 respectively. Lanes 4–17 show the restriction pattern of DNA from individual plaques isolated after transfection of the heteroduplex. dm, Double mutant recombinant; wt, wild type recombinant.

Methods of Heteroduplex Preparation. To test the effects of different methods of heteroduplex preparation on the outcome of the repair experiments, we compared three different construction protocols: heat-denaturation of full-length linears, NaOH-denaturation of full-length linears, and NaOH-denaturation of overlapping restriction fragments (Fig. 3 and *Materials and Methods*).

As indicated in Fig. 3, the three heteroduplex preparations, which each contained a single-stranded loop 247 nucleotides long, yielded quite similar results by three criteria. (*i*) All plaques contained one or the other of the parental genotypes or both; no new mutants were generated. (*ii*) The proportion of plaques containing only one genotype ("pure" plaques) ranged from ≈85% to 95%. (*iii*) The ratio of parental genotypes was about ≈2:1 in favor of the deletion. As is discussed, these criteria reflect, respectively, the accuracy, efficiency, and strand bias of repair. We conclude from these results that the method of preparation had little effect on the cellular repair of single-stranded loops.

Repair Products from Different Heteroduplexes. To assess the effect of loop size and the effects of loop separation and configuration on the outcome of the repair process, we analyzed 1123 progeny plaques resulting from transfection of 13 different heteroduplexes, which contained one, two, or three loops in several different configurations (Fig. 1*B*).

The results were similar to those obtained with the single-loop heteroduplex shown in Fig. 3. The percentage of pure, mixed, and mutant plaques is summarized in Table 1. The proportion of plaques containing a single genotype ranged from 72% to 100% with a mean of 90%. However, in this larger data set, ≈2% of the plaques contained nonparental mutant genomes. (Their sequences will be described elsewhere.) Overall, the frequency of pure, mixed, and mutant

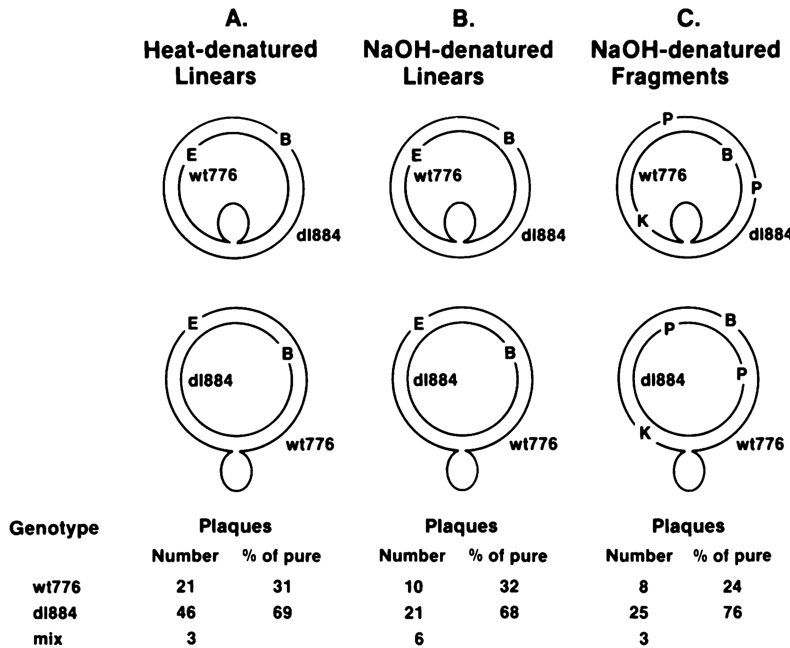

FIG. 3. Comparison of three methods of heteroduplex preparation. Each reannealing reaction produced two heteroduplex products due to the two possible combinations of single strands. The line with the single-stranded loop represents wild-type 776 (wt776); the line without the loop represents dl884. Nicks at restriction sites *Bam*HI, *Eco*RI, *Pst* I, and *Kpn* I are indicated by B, E, P, and K, respectively. Heat-denatured heteroduplexes were prepared as described in *Materials and Methods*. The circular heteroduplexes in *C* were formed from four overlapping fragments after digestion of dl884 with *Pst* I and wt776 with *Kpn* I and *Bam*HI (10). NaOH-denatured heteroduplexes were prepared according to Shortle (28).

plaques was influenced very little by loop size, loop separation, or loop configuration.

