MOLECULAR AND CELLULAR BIOLOGY, Jan. 1985, p. 59–69
0270-7306/85/010059-11$02.00/0

Vol. 5, No. 1

Nonreciprocal Exchanges of Information Between DNA Duplexes Coinjected into Mammalian Cell Nuclei

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Received 31 August 1984/Accepted 8 October 1984

We have examined the mechanism of homologous recombination between plasmid molecules coinjected into cultured mammalian cells. Cell lines containing recombinant DNA molecules were obtained by selecting for the reconstruction of a functional Neo^r gene from two plasmids that bear different amber mutations in the Neo^r gene. In addition, these plasmids contain restriction-length polymorphisms within and near the Neo^r gene. These polymorphisms did not confer a selectable phenotype but were used to identify and categorize selected and nonselected recombinant DNA molecules. The striking conclusion from this analysis is that the predominant mechanism for the exchange of information between coinjected plasmid molecules over short distances (i.e., <1 kilobase) proceeds via nonreciprocal homologous recombination. The frequency of homologous recombination between coinjected plasmid molecules in cultured mammalian cells is extremely high, approaching unity. We demonstrate that this high frequency requires neither a high input of plasmid molecules per cell nor a localized high concentration of plasmid DNA within the nucleus. Thus, it appears that plasmid molecules, once introduced into the nucleus, have no difficulty seeking each other out and participating in homologous recombination even in the presence of a vast excess of host DNA sequences. Finally, we show that most of the homologous recombination events occur within a 1-h interval after the introduction of plasmid DNA into the cell nucleus.

We previously demonstrated that mammalian somatic cells in culture contain the enzymatic machinery to mediate recombination between newly introduced, nonreplicating plasmid DNA molecules with a frequency approaching unit (5). This was a surprising result because the frequency of homologous recombination in cultured somatic cells between given markers on a chromosome is low, 1 per 10^6 to 10^7 cells per generation (10, 14, 19, 23). Our previous analysis of homologous recombination in cultured mammalian cells involved demonstrating that the cells' propensity to incorporate multiple copies of a given plasmid into head-to-tail concatemers resulted from reciprocal homologous recombination between the newly introduced plasmid DNA molecules. Injected linear and supercoiled plasmid DNAs were equally efficient in entering the head-to-tail concatemers. Once in the chromosome the head-to-tail concatemers were stable even when grown for hundreds of generations in nonselective medium (5). This observation is consistent with the much lower rate of homologous recombination observed between markers in the chromosome. Thus, it appears that, whereas newly introduced naked DNA is extremely susceptible to manipulation by the cellular recombination machinery, once in chromatin structure the same DNA sequences become refractory to the action of the recombination machinery.

Studies on homologous recombination in mammalian cells, using nonreplicating plasmids, have been extended by monitoring the reconstruction of a functional gene from plasmids bearing different mutations in the gene (4, 9, 15, 16). These studies utilized deletion and insertion mutations. The frequency of recombination between the cotransferred mutant plasmids was again found to be very high and roughly proportional to the distance between the mutations.

An alternative, but very attractive system for analyzing recombination in mammalian cells uses mammalian viruses. This system has been used to study both inter- and intramolecular recombination (17, 18, 20–22). Initially, it was not clear whether or not these recombination events were dependent on viral replication or on virus-coded functions or both. More recent experiments indicate that recombination will occur in the absence of viral replication, although it occurs at a lower frequency in the absence of replication, as expected. One frequent feature of the viral system is that the recombinant is a replicating virus, automatically amplifying the recombinant product and thereby facilitating analysis of the recombinant product. Subramani and Berg (17) have exploited this system to study homologous and nonhomologous recombination in cultured monkey cells at the DNA sequence level. Using a simian virus 40-pBR322 hybrid plasmid that generated simian virus 40 molecules by intramolecular excision, they found that homologous and nonhomologous recombination events occurred with roughly equal frequency.

In this paper, we examined three issues. The first question concerned the general mechanism of homologous recombination between coinjected plasmid molecules in mammalian cells. Does it involve reciprocal or nonreciprocal exchanges of information between DNA duplexes? The second question that we addressed was whether the high recombination frequency observed between coinjected plasmid molecules resulted from a localized high concentration of plasmid molecules. Third, we determined the time interval between when plasmid molecules were introduced into the nucleus and when recombination between the coinjected molecules occurred.

MATERIALS AND METHODS

The methods used for culturing mammalian cells, plasmid DNA preparation, Southern transfer analysis, microinjection, and the isolation and characterization of the amber mutations in the Tn*5* Neo^r gene have been described else-

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where (2, 5, 8; K. Folger, K. Thomas, and M. R. Capecchi, Cold Spring Harbor Symp. Quant. Biol., in press).

