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Accurate modification of a chromosomal plasmid by homologous recombination in human cells

(transfection/gene targeting/plasmid rescue)

KYU-YOUNG SONG*, FAINA SCHWARTZ*, NOBUYO MAEDA†, OLIVER SMITHIES†, AND RAJU KUCHERLAPATI*

*Center for Genetics, University of Illinois College of Medicine, Chicago, IL 60612; and †Department of Genetics, University of Wisconsin, Madison, WI 53706

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ABSTRACT **We have examined the consequences of modifying mammalian cellular DNA sequences by homologous recombination. A plasmid carrying a 248-base-pair deletion in the neomycin phosphotransferase (*neo*) gene was introduced into hamster and human cells. The integrated, defective *neo* gene was used as a target for modification by a second round of transfection with a plasmid carrying a different (283-base-pair) deletion in the *neo* gene. Recombinants resulting in an intact *neo* gene were selected by their G418 resistance phenotype. The best ratio of homologous to nonhomologous recombination events was about 1:80. Analyses of the functional *neo* genes in various independent cell lines establish that simple crossovers (single and double) generated the wild-type *neo* genes.**

Homologous recombination between specific cellular sequences and their counterparts introduced by transformation is an important genetic tool in yeast (1) and is routinely used for site-specific gene modification and gene disruption. Mammalian somatic cells can also catalyze recombination between homologous DNA molecules (2–7). Recombination events resulting from single and double crossovers (gene conversions) have been observed in various systems (8–12) but the relative frequencies of these two types of events have not been well documented. In addition, there has been one report (13) that recombination between a chromosomal plasmid and its homologous DNA introduced by microinjection can lead to a high frequency of mutational events not obviously recombinational in nature. In an attempt to understand the various types of recombinational events that occur in mammalian cells, we have introduced a partially deleted, dominant, selectable gene into the human genome and have used it as a target for modification by a second round of transfection (see also refs. 8–11). The results of these chromosomal plasmid–plasmid recombination experiments are described here. They indicate that a high efficiency of gene modification can be obtained by transfection and that single- and double-crossover events play a role in the reconstruction of an intact gene in the treated cells. All the corrected genes we have studied are the consequence of simple and precise recombination events that do not introduce any mutations into the genes.

MATERIALS AND METHODS

Cells. 743R is a hypoxanthine phosphoribosyltransferase-deficient (*HPRT*$^-$) derivative of the Chinese hamster lung cell line V79. It is a gift from S. Warren (Emory University). The human bladder carcinoma cell line EJ was a gift from R. Weinberg (MIT). COS-1 cells are simian virus 40-transformed monkey cells (14). Cells were grown in Dulbecco's modified Eagle's medium with 10% (vol/vol) fetal calf serum.

Selection Systems. Hamster and human cells having the bacterial guanine/xanthine phosphoribosyltransferase (*gpt*) gene were, respectively, selected by the use of HAT medium (15) or with medium containing mycophenolic acid, adenine, and xanthine (MAX) (16). Cells with the bacterial neomycin phosphotransferase (*neo*) gene were selected by using Geneticin (G418, GIBCO) at 200 μg/ml (743R) or 400 μg/ml (EJ).

Cell Fusions. Approximately 3×10^6 cells of each type were mixed and plated in 100-mm Petri dishes. Sixteen hours later, the monolayer was treated with 50% (wt/vol) polyethylene glycol 1000 according to the method of Davidson *et al.* (17). Forty-eight hours after the fusion, low molecular weight DNA was isolated by the method of Hirt (18).

Plasmids. The plasmid pSV2neo-SV2gpt (19) was a gift from P. Southern (Scripps Institute). A deletion was introduced into the left (5′) end of the *neo* gene by deleting a 248-base-pair (bp) *Nar* I fragment to give pSV2neo-DL-SV2gpt, where DL is deletion left (Fig. 1). The deletion renders the *neo* gene inactive in bacterial and mammalian cells (5). pSV2neo-DR, where DR is deletion right, was obtained by deleting a 283-bp *Nae* I fragment spanning the right (3′) end of the *neo* gene (5). Several restriction enzyme recognition sites flanking the deletion *neo* gene in pSV2neo-DR were modified to generate DR-pLCX102 (7). The *neo* genes in pSV2neo-DL-SV2gpt and DR-pLCX differ at three sites in addition to the deleted regions (see Fig. 1). The markers on pSV2neo-DL-SV2gpt are *Hin*dIII, DL, *Sma* I, and *Bam*HI. The corresponding region in DR-pLCX contains *Sma* I, DR, *Xba* I, and *Bam*HI/*Xho* I (both enzymes cut).

