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Efficient Correction of Mismatched Bases in Plasmid Heteroduplexes Injected into Cultured Mammalian Cell Nuclei

KIM R. FOLGER, KIRK THOMAS, AND MARIO R. CAPECCHI*
Department of Biology, University of Utah, Salt Lake City, Utah 84112

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Heteroduplexes were prepared from two plasmids, pRH4-14/TK and pRH5-8/TK, containing different amber mutations in the neomycin resistance gene (Neo^r). The Neo^r gene was engineered to be expressed in both bacterial and mammalian cells. A functional Neo^r gene conferred kanamycin resistance to bacteria and resistance to the drug G418 to mammalian cells. In addition, the plasmids contained restriction site polymorphisms which did not confer a selectable phenotype but were used to follow the pattern of correction of mismatched bases in the heteroduplexes. In a direct comparison of the efficiency of transforming mouse LM*tk*$^-$ cells to G418^r, the injection of heteroduplexes of pRH4-14/TK–pRH5-8/TK was 10-fold more efficient than the coinjection of pRH4-14/TK and pRH5-8/TK linear plasmid DNA. In fact, injection of 5 to 10 molecules of heteroduplex DNA per cell was as efficient in transforming LM*tk*$^-$ cells to G418^r as the injection of 5 to 10 molecules of linear plasmid DNA per cell containing a wild-type Neo^r gene. To determine the pattern of mismatch repair of the injected heteroduplexes, plasmids were "rescued" from the G418^r cell lines. From this analysis we conclude that the generation of wild-type Neo^r genes from heteroduplex DNA proceeds directly by correction of the mismatched bases, rather than by alternative mechanisms such as recombination between the injected heteroduplexes. Our finding that a cell can efficiently correct mismatched bases when confronted with preformed heteroduplexes suggests that this experimental protocol could be used to study a wide range of DNA repair mechanisms in cultured mammalian cells.

Correction of mismatched bases in *Escherichia coli* has been studied extensively, using transfection of heteroduplexes prepared from bacteriophage λ (13, 16, 17, 19, 20), φX174 (1), fd (5), and T7 (2). Such experiments have demonstrated that mismatched bases can be eliminated from the heteroduplexes before replication and in the absence of recombination. *E. coli* enzymes appear to recognize and correct mismatched base pairs by a process akin to excision repair.

For mismatched bases introduced during replication, it would be advantageous if repair systems not only detected the base pair mismatches but also discriminated between the parental and newly synthesized strands. Wagner and Meselson (17) proposed that the transient undermethylation of the newly synthesized strand could provide the basis for such discrimination. Consistent with this hypothesis was the finding by Pukkila et al. (14) that hemimethylated λ heteroduplexes when transfected into *E. coli* were almost exclusively mismatch repaired on the unmethylated strand to yield the genotype of the methylated strand. These studies have been extended to the analysis of mismatch repair in cell-free extracts of *E. coli* (10).

In mammalian cells repair of mismatches has been studied by using heteroduplexes of simian virus 40 and polyoma virus (9, 12). The results of these experiments were consistent with mismatch repair occurring in mammalian cells. However, the interpretation of these experiments may have been complicated by contributions of recombination either between the reciprocal heteroduplexes or between progeny virus produced by replication. These problems can in part be obviated by using heteroduplexes prepared from nonreplicating plasmids.

In the present study we prepared heteroduplexes from two plasmids incapable of self-replication in mammalian cells. These two plasmids bore two different amber mutations in the neomycin resistance gene (Neo^r) coded for by the Tn*5* transposon. These plasmids also contained restriction site polymorphisms which provided additional mismatches for following the pattern of repair. Previously we examined homologous recombination between these two plasmids in cultured mammalian cells under experimental conditions comparable to those for heteroduplex analysis (7). Therefore, we were able to directly compare the frequencies and patterns of mismatch repair of the heteroduplexes with the frequencies and patterns of homologous recombination between these two plasmids.