Plasmid rescue. Total DNA from cultured mammalian cells was purified as described previously (5) and digested to completion with *Bam*HI restriction endonuclease. A 4-μg portion of the digested DNA was ligated at 4°C for 16 h in a 400-μl reaction mixture consisting of 50 mM Tris-hydrochloride (pH 8.0), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, and 400 U of T4 DNA ligase (New England Biolabs). The ligated DNA was precipitated by the addition of 2 volumes of 100% ethanol and suspended in 40 μl of 10 mM Tris-hydrochloride (pH 8.0)–1 mM EDTA. A 10-μl portion of this sample was used to transform 90 μl of $CaCl_2$-shocked *Escherichia coli* strain MH1 (6). The transformation mixture was plated on L-broth agar plates (13) supplemented with 100 μg of ampicillin per ml.

Plasmid analysis. To assay for the presence of a functional Neo^r gene, plasmid-transformed bacterial cells were streaked from single colonies on M63 plates (13) supplemented with 0.2% glucose, 1 μg of vitamin B_1 per ml, 50 μg of ampicillin per ml, and 100 μg of kanamycin (Sigma Chemical Co.) per ml. After 36 h of incubation at 37°C, the size of individual

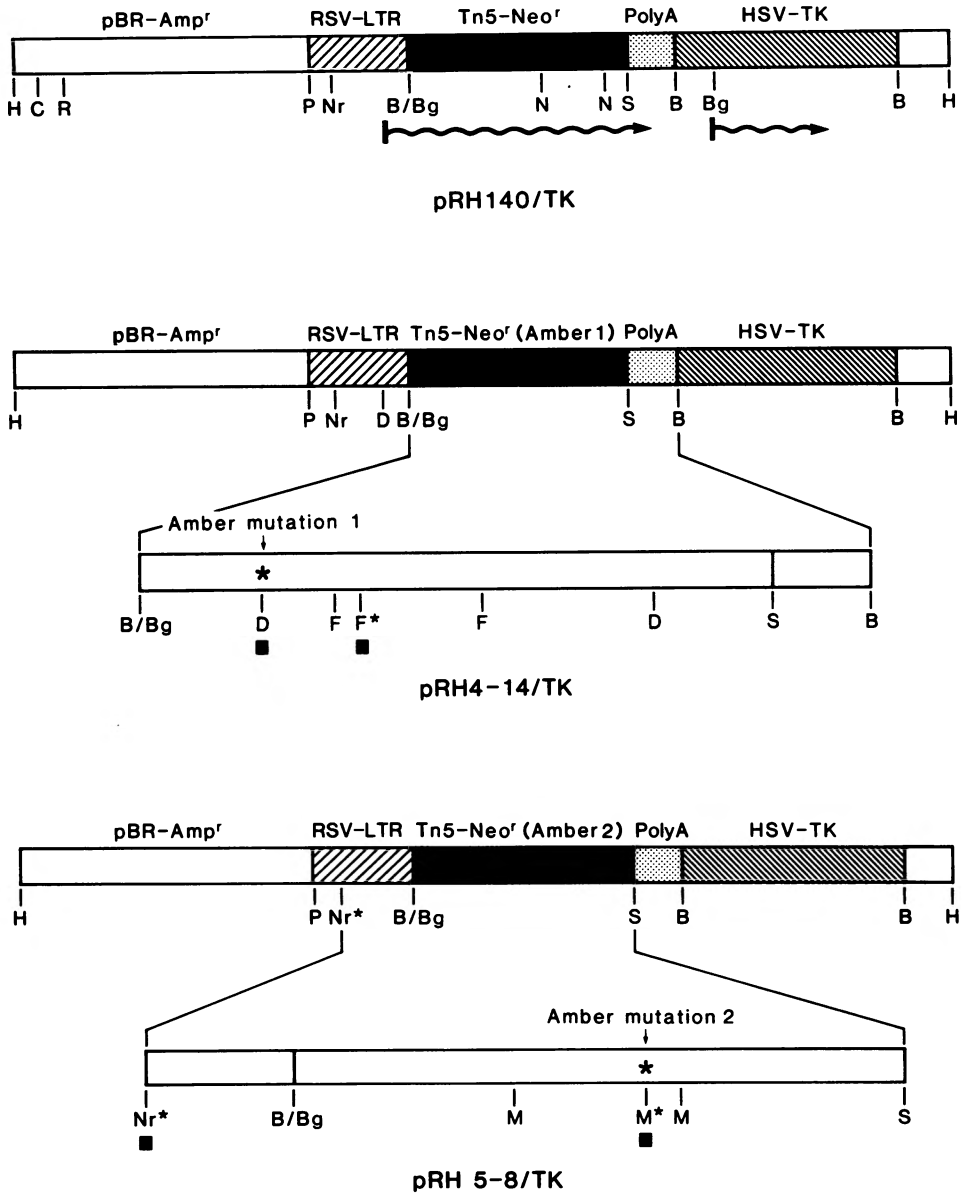

FIG. 1. Maps of the three Neo^r/thymidine kinase vectors pRH140/TK, pRH4-14/TK, and pRH5-8/TK. The three vectors were derived from pRH140 (8). The RSV-LTR provides an enhancer (11) and a bifunctional promoter which allows expression of the Neo^r gene in both *E. coli* and mammalian cells (8). pRH4-14/TK and pRH5-8/TK contain amber mutations in the Neo^r gene. The HSV tk gene was inserted into the above vectors at the unique *Bam*HI site in the pBR322 sequence as a 3.4-kb *Bam*HI fragment. Each of the vectors is represented in linear form from the unique *Hin*dIII site. The restriction sites are as designated: 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*4H1; M = *Mbo*I. Mutations resulting in the gain or loss of a restriction site are designated by a box under the site. The asterisk next to the restriction site abbreviation indicates the loss of the site. pRH4-14/TK and pRH5-8/TK each contain two mutations which create changes in the restriction sites compared to the wild-type gene. The nature of these mutations is summarized in Fig. 2 to 5. Poly A, Polyadenylated.

colonies was scored (see Fig. 6). Restriction enzyme analysis of rescued plasmids was performed on plasmid DNA purified from bacterial cells by the boiling-lysis method of Maniatis et al. (12). Plasmid DNAs were analyzed by digestion with *Bam*HI, *Bam*HI + *Hin*dIII, *Eco*RI, *Bam*HI + *Nru*I, and *Dde*I.

Analysis of restriction-length polymorphism. The *Dde*I and *Nru*I restriction-length polymorphisms present in recombinant molecules were visualized on agarose gels of digested rescued plasmid DNA or by Southern transfers (see Fig. 2 and 5). To visualize the *Fnu*4H1 polymorphism present in plasmids rescued from G418^r-transformed cell lines, plasmid DNA, digested with *Fnu*4H1, was denatured and resolved on 8% polyacrylamide gels. The resolved fragments were then transferred onto Gene Screen filters (New England Nuclear Corp.) by electroelution and processed by the method of Church and Gilbert (3). The filters were hybridized with a ^{32}P-labeled probe copied from an insert in M13mp8 containing a 295-base pair (bp) *Pst*I-*Eco*RI fragment from the long terminal repeat (LTR)-Neo^r region of pRH140 (see Fig. 3). To determine the presence of the *Mbo*I polymorphism present in rescued plasmids, the DNA was digested with *Mbo*I and end-labeled with DNA polymerase "Klenow fragment," using [α-^{32}P]dATP, in the presence of unlabeled dCTP, dGTP, and dTTP. The labeled fragments were separated on 6% polyacrylamide-urea gels and the fragments were visualized by exposure to XAR5 film (see Fig. 4).