Transfections. For each transfection 7×10^5 cells were plated in 60-mm dishes. Calcium phosphate DNA coprecipitates prepared as described by Lowy *et al.* (20) were added to the monolayer without carrier DNA. Four hours later, the medium was removed, and the cells were treated with dimethyl sulfoxide [10% (vol/vol) for 743R and 20% (vol/vol) for EJ] for 2 min. On the next day the cells were transferred to 100-mm plates containing selective medium. Primary transfectants were obtained by transfecting 1 μg of supercoiled pSV2neo-DL-SV2gpt DNA per plate of hamster or EJ cells. To obtain secondary transfectants, 4–5 μg of pLCX DNA (linearized at the site of deletion) was used per plate.

DNA Manipulations. Total high molecular weight cellular DNA was obtained as described (21). The DNA was digested to completion with appropriate restriction endonucleases, separated on 1% agarose gels, and hybridized as described by Southern (22). Radiolabeled probes were prepared by the primer extension method of Feinberg and Vogelstein (23). To rescue the integrated plasmid sequences, cells were fused with COS-1 cells as described above, and low molecular

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Abbreviations: R, resistant; S, sensitive; Amp, ampicillin; Kan, kanamycin; DL, deletion left; DR, deletion right.

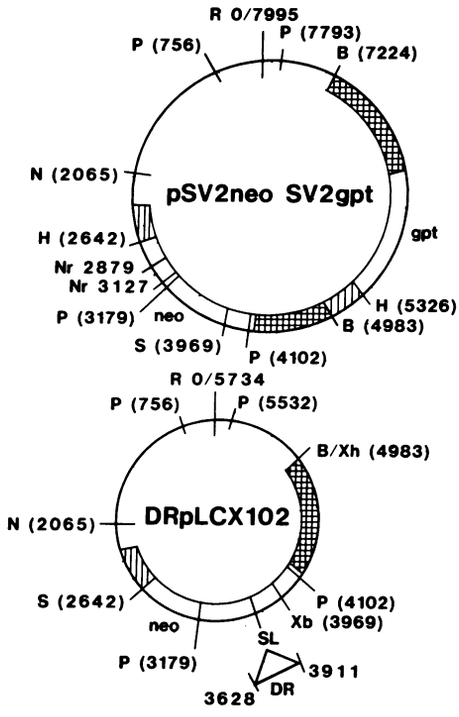

FIG. 1. Plasmids used for gene modification. (*Upper*) pSV2neo-SV2gpt. (*Lower*) pSV2neo-DR (DR-pLCX102). For each, key restriction enzyme recognition sites along with the nucleotide number are indicated. B, *Bam*HI; H, *Hin*dIII; N, *Nde* I; Nr, *Nar* I; P, *Pst* I; S, *Sma* I; SL, *Sal* I; Xb, *Xba* I; Xh, *Xho* I. Thin lines represent pBR322 sequences. Open boxes, *neo* or *gpt* sequences. Cross-hatched boxes, simian virus 40 origin and early promoter sequences. Hatched boxes, simian virus 40 splicing and polyadenylylation signals.

weight DNA isolated from the fusion products was used to transform the DH1 strain of *Escherichia coli*, which is *recA*$^-$. A second method of rescuing the plasmids involved digesting the cellular DNA to completion with the restriction endonuclease *Xba* I and incubating the digested DNA with T4 DNA ligase at a DNA concentration of 1 μg/ml. The resulting DNA, which contains circular molecules, was used to transform the *recA*$^-$ *E. coli* strain DH1. Colonies that can grow in ampicillin (Amp) or kanamycin (Kan) were selected. DNA sequencing was conducted on supercoiled plasmid DNA preparations by the primer extension method described by Chen and Seeburg (24).