MATERIALS AND METHODS

The methods used for culturing mammalian cells, microinjection, and plasmid DNA preparation have been described elsewhere (4, 6, 8). The methods used for rescuing the corrected plasmids from the transformed mammalian cell lines and for analyzing these rescued plasmids have also been described (7).

pRH4-14/TK–pRH5-8/TK heteroduplexes. pRH4-14/TK and pRH5-8/TK plasmid DNAs were digested with *Hin*dIII, mixed in a ratio of 1:1, and heat denatured at 100°C for 5 min in 10 mM Tris (pH 7.4)–5 mM EDTA. The denatured plasmid mixture was reannealed at 68°C in 0.6 M NaCl–20 mM Tris (pH 7.0)–5 mM EDTA. The extent of denaturation and reannealing was monitored on agarose gels. The linear double-stranded DNA was separated from unhybridized DNA on a 1% preparative agarose gel and purified on ground glass beads.

RESULTS

Recombinant plasmids. The recombinant plasmids used to prepare the heteroduplexes are illustrated in Fig. 1. pRH4-14/TK and pRH5-8/TK were derived from the parental plasmid pRH140 (7, 8), which contains sequences from the

* Corresponding author.

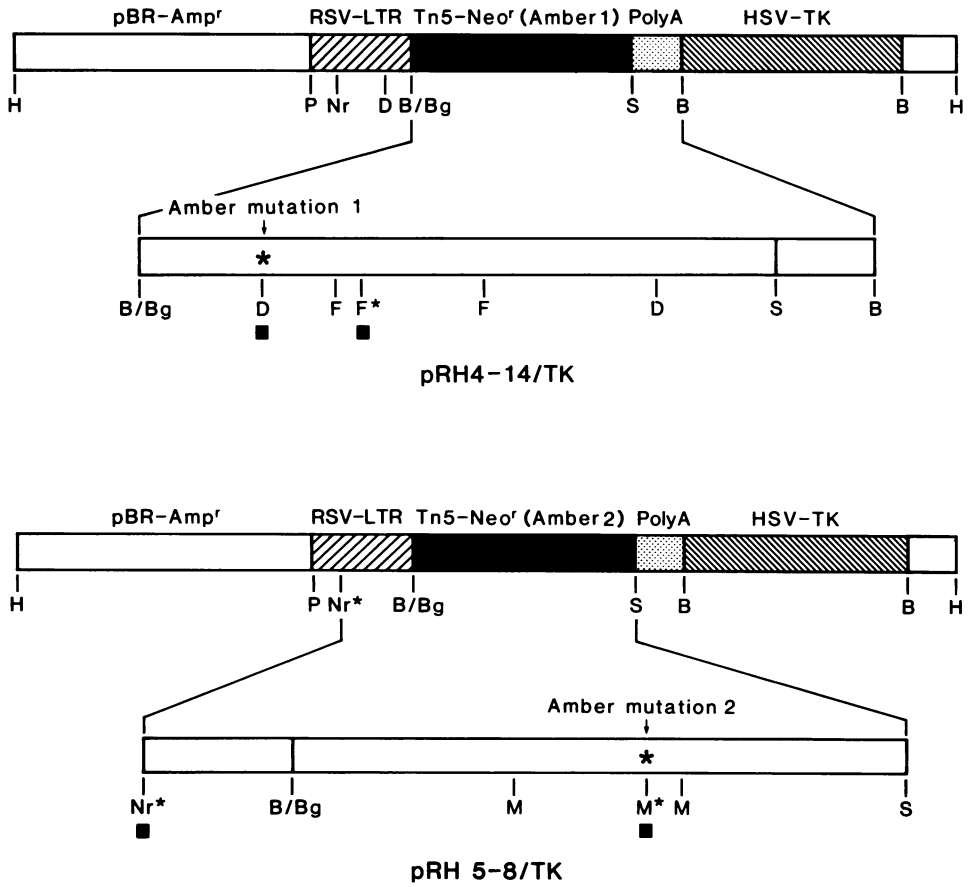


FIG. 1. Maps of plasmids pRH4-14/TK and pRH5-8/TK. These plasmids contain sequences derived from pBR322, the Neo^r gene coded for by the bacterial Tn*5* transposon, and the herpes simplex virus thymidine kinase gene (HSV-TK). The Neo^r gene is expressed from a bifunctional