RESULTS

Recombinant plasmids. In Fig. 1 we illustrate the recombinant plasmids used in the experiments we describe. The parental plasmid, pRH140/TK, contains sequences derived from the bacterial plasmid pBR322, the herpes simplex virus thymidine kinase gene (HSV TK), and the neomycin resistance gene (Neo^r) 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 neomycin resistance gene is transcribed from a promoter isolated from an avian retrovirus LTR (RSV-LTR). This promoter functions in both bacterial and mammalian cells (8). To allow expression of the Neo^r gene in mammalian cells, a polyadenylic acid addition site, derived from the HSV TK gene, was added to the 3′ terminus of the gene. Transfection of bacteria with pRH140/TK DNA confers ampicillin and kanamycin resistance. DNA-mediated transfer of pRH140/TK DNA into a thymidine kinase-deficient mouse cell line, LM*tk*$^-$, renders the cells resistant to the drug G418 and to hypoxanthine-aminopterine-thymidine selection (8).

The derivative plasmid pRH4-14/TK contains an amber mutation in the Neo^r gene. This premature termination signal renders the gene product defective in both bacterial and mammalian cells (8). The mutation was induced by in vitro mutagenesis of pRH140 with hydroxylamine. Sequence analysis, when compared with the wild-type sequence (1), showed that the amber mutation arose as a result of a G → A transition in a tryptophan codon (TGG; amino acid residue 15) converting it to an amber code, TAG (L. Fraser, J. Goddard, and M. R. Capecchi, unpublished data). The G → A transition that created the amber mutation concomitantly created a new *Dde*I site which could be used as a diagnostic test for the presence of the amber mutation in recombinant molecules (Fig. 2). The hydroxylamine mutagenesis also induced a silent mutation in the Neo^r gene, resulting in the destruction of an *Fnu*4H1 restriction enzyme recognition site within the coding region (Figs. 1 and 3). The silent mutation resulting in a restriction-length polymorphism wasused as a marker to follow the transfer of information from pRH4-14/TK to other DNA duplexes.

A second derivative plasmid, pRH5-8/TK, contains a different amber mutation 417 bp downstream from the 4-14 amber mutation. This mutation resulted from a C → T transition of a glutamine codon (CAG) to the amber codon and at the same time destroyed an *Mbo*I site which could be used as a diagnostic test for the presence or absence of the 5-8 amber mutation (Fig. 4). In addition, a restriction site polymorphism was introduced into this plasmid by the removal of a single base pair in the *Nru*I site contained in the RSV-LTR (Fig. 5). This single-base pair deletion does not affect either the promoter function or the "enhancer" function of the RSV-LTR (11). All of the above polymorphisms were identified by sequence analysis and confirmed by restriction enzyme analysis.