Table 1 also summarizes the classification of genotypes found in progeny plaques. These results resemble those in Fig. 3 as well: at each loop, the overall ratio of progeny genotypes was biased nearly 2 to 1 in favor of the nonlooped strand (N), which carried the deletion at the position of the loop. For this series of loops, which ranged from 25 to 247 nucleotides in length, loop size had little influence on the bias of genotypes.

Heteroduplexes with multiple loops often produced plaques that contained genotypes representing new combinations of the mutations present in the parents. The proportions of parental and "recombinant" genotypes are shown in Table 2. The overall frequency of recombinants was ≈25%. However, there was considerably more variability in this parameter than in those discussed above. The frequency of recombinants showed a clear dependence on loop configuration and loop separation. Cis loops always generated a significantly lower frequency of recombinants than the corresponding trans loops. In addition, among heteroduplexes with the same configuration, the proportion of recombinants increased with increasing loop separation. The dependence of recombinant frequency on loop separation and loop configuration is shown in Fig. 4.

Table 1. Classification of plaques arising from transfected heteroduplexes and the genotypic bias at individual heteroduplex sites

							Loop size, nucleotides							
							25		27		57		247	
			Total	Plaque types, % of total			N§	L§	N	L	N	L	N	L
Heteroduplex*		Configuration	plaques†	Pure	Mixed	Mutant‡	Genotype bias in progeny, % of pure plaques							
A	25	Single	120	72	27	1	56	44						
B	57	Single	132	87	11	2					57	43		
C_1¶	247	Single	70	96	4	0							69	31
C_2	247	Single	37	84	16	0							68	32
C_3	247	Single	36	92	8	0							76	24
D	25–27	Double cis	48	94	6	0	73	27	73	27				
E	27–57	Double cis	42	95	5	0			80	20	67	33		
F	25–57	Double cis	50	98	2	0	80	20			71	29		
G	25/247	Double trans	58	100	0	0	69	31					48	52
H	25/27	Double trans	59	89	8	3	62	38	48	52				
I	27/57	Double trans	94	94	6	0			75	25	50	50		
J	25/57	Double trans	121	89	9	2	58	42			77	23		
K	25/57	Double trans	91	87	8	5	77	23			73	27		
L	25–27/57	Triple cis–trans	80	93	3	4	65	35	65	35	58	42		
M	25/27–57	Triple cis–trans	85	87	6	7	55	45	46	54	54	46		
			Mean	90	8	2	66	34	65	35	63	37	65	35
						N/L ratio	1.9		1.9		1.7		1.9	

*Heteroduplexes designated A–M were constructed from SV40 DNAs as described below. For each heteroduplex, the mutation indicated first is located closer to the origin of replication. A, dl891/wt776; B, dl883/wt776; C, dl884/wt776; D, dl891–890/wt776; E, dl890–883/wt776; F, dl891–883/wt776; G, dl891/dl884; H, dl891/dl890; I, dl890/dl883; J, dl891–890/dl890–883; K, dl891/dl883; L, dl891–890/dl883; M, dl891/dl890–883. The number of nucleotides in single-stranded loops is indicated in the adjacent column.

†Each heteroduplex was transfected two or three times, yielding similar results.

‡Pure plaques with a genotype unlike either parent or recombinant.

§N and L refer to the genotype corresponding to the nonlooped and looped heteroduplex strand, respectively.

¶Numbers 1, 2, and 3 of heteroduplex C refer to the different methods of heteroduplex preparation shown in Fig. 3 *A*, *B*, and *C*, respectively.