RESULTS

Homologous Recombination with the Chromosomal Plasmid. Hamster and human cells were transfected with circular pSV2neo-DL-SV2gpt in the absence of carrier DNA. The RK series of primary transfectant cell lines and the KY34a and KY36a cell lines are hamster cells. The KY21b and -22b cell lines are human cells. The primary cell lines all contain one and sometimes more than one integrated copy of the input plasmid. The primary lines were transfected with each of the following plasmids: pSV2neo, pSV2neo-DL, and pSV2neo-DR (or DR-pLCX). Following this secondary transfection, cells were selected in HAT/G418 or MAX/G418 medium.

Results from several such experiments are summarized in Table 1. The data in the wild-type column show that the cell lines differ from each other in their transfection efficiencies with supercoiled pSV2neo. When transfected with circular pSV2neo-DL, none of the cell lines yielded G418-resistant (G418^R) colonies, but, when transfected with pSV2neo-DR or DR-pLCX linearized at the site of the deletion, several cell lines yielded G418^R colonies. When pSV2neo (wild type) is used as the exogenous DNA, G418^R colonies result from integration of the plasmid at many sites in the recipient cell genome (nonhomologous recombination). On the other hand, G418^R colonies obtained after transfection with the DR plasmids result from homologous recombination between the chromosomal plasmid and the incoming plasmid (see below). Thus the ratio of G418^R colonies obtained from use of DR-pLCX to those obtained from pSV2neo provides a measure of the ratio of homologous to nonhomologous events. This ratio ranged from a high of 1:76 in KY22b to a low of 1:500 in KY21b. Combining the successful experiments gives a ratio in hamster cells of 1:174 and in human cells 1:78.

Characterization of the G418^R Colonies. There are three simple mechanisms by which the secondary transfectants could have acquired a G418^R phenotype. They are (*i*) a double-crossover or gene-conversion event between the incoming DNA and the chromosomal plasmid that results in correction of the 248-bp deletion in the chromosomal sequence, (*ii*) correction of the 283-bp deletion in the incoming DR plasmid by a similar mechanism followed by integration of the corrected plasmid elsewhere in the genome, and (*iii*) a single-crossover event that results in the generation of an intact *neo* gene accompanied by integration of the incoming plasmid into the homologous chromosomal site. Secondary cell lines generated by any of these mechanisms should have a wild-type *neo* gene that can be identified by Southern blot analysis at the genome level or by rescuing the integrated gene and analyzing the resulting rescued plasmid. Since the DL *neo* gene and the exogenous plasmid DR-pLCX used in generating G418^R colonies from KY21b and KY22b have different flanking markers, analysis of the rescued plasmids allows the deduction of mechanisms by which the wild-type *neo* genes were generated. Most of the experiments we are reporting here were with secondary cell lines derived from the primary transfectants KY21b and KY22b.

Table 1. Frequency of homologous recombination in hamster and human cells

	G418^R colonies with sequence indicated						
	WT		DL		DR		
Primary cell line	Col. per μg of DNA	Freq.	Col. per μg of DNA	Freq.	Col. per μg of DNA	Freq.	Freq. with DR/ freq. with WT
RK47a	14/1	14	0/12.5	0	0/19.5	0	—
RK47b	68/1	68	0/12.5	0	16/19.5	0.8	1:85
RK47c	10/1	10	0/12.5	0	0/19.5	0	—
RK48d	145/1	145	0/12.5	0	7/19.5	0.36	1:400
KY34a	NT		NT		74/14	5.3	—
KY36b	170/8	21	0/100	0	10/114	0.09	1:230
KY21b	163/8	20	0/32	0	4/90	0.04	1:500
KY22b	648/11	59	0/130	0	155/200	0.78	1:76

WT, wild type; DL, PSV2neo-DL (deletion left); DR, PSV2neo-DR (deletion right); col., colonies; freq., frequency.