promoter, RSV-LTR, which allows expression of the Neo^r gene in both *E. coli* and mammalian cells. pRH4-14/TK contains two point mutations in the Neo^r gene, an amber mutation and a silent mutation. The amber mutation creates a new *Dde*I site; the silent mutation destroys an *Fnu*4HI site. pRH5-8/TK contains a different amber mutation in the Neo^r gene and a single-base pair deletion in the RSV-LTR. The 5-8 amber mutation destroys an *Mbo*I site and the single-base pair deletion destroys an *Nru*I site. Each vector is represented in linear form from the unique *Hin*dIII site. The restriction sites are designated as follows: H = *Hin*dIII; C = *Cla*I; B = *Bam*HI; Bg = *Bgl*II; Nr = *Nru*I; R = *Eco*RI; P = *Pvu*II; N = *Nae*I; S = *Sma*I; D = *Dde*I; F = *Fnu*4HI; M = *Mbo*I.

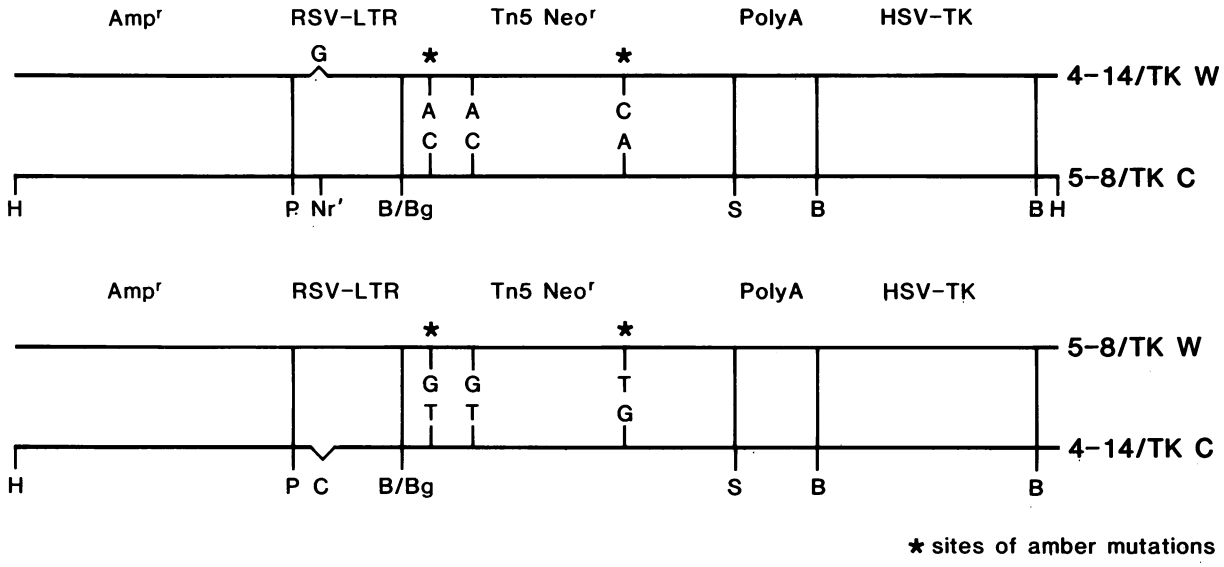
bacterial plasmid pBR322 and the Neo^r gene coded for by the bacterial Tn*5* transposon. The pBR322 sequences supply an ampicillin resistance gene (Amp^r) and an origin of DNA replication which functions in bacteria. The Neo^r gene was engineered to be expressed both in bacteria and in mammalian cells. In bacteria the Neo^r gene confers kanamycin resistance; in mammalian cells the Neo^r gene confers resistance to the drug G418 (GIBCO). The herpes simplex virus thymidine kinase gene was introduced into the above plasmids at the unique *Bam*HI site as a 3.4-kilobase (kb) *Bam*HI fragment.