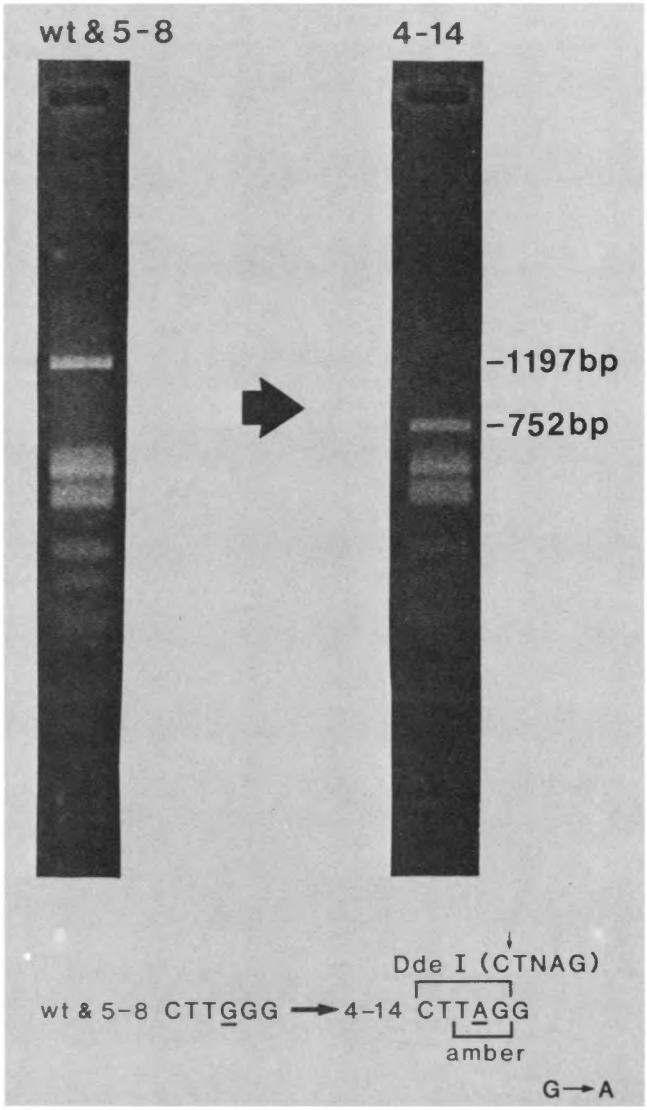

FIG. 2. *Dde*I polymorphism. The amber mutation in the Neo^r gene of pRH4-14/TK resulted from a G → A transition and concomitantly created a new *Dde*I site. The new *Dde*I site causes a shift of a 1,197-bp fragment into two fragments 752 and 445 bp in size. The shift can be visualized on agarose gels of digested plasmid DNA (see above) or by Southern transfers with a nick-translated *Eco*RI-*Pst*I probe (Fig. 3).

Homologous recombination between coinjected plasmids. LM*tk*$^-$ cells receiving an injection of either pRH4-14/TK or pRH5-8/TK alone have never been observed to become transformed to G418^r (Table 1). By comparison, 10 to 15% of the cells injected with plasmid DNA containing the wild-type Neo^r gene become G418^r (Table 1). Coinjecting either pRH4-14/TK and pRH5-8/TK supercoiled molecules at 25 to 50 copies per cell or pRH4-14/TK and pRH5-8/TK linear mol-