Total cellular DNA from primary and secondary transfectants was digested to completion with *Xba* I, transferred to nitrocellulose, and hybridized using pSV2neo as the probe. Representative results with KY22b and its derivatives are presented in Fig. 2. *Xba* I does not cut within the chromosomal pSV2neo-DL-SV2gpt but does cut once within the exogenous plasmid DR-pLCX. KY22b has five bands (Fig. 2, lane C) ranging in size from 13 to >26 kilobases (kb), indicating that this primary cell line contained five integrations of the plasmid. All of the secondary cell lines (lanes D–I) shared one band at 13 kb with the primary cell line, but in addition each secondary cell line lost some bands and acquired new bands of various sizes. Since survival under selection requires the retention of only one intact bacterial *gpt* gene and the reconstitution of only one intact *neo* gene, it is not surprising that the secondary cell lines lose some of the sequences originally present in KY22b. Because most of the new bands are in the 19- to >26-kb range, we were unable to deduce the nature of the events leading to their formation from changes in their sizes. We, therefore, proceeded to rescue the integrated plasmids by fusing the transformants derived from KY21b and KY22b with monkey COS cells and 48 hr later isolating low molecular weight DNA from the fusion products. This DNA was then used to transform *recA*$^-$ *E. coli*, and colonies were selected on Amp- or Kan-containing plates. Each of the colonies was characterized for its drug resistance pattern by replica plating.

The results from a representative set of experiments are shown in Table 2. As expected, plasmids rescued from the parental cell lines KY21a and KY22b yielded only Amp^R,-Kan^S colonies, where R is resistant and S is sensitive. Plasmids rescued from all but one of the secondary transfectants yielded both Amp^R,Kan^R and Amp^R,Kan^S colonies. KY22b-2A gave only Amp^R,Kan^R colonies.

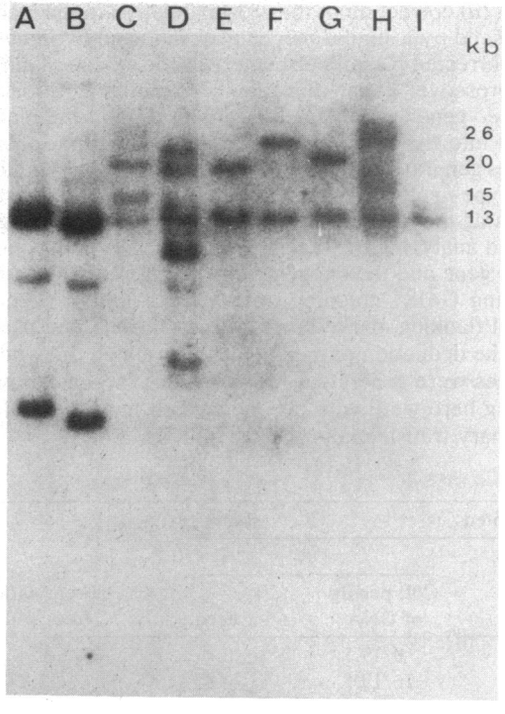

FIG. 2. Blot hybridization of DNA from KY22b and secondary transfectants derived from KY22b. DNA was digested with *Xba* I and blot hybridized using pSV2neo as the probe. Lanes: A, pSV2neo-SV2gpt; B, pSV2neo-DL-SV2gpt; C, primary transfectant KY22b. Secondary transfectants (lanes D–I). Lanes: D, KY22b-1B; E, KY22b-2A; F, KY22b-3B; G, KY22b-4B; H, KY22b-5A; I, KY22b-6A. Lanes A and B show form I, II, and III DNA.

Table 2. Drug resistance patterns of plasmids rescued by COS cell fusion of secondary transfectants

Cell line	Transfectant	Colonies, no. Amp^R,Kan^R	Colonies, no. Amp^R,Kan^S	% Kan^R colonies
KY21b	1°	0	24	0
KY21b-2a	2°	5	10	33
KY22b	1°	0	24	0
KY22b-1B	2°	13	47	22
KY22b-2A	2°	62	0	100
KY22b-3B	2°	7	20	26
KY22b-4B	2°	29	1	97
KY22b-5A	2°	37	23	62
KY22b-6A	2°	8	3	73

1°, primary; 2°, secondary.