Table 2. Frequencies of parental and recombinant genotypes among plaques from multiple loop heteroduplexes

Heteroduplex*		Loop separation, base pairs	Progeny types,† % of pure plaques			
			Parental		Recombinant	
			NN	LL	NL	LN
Cis loops						
D	25–27	38	73	27	0	0
L‡	25–27	38	64	34	1	1
E	27–57	131	65	17	15	3
M‡	27–57	131	42	42	4	12
F	25–57	196	69	19	10	2
		Mean	62	28	6	4
			NL	LN	NN	LL
Trans loops						
G	25/247	36	52	31	17	0
H	25/27	38	44	33	17	6
M‡	25/27	38	52	42	3	3
I	27/57	131	46	23	28	3
L‡	27/57	131	36	30	29	5
J	25/57	169	22	40	36	2
K	25/57	196	19	15	58	8
		Mean	39	30	27	4

*Heteroduplex designations correspond to those of Table 1.
†Parental progeny plaques reflect the genotype of one or the other of the two heteroduplex strands. Recombinant plaques represent those repair products that contain a reciprocal combination of the parental mutations.
‡The cis and trans intervals of each triple-loop heteroduplex are listed separately under the respective categories.

DISCUSSION

Mammalian cells can efficiently repair mismatched bases in transfected heteroduplexes (9, 11), but their capacity for repair of deletion loops has not been well-documented. Previous studies have suffered from two limitations: low numbers of analyzed repair events and the potentially complicating effects of other types of mismatches in the same heteroduplex (9–11).

In this study, we prepared a variety of heteroduplexes that contained one, two, or three single-stranded loops but no other mispaired bases. Because these loops were located in the T-antigen intron, which is nonessential, the progeny plaques should contain an unbiased collection of all the potential products of repair. By measuring the distribution of genotypes among 1123 plaques derived from 13 different heteroduplexes, we have been able to assign numerical values to several parameters of single-stranded loop repair in mammalian cells.

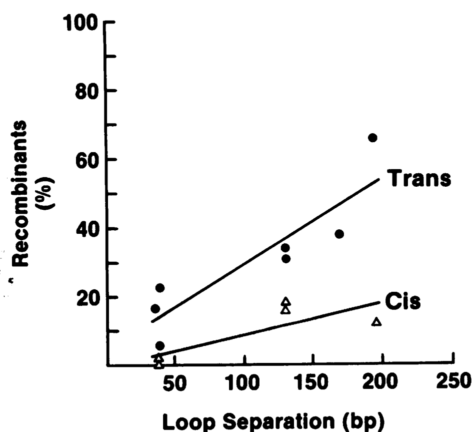

FIG. 4. Frequency of recombinants as a function of loop separation. Data are from Table 3. bp, Base pairs.

Efficiency of Repair. One estimate of the efficiency of repair is the proportion of plaques containing one genotype, which averaged 90% for all heteroduplexes. This estimate is based on the expectation that unrepaired mismatches would, upon replication, generate a mixed plaque.

The high frequency of apparent repair could be mimicked by strand loss (13), which is a catchall term indicating the possibility that one strand might not contribute to the progeny for reasons unrelated to repair. For example, one strand could have been degraded during transfection or one of the two genomes represented in a nonrepaired heteroduplex could have outreplicated the other. In either case, the resulting plaque would appear pure even though the heteroduplex was not repaired. Strand loss must be minimal in this system because heteroduplexes with multiple loops generated a high frequency of recombinant progeny, which could not have arisen by prerepair or postreplication strand loss. Independent unbiased repair of adjacent loops should produce 50% recombinants and 50% parentals. As shown in Table 2, adjacent loops in the trans configuration, which were separated by >150 base pairs, produced an average of 52% recombinants. Thus, we conclude that strand loss is rare and that repair of single-stranded loops is very efficient in monkey cells.

Accuracy of Repair. As judged by the electrophoretic mobility of diagnostic restriction fragments, repair of single-stranded loops is ≈98% accurate. The observed 2% frequency of mutations is quite high relative to the frequency of spontaneous cellular mutations and ≈10-fold higher than observed in transfection experiments with similar sized targets (29, 30). The sequences of the mutants (unpublished data) suggest that they were induced by the heteroduplex and, thus, may have resulted from an error-prone repair process (11, 31).