pRH4-14/TK contains two point mutations in the Neo^r gene which were induced by in vitro mutagenesis of pRH140 with hydroxylamine (8). The first mutation introduced an amber codon near the NH_2 terminal end of the Neo^r gene and rendered the gene product defective both in bacteria and in mammalian cells. This mutation concomitantly created a new *Dde*I site which was used as a diagnostic test for the presence of the amber mutation. The second point mutation converted a leucine codon to a threonine codon and concomitantly destroyed an *Fnu*4HI site. This mutation appears to be silent since Neo^r genes lacking the amber mutation but still retaining the *Fnu*4HI mutation encode a product with wild-type enzymatic activity (K. Folger, K. Thomas, and M. R. Capecchi, Cold Spring Harbor Symp. Quant. Biol., in press).

pRH5-8/TK contains a different amber mutation 417 base pairs (bp) downstream from the 4-14 mutation. This mutation destroyed an *Mbo*I site which was used as a diagnostic test for the presence of the 5-8 amber mutation. pRH5-8/TK also contains a single-base pair deletion in the avian retrovirus long terminal repeat (RSV-LTR) which destroyed an *Nru*I site. This deletion does not affect either the promotor function or the "enhancer" function of the RSV-LTR (11). The above mutations were characterized by sequence analysis (7).

Injection of heteroduplexed DNA. Heteroduplexes of pRH4-14/TK and pRH5-8/TK were prepared by mixing the linear plasmids in a ratio of 1:1, heat denaturing, and renaturing the mixture. The extent of denaturation and renaturation was monitored by agarose gel electrophoresis. The extent of heteroduplex formation was judged to be complete since the ratio of *Nru*I-susceptible/*Nru*I resistant molecules shifted from 1:1 before treatment to 1:3 after treatment.

A schematic diagram of the position and nature of the different mismatched bases is illustrated in Fig. 2. Since the 4-14 and 5-8 amber mutations are on opposite strands, generation of a functional Neo^r gene by correcting the



Heteroduplexes

FIG. 2. Schematic diagram of the pRH4-14/TK–pRH5-8/TK heteroduplexes illustrating the different mismatched bases. The preparation and purification of the pRH4-14/TK–pRH5-8/TK heteroduplexes are described in the text. W and C refer to the "Watson" and "Crick" strands of DNA, respectively. The top heteroduplexes contain three base pair mismatches and a single-base insertion-deletion mismatch designated by the extended G or C in the RSV-LTR. Poly A, Polyadenylated; HSV-TK, herpes simplx virus thymidine kinase gene.

heteroduplex requires using information from both strands. If the selection of which base to correct were chosen at random, then one-fourth of the corrected heteroduplexes should yield a functional Neo^r gene. In addition to the two heteroduplexes, the reannealed mixture also contained parental molecules. To reduce contributions resulting from recombination between the injected plasmid molecules, these experiments were done at a low input of plasmid DNA per cell, approximately 5 to 10 molecules per cell, and used plasmids that were incapable of self-replication.

In Table 1 we show the transformation frequency obtained by injecting pRH4-14/TK–pRH5-8/TK heteroduplexes into LM*tk*$^-$ cells and selecting for G418^r colonies. As controls we also injected heat-denatured and renatured pRH140/TK, pRH4-14/TK, and pRH5-8/TK plasmid DNA by themselves. Denaturation and renaturation do not reduce the DNA-mediated transforming efficiency of pRH140/TK. The surprising result is that pRH4-14/TK–pRH5-8/TK heteroduplexes transform LM*tk*$^-$ cells to G418^r as efficiently as the wild-type plasmid DNA. pRH4-14/TK–pRH5-8/TK heteroduplexes also transform LM*tk*$^-$ cells to G418^r 10-fold more efficiently than coinjected pRH4-14/TK and pRH5-8/TK linear molecules (i.e., 120 transformants per 10^3 cells receiving an injection compared with 11 transformants per 10^3 cells receiving an injection [7]). The injections of heteroduplexes and linears of pRH4-14/TK and pRH5-8/TK DNA into nuclei of LM*tk*$^-$ cells were done under comparable conditions.