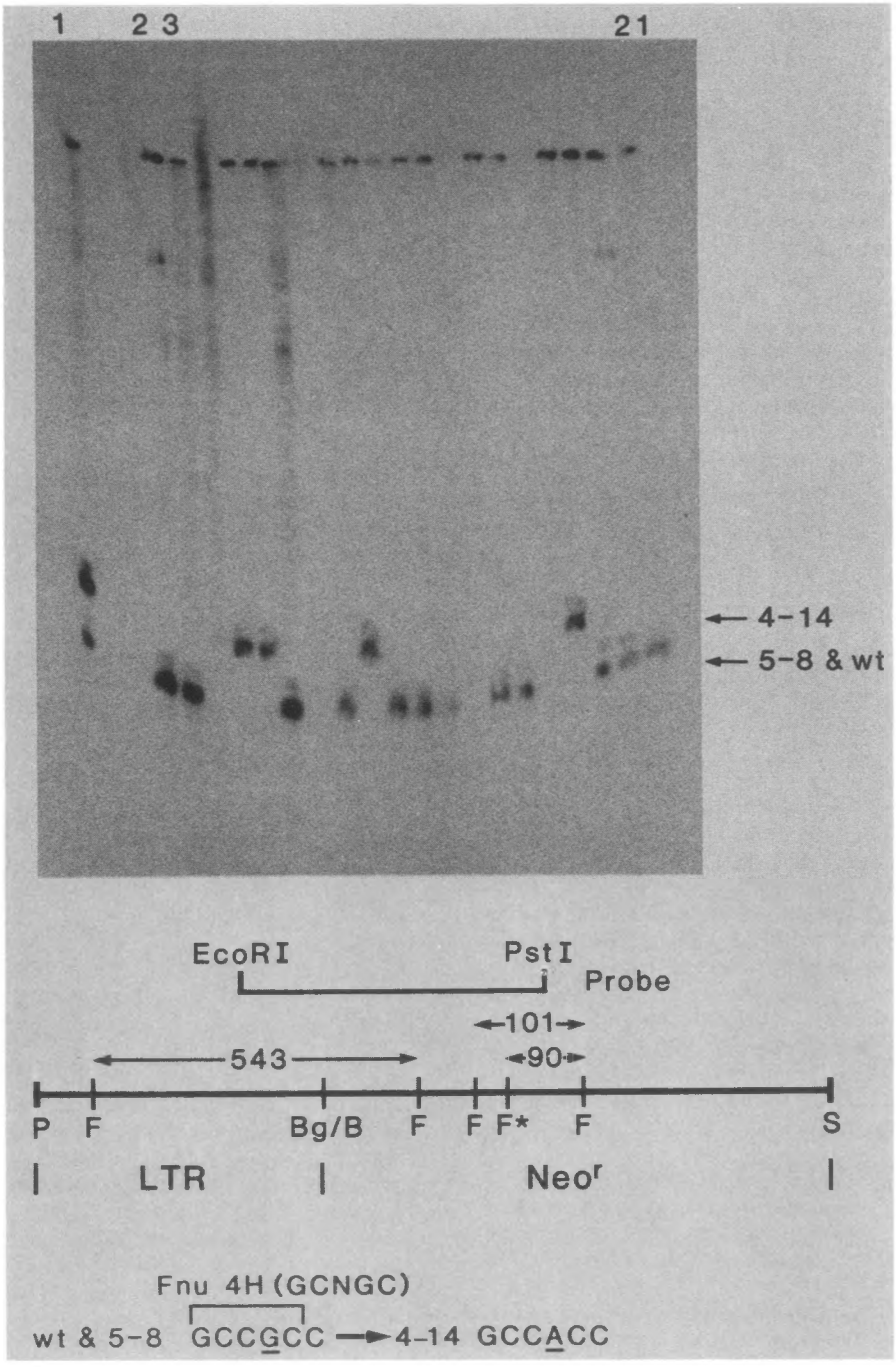

FIG. 3. *Fnu*4H1 polymorphism. The pRH4-14/TK plasmid contains a silent mutation within the Neo^r coding sequence. This G → A transition converts a leucine codon to a threonine codon and results in the destruction of an *Fnu*4H1 site. The loss of the site causes the shift of two fragments, 90 and 11 bp in length, to one 101-bp fragment. The shift can be visualized on transfer of digested plasmid DNA from acrylamide gels onto filters, using a Klenow fragment-labeled *Eco*RI-*Pst*I probe (see text). Lane 1 contains a mixture of pRH4-14/TK and pRH5-8/TK digested with *Fnu*4H1. Lanes 2 through 21 contain digestions of plasmid DNA rescued from cells transformed with a mixture of 4-14 and 5-8 supercoiled plasmids. The 4-14 arrow indicates the position of the 101-bp band and the 5-8 or wild-type (wt) arrow indicates the position of the 90-bp band. The band near the top is the 543-bp *Fnu*4H1 fragment containing LTR and Neo^r sequences that overlaps the probe. The 52-bp fragment (between the 543- and 101-bp fragments) and the 11-bp fragment were electrophoresed off the bottom of the gel to resolve the 101-bp band from the 90-bp band.