Representative plasmids rescued from each cell line were mapped by restriction enzyme digestions. Results with *Pst* I and *Nde* I/*Bam*HI are shown in Fig. 3, and the restriction maps obtained with other enzymes are summarized in Fig. 4. Digestion of pSV2neo-SV2gpt with *Pst* I yields four bands (Fig. 3, lane C) 3.8, 2.4, 0.96, and 0.92 kb long. In pSV2neo-DL-SV2gpt the 2.4-kb band is reduced by the deletion to 2.2 kb (Fig. 3, lane B). Plasmids rescued from KY22b carried the 2.2-kb band diagnostic of the deleted *neo* gene (Fig. 3, lane D), while all but one of the Kan^R colonies rescued from secondary transfectants derived from KY22b show the 2.4-kb *Pst* I band (Fig. 3, lanes E–I) diagnostic of the intact *neo* gene. Because the plasmids rescued from each cell line vary in size, the other bands show variability in size. This demonstration that the secondary transfectants carry an intact *neo* gene is confirmed with other restriction enzymes. *Nde* I/*Bam*HI digestions show that a 2.9-kb band is diagnostic of the normal *neo* gene (Fig. 3, lane L) while the DL deletion is characterized by a 2.7-kb band (Fig. 3, lane K). As expected, the KY22b-derived plasmid carries the 2.7-kb band (Fig. 3, lane M), while all of the Kan^R plasmids from the secondary transfectants contain the 2.9-kb band (Fig. 3, lanes N–S).

Since the *neo* gene in the chromosomal plasmid and in the input plasmid are flanked by different genetic markers, these markers in the rescued plasmids can reveal the nature of the recombination event that led to the generation of a wild-type *neo* gene. Results of this analysis are shown diagramatically

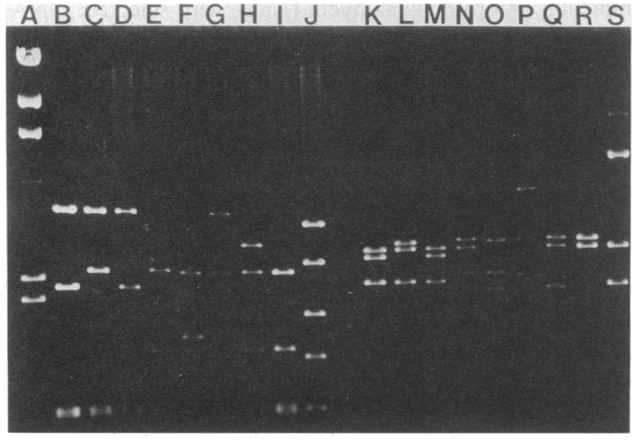

FIG. 3. Restriction enzyme digestion of plasmids rescued from KY22b and its G418^R derivatives. λ marker phage DNA digested with *Hin*dIII (lane A). DNA digested with *Pst* I (lanes B–J). DNA digested with *Nde* I/*Bam*HI (lanes K–S). Lanes: B and K, pSV2neo-DL-SV2gpt; C and L, pSV2neo-SV2gpt; D and M, plasmids rescued from KY22b; E and N, KY22b-1B; F and O, KY22b-2A; G and P, KY22b-3B; H and Q, KY22b-4B; I and R, KY22b-5A; J and S, KY22b-6A.