TABLE 1. DNA-mediated transformation frequencies to G418^r obtained by injecting LM*tk*$^-$ cells with heteroduplexes of pRH4-14/TK and pRH5-8/TK

DNA injected	No. of transformants/10^3 cells receiving an injection	No. of cells injected
pRH140/TK	112	10^3
pRH4-14/TK	0	10^3
pRH5-8/TK	0	10^3
pRH4-14/TK + pRH5-8/TK	120	10^3

[a] LM*tk*$^-$ cells were injected with heteroduplexes of pRH4-14/TK–pRH5-8/TK at a concentration of 5 to 10 copies per cell. The heteroduplexes were prepared and purified as described in the text. The injected cells were processed and subjected to G418^r selection as previously described (7). As controls, pRH140/TK, pRH4-14/TK, and pRH5-8/TK, linearized at the unique *Hin*dIII site, were separately heat denatured, reannealed, injected into LM*tk*$^-$ cells, and subjected to selection for G418^r.

To determine the pattern of mismatch repair in any given molecule, plasmids were "rescued" from the genome of the G418^r cells (7). Genomic DNA from five G418^r cell lines was digested with the restriction endonuclease *Bam*HI and ligated under conditions that favored intramolecular ligation. This DNA was used to obtain ampicillin-resistant bacteria by transfection. Note that we did not select for rescued plasmid molecules which were corrected to yield a functional Neo^r gene (i.e., Kan^r bacterial colonies), but rather rescued all plasmids containing a functional Amp^r gene and a bacterial origin of DNA replication.

In Table 2 we summarize the characterization of 107 plasmids rescued from the five G418^r cell lines. The data were pooled into classes of plasmids sharing all of the tested characteristics.

Corrected plasmids containing a wild-type Neo^r gene were identified by streaking bacteria containing rescued plasmids on kanamycin plates. As described previously (7), growth on kanamycin plates also allowed us to distinguish rescued plasmids which contained the 4-14 amber mutation from plasmids with the 5-8 amber mutation. The phenotypic assignment for each rescued plasmid by its growth pattern on kanamycin plates was then confirmed by determining the source (4-14 or 5-8) of the *Dde*I and *Mbo*I restriction-length polymorphisms. The methods used to identify each restriction-length polymorphism present in the rescued plasmids,

*Nru*I, *Dde*I, *Fnu*4H1, and *Mbo*I, were described in detail in the accompanying paper (7). The rescued plasmids were also characterized with respect to size. If in the mammalian genome the 5′ neighbor of a rescued plasmid was another plasmid, then the plasmid was of unit length (i.e., 5.8 kb). Such plasmids were designated with an L (see Table 2). If the 5′ neighbor was genomic sequences, then the rescued plasmid was longer than 5.8 kb and of variable length depending on the position of the next *Bam*HI restriction site. These plasmids were designated with a J. J, J′, and J″ denote plasmids of a given class containing different junction fragments, that is, plasmids that integrated into different sites in the host genome. The assignment of rescued plasmids to the L or J class was further corroborated by analysis of *Dde*I fragments on agarose gels. One of the *Dde*I fragments, 603 bp in length, spans the *Bam*HI junction in unit-length plasmids. Thus, a change in this fragment length was an indicator of the presence of genomic sequences in the rescued plasmids.

TABLE 2. Classes of plasmids rescued from cell lines injected with pRH4-14/TK–pRH5-8/TK heteroduplexes[a]

Cell line/ class	Kan^{r}[b]	Polymorphisms[c]				Size[d]	No. rescued
		*Nru*I	*Dde*I	*Fnu*4H1	*Mbo*I		
LM3							
1	+++	X	X	X	0	L	6
2	−	0	0	0	0	J	9
3	±	X	X	X	X	J′	6
4	±	X	X	X	X	L	1
5	±	0	X	X	X	(L)	3
LM6							
1	−	0	0	0	X	L	15
2	±	0	X	X	0	(L)	5
3	+++	0	X	X	0	(L)	7
LM9							
1	+++	0	X	X	0	L	1
2	±	0	X	X	X	(L)	5
3	−	0	0	0	0	J	2
4	−	0	0	0	0	J′	2
5	±	X	X	X	X	J″	1
6	±	X	X	X	X	L	2
LM27							
1	±	X	X	X	X	J	9
2	+	X	X	X	X	J′	5
3	−	0	0	X	X	J″	7
LM33							
1	±	X	X	X	0	J	3
2	−	0	0	0	X	L	17
3	+++	0	X	X	0	(L)	1
pRH4-14	−	0	0	0	0	L	
pRH5-8	±	X	X	X	X	L	