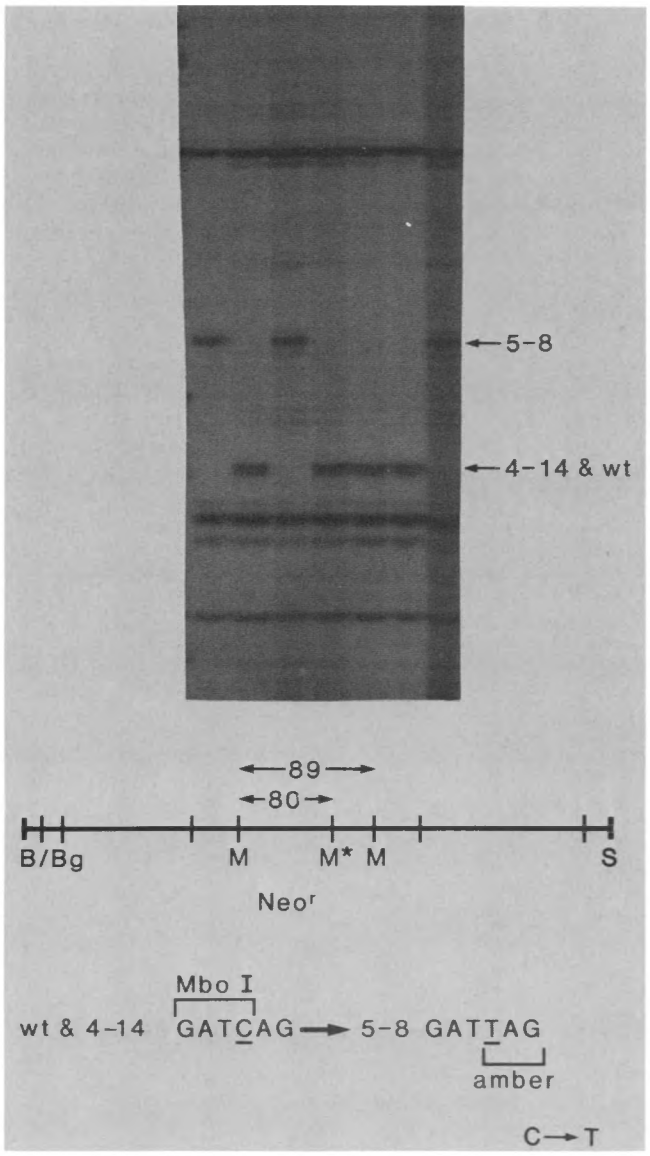

FIG. 4. *Mbo*I polymorphism. The amber mutation in the Neo^r gene of pRH5-8/TK resulted from a C → T transition which concomitantly destroyed an *Mbo*I site. The loss of the site causes the shift of two fragments, 80 and 9 bp in size, to one 89-bp fragment. The 80- to 89-bp shift was visualized by autoradiography of *Mbo*I fragments of plasmid DNA, end labeled with a Klenow fragment and [α-^{32}P]ATP and resolved on polyacrylamide gels. (Top) Analysis of the *Mbo*I polymorphism present in a series of plasmids rescued from the G418^r-transformed cell lines. The positions of the 4-14 and 5-8 restriction fragments, 84 and 93 bp in length after Klenow fill-in, are indicated by the arrows. wt, Wild type.

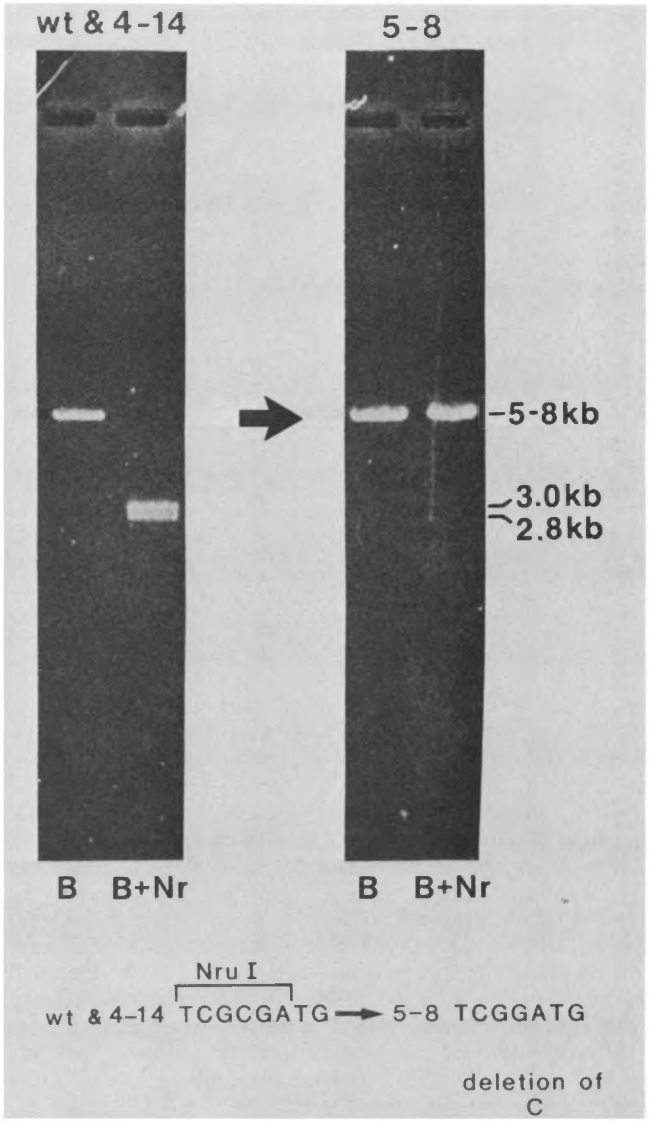

FIG. 5. *Nru*I polymorphism. The single-base pair deletion in the RSV-LTR region of pRH5-8/TK results in the destruction of an *Nru*I site. This deletion was obtained by ligating *Nru*I fragments of pRH5-8 in the presence of *Nru*I and transfecting *E. coli*. This mutation does not effect either the "enhancer" or the promoter function of the RSV-LTR. The loss of the site causes a shift of two fragments 2.8 and 3.0 kb in size to a 5.8-kb fragment in plasmids rescued from the G418^r-transformed cell lines and digested with *Bam*HI plus *Nru*I.

ecules at 5 copies per cell renders 0.7 and 1.1% of the cells receiving an injection resistant to G418, respectively (Table 1).