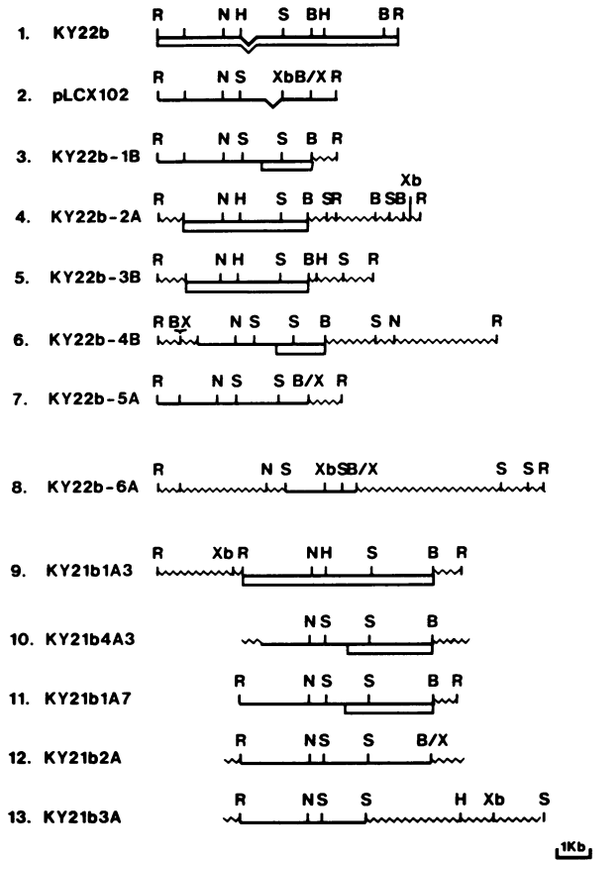

FIG. 4. Restriction enzyme maps of plasmids rescued from different cell lines. Maps were deduced from single and double digests with different restriction endonucleases. The presence of *Bam*HI or *Bam*HI/*Xho* I sites at the 3′ end of the *neo* gene was ascertained by primer directed DNA sequencing. Straight lines, sequences that are definitely derived from the *neo* plasmids. Wavy lines, DNA of plasmid or chromosomal origin. Double lines, sequences derived from chromosomal plasmid sequences. Single straight lines, sequences derived from DR-pLCX. B, *Bam*HI; H, *Hin*dIII; N, *Nde* I; R, *Eco*RI; X, *Xho* I; Xb, *Xba* I.

in Fig. 4. The DL *neo* gene in the chromosomal plasmid (Fig. 4, line 1) is flanked on its 5′ side by a *Hin*dIII site and on its 3′ side by *Sma* I and *Bam*HI sites. In DR-pLCX (Fig. 4, line 2) the DR *neo* gene is flanked by a *Sma* I site on its 5′ end and by *Xba* I and *Bam*HI/*Xho* I sites on its 3′ end. Single- and double-crossover events involving these two plasmids yield wild-type genes with distinctive flanking markers. Thus correction of the chromosomal plasmid by a double crossover will yield a wild-type *neo* gene flanked by *Hin*dIII on one side and *Sma* I/*Bam*HI on the other side (class 1). Correction of the deletion in pLCX by a double crossover will yield a plasmid carrying a wild-type *neo* gene flanked by a *Sma* I site at the 5′ end and a *Bam*HI/*Xho* I site at its 3′ end (class 2). A single crossover between the chromosomal and input plasmid sequence will yield a *neo* gene with a *Sma* I site on its 5′ side and a *Bam*HI site on its 3′ side (class 3).

We analyzed plasmids carrying a wild-type *neo* gene from 11 secondary transfectants (Fig. 4, lines 3–13). Of these, 3 are of class 1 (22b2A, 22b3B, and 21b1A3), 3 are of class 2 (22b5A, 22b6A, and 21b2A), and 4 are of class 3 (22b1B, 22b4B, 21b4A3, and 21b1A7). The origin of KY21b3A was ambiguous; the Kan^R plasmid from it (line 13) contained an intact wild-type *neo* gene with a *Sma* I site at its 5′ end and another *Sma* I site at its 3′ end, but it did not carry a *Bam*HI or *Bam*HI/*Xho* I site. This *neo* gene could be from either a single-crossover event (class 3) or a double-crossover event correcting a DR-pLCX-derived gene (class 2). These overall results indicate that the wild-type *neo* genes were generated by the three expected mechanisms at close to equal frequencies.

To establish that the plasmids rescued by the COS cell fusions correctly represent the original *neo* genes in the cell, we rescued the gene from the secondary transfectant KY22b1B by a method not involving COS cells. Total cellular DNA from KY22b1B was digested to completion with *Xba* I and ligated under cyclization conditions. The resulting DNA was used to transform *recA*$^-$ *E. coli*. Using 0.75 μg of DNA we obtained four Amp^R colonies. One of these was Kan^R. The restriction map of this Amp^R,Kan^R plasmid showed that the structure of this plasmid around the *neo* gene is identical to that of the equivalent plasmid rescued from COS cells (results not shown). These results validate COS cell fusions for rapidly analyzing the recombination event.