[a] Plasmids were rescued, as *Bam*HI fragments, from five G418^{r} cell lines obtained by injection of pRH4-14/TK–pRH5-8/TK heteroduplex DNA into LM*tk*$^{-}$ cells (see Table 1). The rescued plasmids were analyzed for conferring Kan^{r} to bacteria, size, and restriction site polymorphisms. For comparison, the characteristics of linear pRH4-14 and pRH5-8 are shown.

[b] +++, Wild-type pRH140 Kan^{r} phenotype; ±, pRH5-8 phenotype; −, pRH4-14 phenotype.

[c] The *Nru*I, *Dde*I (4-14 amber), *Fnu*4H1, and *Mbo*I (5-8 amber) restriction site polymorphisms were assayed as previously described (7). 0, The restriction site polymorphism originated from pRH4-14; X, the restriction site polymorphism originated from pRH5-8.

[d] L, Plasmids of unit length, 5.8 kb, the expected size of a plasmid from an internal positiin in a tandem array; (L), plasmids smaller than unit length (the reduction in size resulted from removal of approximately 150 bp from both ends of the heteroduplex [*Hin*dIII sites] before integration); J, the plasmid contains a chromosomal junction sequence; J, J′, and J″ indicate that the rescued plasmid from a given cell line contained different chromosomal junction fragments; that is, these plasmids had integrated at different sites in the host chromosome.

The 107 rescued plasmids were subdivided into 20 classes that shared the tested characteristics. The four markers did not segregate completely independently. Of the 16 possible distributions of the four markers in the rescued plasmids, we identified 7. However, as expected, the restriction site polymorphisms did segregate more independently in cell lines obtained by injecting heteroduplexes than in cell lines obtained by coinjecting pRH4-14/TK and pRH5-8/TK plasmid DNA (see Discussion). Also, two new types of rescued plasmids emerged in cells injected with heteroduplexes not observed in cells coinjected with pRH4-14/TK and pRH5-8/TK DNA. The first type of plasmid was observed in four of the five G418^{r} cell lines analyzed. Rescued plasmids which received their *Nru*I polymorphism from the 4-14 strand, LM3 class 5, LM6 class 2, LM6 class 3, LM9 class 2, and LM33 class 3, were shorter than unit length, 5.8 kb. The reduction in size resulted from losing approximately 150 bp from both ends of the heteroduplex molecule (i.e., from the *Hin*dIII site). The position and extent of the deletions in this type of plasmid were determined by fine-structure restriction enzyme mapping. The second unique type of plasmid, rescued from two of the G418^{r} cell lines, LM6 class 2 and LM33 class 1, did not contain either the 4-14 or the 5-8 amber mutation, yet bacteria transfected with these plasmids grew poorly on kanamycin plates.

DISCUSSION

We have demonstrated that heteroduplexes of pRH4-14/TK–pRH5-8/TK are corrected with remarkable efficiency when injected into nuclei of cultured mammalian cells. Injection of 5 to 10 molecules of heterodulex DNA per cell is as efficient for transforming LM*tk*$^{-}$ cells to G418^{r} as injecting 5 to 10 molecules of linear plasmid per cell containing a wild-type Neo^{r} gene. This implies that nearly every heteroduplex molecule that was injected into these mouse cells was corrected. Furthermore, direct comparison of the efficiency of transforming LM*tk*$^{-}$ cells to G418^{r} by injecting heteroduplex DNA with coinjecting pRH4-14/TK and pRH5-8/TK linear plasmid DNA showed that transformation by heteroduplexes is 10-fold more efficient. These results indicate that the generation of a wild-type Neo^{r} gene from heteroduplex DNA proceeds directly by correction of the mismatched bases rather than by alternative mechanisms such as recombination between injected heteroduplex or parental molecules. This conclusion is further strengthened by an analysis of the rescued plasmids (see below).