Strand Bias of Repair. Although either the looped or the nonlooped strand at a mismatch can be used as the template strand for repair, the choice evidently is not random. Among the progeny from all heteroduplexes, there was a consistent bias of ≈2 to 1 in favor of using the nonlooped strand as the template strand for repair (Table 1). Since the efficiency and strand bias of repair were essentially the same for single-stranded loops ranging from 25 to 247 nucleotides, the mechanism of repair appears to be independent of loop size within the range tested.

The basis for the observed strand bias is unclear. It seems unlikely that the strand bias is directed by the nicks in the heteroduplexes that remain from construction, since these nicks are >2 kilobases away. In more extensive studies, we have observed that even nicks as close to the loop as 71 base pairs have only a minor effect on the direction of repair (unpublished observations). It also seems unlikely that the observed bias is due to a replicative advantage by the shorter strand. If such an advantage did exist, it should be reflected in the proportions of long and short genomes present in mixed plaques. Contrary to this expectation, the majority of mixed plaques with an unequal mixture of two genomes contain an overrepresentation of the longer strand.

Independence of Repair. If adjacent single-stranded loops are repaired independently of one another, recombinant genotypes can be generated. By contrast, if adjacent loops are repaired in one event (co-repaired), only parental genotypes can be generated. Thus, the frequency of recombinants is one measure of the independence of repair. These recombinants are unlikely to have arisen by conventional genetic recombination for several reasons. First, heteroduplex DNA

was transfected at very low concentration to ensure that cells were infected by single molecules. Second, recombination between unrepaired parental genotypes in a replicating pool would produce a mixed plaque. Finally, our previous measurements of intramolecular recombination (which is more frequent than intermolecular recombination) in monkey cells indicate a maximum of 1% recombination per 100 base pairs (24). This value is much too low to account for the frequency of recombinant genotypes generated by transfected heteroduplexes.

If repair of adjacent loops is assumed to be fully independent, the expected proportions of parental and recombinant genotypes can be calculated. Given independent repair with a strand bias of 2 to 1 in favor of the nonlooped strand at each mismatch, the ratio of genotypes in the progeny should be 4NN:2NL:2NL:1LL for any pair of loops. For trans loops, the frequency of recombinants (NN and LL) should be 56%; for cis loops, the frequency of recombinants (NL and LN) should be 44%. As shown in Fig. 4, trans-loop recombinants reach the proportion expected from independent repair at a loop separation of ≈200 base pairs. Extrapolation of the line for cis-loop recombinants in Fig. 4 suggests independent repair at a loop separation of ≈400 base pairs.

These results indicate that repair at one single-stranded loop can influence the repair at an adjacent loop if it is <200–400 base pairs away. Distance-dependent correction of adjacent mismatches is taken as an indication of co-repair (6, 9, 13, 16). Co-repair is thought to result when an excision tract, which was initiated at one mismatch, extends far enough to include an adjacent mismatch. This reasoning suggests that excision tracts initiated at single-stranded loops are rarely longer than 200–400 base pairs. This estimate is similar to other more limited studies suggesting that heteroduplex markers separated by 90 base pairs are readily co-repaired, whereas markers separated by 600 base pairs are rarely co-repaired (9). It is also of interest that gene conversion in mouse cells involves contiguous stretches of DNA that are usually <358 base pairs long (32).

In conclusion, the data presented provide evidence for efficient correction of single-stranded loops in transfected heteroduplex DNA. In addition, they suggest that the repair process favors the shorter strand as the template for repair and proceeds by formation of excision tracts. We cannot formally exclude the possibility that the apparent bias in the repair process is introduced by destruction of single-stranded loops during transit. If single-stranded loops, however, were broken at the same rate as double-stranded breaks are introduced into transfected DNA (roughly one break per 10 kilobases (33), most single-stranded loops should remain intact. Nevertheless, the rate of breakage of single-stranded DNA is unknown, and the resolution of this point must await further experiments in which heteroduplexes are delivered directly into the nucleus by microinjection.

Note Added in Proof. Ayares *et al.* (35) have also demonstrated that monkey cells efficiently repair heteroduplexes containing single-stranded loops.

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