DISCUSSION

We have shown here that homologous recombination can occur at high efficiency between a chromosomal plasmid in a primary transfectant and a plasmid introduced by a calcium phosphate coprecipitate. In each of the primary cell lines studied, the chromosomal plasmid is at a different site within the genome. We observe that the efficiency with which recombination occurs in the different cell lines varies over a 10-fold range. Since each of the cell lines carries only two–five copies of integrated plasmids, this variation cannot be solely attributed to the copy number. It is likely that the variation reflects chromosomal position effects.

In our experiments, the ratio of homologous to nonhomologous recombination events ranged from 1:75 to 1:500. When all of the results are combined this ratio is 1:174 in hamster cells and 1:78 in human cells. Other investigators (8, 9) have reported similar success in modifying a chromosomal plasmid. Using a herpes simplex virus thymidine kinase gene as the target Lin *et al.* (9) observed a recombination frequency of 10^{-6} per cell/per 4 μg of DNA. Smith and Berg (8) using the *neo* gene as the target obtained a ratio of homologous to nonhomologous recombination of 1:1000 to 1:10,000. Both groups used calcium phosphate coprecipitation to introduce the recombination substrate. Thomas *et al.* (11) have demonstrated homologous recombination between a chromosomal *neo* plasmid and its counterpart introduced by microinjection. In their experiments the ratio of homologous to nonhomologous events was 1:100. The similarities of frequencies obtained in this previous (11) and our present study suggest that the method of introducing the secondary plasmid does not greatly influence the frequency of homologous recombination.

Of the 11 recombination events we analyzed by plasmid rescue from $G418^R$ colonies, three appeared to have resulted from correction of the chromosomal sequence by the input plasmid, three from correction of the input plasmid by the chromosomal plasmid followed by integration of the corrected plasmid elsewhere in the genome, and four from single-crossover events leading to integration of the input plasmid at its homologous site. Southern blots of the DNA from the secondary cell lines KY22b1B, KY22b2A, and KY22b3B digested with *Hin*dIII or *Hin*dIII/*Bam*HI supported these interpretations (results not shown). We, therefore, conclude that at least three types of recombination events are possible. Since correction by single-crossover and gene-conversion (double-crossover) events may reflect alternate modes of resolution of recombination intermediates (25, 26), it is not surprising that these two types of events are recovered at approximately equal frequencies. Similar observations have been made in studies of plasmid–plasmid recombination in mammalian cells (e.g., refs. 7 and 27). Smithies *et al.* (12)

observed only single-crossover events in their experiments in which the human β-globin locus was used as a target, but their experimental design only permitted detection of such events. Thomas *et al.* (11) observed only gene-conversion (double-crossover) events in their experiments utilizing a defective *neo* gene as the target, and they suggested that this may be either due to an inherent preference of the mammalian recombination machinery for such events or to a bias introduced by the nature of the substrates they used. Our results show that the first interpretation is not likely to be correct, because we find that mammalian cells are inherently capable of mediating both single-crossover and double-crossover or gene-conversion types of events with no obvious bias in favor of either.

Thomas and Capecchi (13) reported that in some cases they obtained restoration of the Neo^R phenotype by a mechanism other than simple homologous recombination. They showed that an amber mutation at the 5′ end of the integrated *neo* gene remained intact and that a Neo^R phenotype was achieved by insertion of one, four, or seven bases downstream. In the plasmids rescued from secondary transfectants of KY21a and KY22b, we have observed that the original lesion in the *neo* gene was corrected and that the recombination events were accurate at the level of resolution permitted by the methods employed. Possibly the deletion substrates that Thomas and Capecchi (13) used are particularly prone to the insertional events catalyzed by the presence of recombinational intermediates. Possibly our substrates cannot accept such modifications and yield a $G418^R$ phenotype. Whatever the explanation of this difference may be, we stress that our results have not revealed any evidence for mutation events accompanying homologous recombination between the resident and incoming DNA sequences.

Correction of resident sequences by double crossovers, such as we and others have shown, could eventually be very useful for gene therapy, since the cellular mutation can be corrected without introducing any new genetic information into cells. Single-crossover events, such as we have described here and have been reported by Smithies *et al.* (12) with a native cellular gene could be useful for experiments where gene inactivation is desirable. Clearly we need to know how to preferentially promote each of these types of events.

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