After injection of heteroduplex DNA, we selected for molecules which were corrected to yield a functional Neo^{r} gene. However, 14 of the 20 classes of plasmids rescued from these G418^{r} cell lines contained the nonselected Kan^{s} genotype. When we compared the distribution of the four polymorphic markers in these rescued plasmids with the distribution pattern of the same markers in plasmids rescued from G418^{r} cell lines obtained by coinjecting pRH4-14/TK and pRH5-8/TK plasmids, it was apparent that these markers segregated more independently in cell lines obtained by injecting heteroduplexes. For example, in G418^{r} cell lines obtained by coinjecting pRH4-14/TK and pRH5-8/TK plasmids, we did not rescue any plasmids containing both the 4-14 and the 5-8 amber mutations (7). Yet after injection of heteroduplexes two of the five G418^{r} cell lines contained such molecules. Also, from G418^{r} cell lines obtained by coinjecting pRH4-14/TK and pRH5-8/TK we did not observe plasmids in which the *Dde*I and *Fnu*4H1 polymorphisms segregated. Such plasmids were rescued from G418^{r} cell lines obtained by injecting heteroduplexes.

Whether a bias exists for using one strand over the other as a template for correcting the mismatched bases or whether a bias exists for cocorrecting adjacent markers has not been fully explored. We will examine these questions in greater detail, using purified heteroduplexes prepared from separated strands. The latter approach will alleviate complications resulting from the simultaneous analysis of correction products from the two types of heteroduplexes.

A unique type of plasmid was rescued from the transformants obtained by injecting pRH4-14/TK–pRH5-8/TK heteroduplexes. This type of plasmid, which contained the *Nru*I polymorphism from the 4-14 strand, was shorter than unit length. The shorter length resulted from the loss of approximately 150 bp from both ends of the injected heteroduplex. We do not understand how this class arose in four of the five independent transformants. However, it is attractive to speculate that this particular set of molecules was acted upon by a subset of the enzymatic machinery, for example, an exonuclease involved in mismatch repair.

It is interesting that two of the classes of plasmids rescued from these G418^r cell lines contained neither the 4-14 nor the 5-8 amber mutation, yet bacteria containing these plasmids did not grow well on kanamycin plates. These plasmids may contain unidentified mutations in the Neo^r gene. Such mutations could have arisen either during injection of the heteroduplexes into mammalian cell nuclei or more interestingly during the repair process itself (i.e., an error-prone repair process). In other studies we have observed the induction of an error-prone repair system involving homologous recombination between an exogenous gene and a gene residing in the mammalian chromosome (Folger et al., in press). We postulated that the triggering of this error-prone repair system required (i) the formation of heteroduplexes between the newly introduced gene and the chromosomal gene and (ii) the presence of base pair mismatches or deletion-insertion mismatches or both. It is interesting that the class of plasmids lacking either the 4-14 or the 5-8 amber mutation but unable to grow well on kanamycin plates was not rescued from G418^r cell lines obtained by coinjecting pRH4-14/TK and pRH5-8/TK plasmid molecules.

There is also a precedent for exogenous DNA acquiring mutations during gene transfer into mammalian cells (3, 14, 17). As much as 10 to 15% of the plasmid DNA transferred into mammalian cells by calcium phosphate coprecipitation, DEAE-dextran, or protoplast fusion suffer mutations including deletions, rearrangements, and point mutations. Many of these mutations may be incurred during transit from the cyotplasm to the nucleus. We have not observed this level of mutagenesis to molecules introduced into mammalian cell nuclei by microinjection (unpublished data).

In conclusion, we would like to reiterate that the remarkable efficiency with which a cell can repair mismatches when confronted with preformed heteroduplexes suggests that this experimental protocol could be used to study a wide range of DNA repair mechanisms in cultured mammalian cells.

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