The reason that we injected more supercoiled molecules than linear molecules per cell is that we previously demonstrated that at a low input of plasmid DNA per cell linear molecules transform mammalian cells much more efficiently compared with supercoiled molecules, whereas at a high input of plasmid DNA per cell linear and supercoiled molecules transformed mammalian cells with equal efficiency (5). To compensate for the difference in transformation efficiencies of linear and supercoiled molecules in these studies, we injected 5 and 25 to 50 copies of linear and supercoiled molecules, respectively, per cell.

The generation of a functional Neo^r gene from the coinjected plasmid DNAs implies the occurrence of homologous recombination between the 4-14 and 5-8 amber mutations. Considering that the distance between these amber mutations represents only 5% of the plasmid length, the frequency of G418^r after coinjection of the pRH4-14/TK and pRH5-8/TK plasmid DNAs is extremely high.

Analysis of the recombinant molecules in the G418^r cell lines obtained by coinjecting pRH4-14/TK and pRH5-8/TK DNA. We have analyzed the nature of the homologous recombination events occurring between the coinjected plasmid molecules by following the segregation patterns of a series of

TABLE 1. DNA-mediated transformation frequencies to G418^r obtained by injecting LM*tk*$^-$ cells with pRH140/TK and derivative plasmids[a]

DNA injected	No. of transformants/10^3 cells receiving injections	No. of cells injected
pRH140/TK, SC	122	10^3
pRH140/TK, L	141	10^3
pRH4-14/TK, SC	0	10^4
pRH4-14/TK, L	0	10^4
pRH5-8/TK, SC	0	10^4
pRH5-8/TK, L	0	10^4
pRH4-14/TK (SC) + pRH5-8/TK (SC)	7	2×10^3
pRH4-14/TK (L) + pRH5-8/TK (L)	11	2×10^3

[a] LM*tk*$^-$ cells were grown on cover slips (10 by 10 mm) in 35-mm petri dishes. From 5 to 50 cells per dish received nuclear injections with the DNA solution. The number of cells per dish receiving an injection was chosen such that at the conclusion of the experiment many of the petri dishes contained no transformed colonies. After the injections the cells were incubated for 24 h in nonselective medium at 37°C in a 5% CO_2 incubator and then switched to minimum essential medium supplemented with 300 μg of G418 per ml. After 1.5 weeks and then again after 2.5 weeks, the dishes were scored for the presence of large, healthy colonies. The cells received nuclear injections of either 25 supercoiled (SC) molecules per cell or 5 linear (L) molecules per cell of each respective plasmid DNA.

restriction-length polymorphisms contributed by both parental plasmid molecules. To associate a particular restriction-length polymorphic pattern with a given recombinant molecule, the recombinant plasmids were "rescued" from the genome of the G418^r cells. Genomic DNA 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 recombination plasmid molecules (i.e., Kan^r colonies) present in the G418^r cell lines but rather rescued all plasmids that contained a functional Amp^r gene and a bacterial origin of DNA replication derived from pBR322 sequences. By this procedure we isolated both selected (Kan^r) and unselected (Kan^s) recombinant plasmids as well as the input Kan^s parental molecules. Note also that the rescued plasmids do not contain the HSV TK gene (Fig. 1).

Reconstruction experiments indicated that any recombinant plasmid that we rescued was the result of a recombination even in mammalian cells and not in bacteria. The concentration of plasmid sequences in a transformed cell line is so low that the probability of a bacterial cell receiving two or more plasmid molecules during a transfection is vanishingly low. In control experiments, pRH4-14/TK and pRH5-8/TK DNA were added to mammalian DNA at concentrations of 1×, 10×, 100×, and 1,000× relative to the concentration of plasmid sequences present in a G418^r-transformed cell line. This DNA was used to transfect *E. coli* in parallel with our rescue experiments. No recombinant plasmids (i.e., Kan^r plasmids) were obtained.

Since the plasmid sequences in a G418^r-transformed cell line can exist as a head-to-tail concatemer, it could be argued that incomplete digestion of cellular DNA with *Bam*HI could result in two plasmid sequences being delivered to *E. coli* on the same piece of DNA. Since there are two *Bam*HI recognition sites in pRH4-14/TK and pRH5-8/TK (Fig. 1), the probability of rescuing adjacent plasmids on the same piece of DNA is reduced. Further, we have not observed HSV TK sequences in any of our rescued plasmids, indicating that the *Bam*HI digestion of the genomic DNA was indeed complete.

In Table 2 we summarize the characterization of 40 plasmids rescued from four G418^r cell lines obtained by coinjecting pRH4-14/TK and pRH5-8/TK supercoiled molecules. The data are pooled into classes of plasmids which share all of the tested characteristics. In Table 3 we summarize the characterization of 58 plasmids rescued from three G418^r cell lines obtained by coinjecting pRH4-14/TK and pRH5-8/TK linear molecules.

Recombinant plasmids containing a wild-type Neo^r gene were identified by streaking bacteria containing rescued plasmids on kanamycin plates. Growth on kanamycin plates also allows us to distinguish rescued plasmids which contain the 4-14 amber mutation from plasmids which carry the 5-8 amber mutation. Bacteria carrying the pRH5-8/TK plasmid grow at the origin of a streak, whereas bacteria carrying pRH4-14/TK plasmids do not (Fig. 6). The phenotypic assignment for each rescued plasmid by growth 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. All of the assignments were internally consistent.

TABLE 2. Classes of plasmids rescued from cell lines coinjected with pRH4-14/TK and pRH5-8TK as supercoiled molecules[a]

Cell line/ class	Kan^r[b]	Polymorphisms[c]				Size[d]	No. rescued[e]
		*Nru*I	*Dde*I	*Fnu*4H1	*Mbo*I		
LM88							
1	+++	X	X	X	0	L	3
2	−	X	0	0	0	J	5
3	−	0	0	0	0	J′	1
4	±	X	X	X	X	L	1
LM94							
1	+++	X	X	X	0	J	5
2	+++	X	X	X	0	L	4
LM95							
1	+++	0	X	X	0	L	2
2	−	0	0	0	0	L	2
3	−	X	0	0	0	J	2
4	±	0	X	X	X	L	2
LM98							
1	+++	X	X	X	0	L	5
2	−	0	0	0	0	L	2
3	±	X	X	X	X	L	3
4	−	X	(int)	X	X	J	3
pRH4-14	−	0	0	0	0	L	
pRH5-8	±	X	X	X	X	L	

[a] Mouse LM*tk*$^-$ cells were coinjected with 25 copies of pRH4-14/TK and pRH5-8/TK as supercoils per cell and selected for G418^r as described in footnote *a*, Table 1. DNA from four independently isolated G418^r colonies (LM88, -94, -95, and -98) was purified, and the plasmid sequences were rescued as *Bam*HI fragments (see text). Plasmids were analyzed for their Kan^r phenotype in bacteria, for the restriction site polymorphisms, and for size.

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

[c] *Nru*I, *Dde*I (4-14 amber), *Fnu*4H1, and *Mbo*I (5-8 amber) restriction site polymorphisms were assayed as described in the text and in the legends to Fig. 2 to 5. 0, the site comes from pRH4-14; X, the site comes from pRH5-8. (int), The plasmid integrated adjacent to the *Dde*I site, changing the size of the diagnostic fragments.

[d] L, plasmid is 5.8 kb in length, the expected size for a plasmid from an internal position in a tandem array; J, plasmid contains a chromosomal junction sequence (J and J′ indicates that the rescued plasmids contained different chromosomal junction fragments).

[e] Number of plasmids rescued from the G418^r cell lines sharing the characteristics of each particular class. For comparison, the characteristics of linear pRH4-14 and pRH5-8 plasmids are shown.

TABLE 3. Classes of plasmids rescued from cell lines coinjected with pRH4-14/TK and pRH5-8/TK as linear molecules[a]

Cell line	Kn^r	Polymorphisms				Size	No. rescued
		*Nru*I	*Dde*I	*Fnu*4H1	*Mbo*I		
LM37							
1	+++	X	X	X	0	L	2
2	−	0	0	0	0	J	2
3	−	0	0	0	0	L	1
4	±	X	X	X	X	J′	5
5	±	X	X	X	X	L	6
LM47	+++	X	X	X	0	L	5
2	±	X	X	X	X	J	14
3	±	X	X	X	X	L	5
LM54							
1	+++	0	X	X	0	L	2
2	−	0	0	0	0	J	1
3	−	0	0	0	0	L	4
4	±	X	X	X	X	L	11

[a] Mouse LM*tk*$^-$ cells were injected with five copies of pRH4-14/TK and pRH5-8/TK linearized at their unique *Hin*dIII sites and selected for G418^r as described in footnote *a*, Table 1. Plasmid sequences were rescued and analyzed as described in footnote *a*, Table 2. 0, The polymorphism comes from pRH4-14; X, the site comes from pRH5-8. See footnotes *b* to *e*, Table 2, for further details.

The 4-14 and 5-8 *Nru*I and *Dde*I polymorphisms present in any given rescued plasmid were identified by agarose gel electrophoretic analysis of the respective plasmid restriction enzyme digests (see Fig. 2 and 5). The identity of the *Fnu*4H1 polymorphic fragment present in each rescued plasmid was determined by a modification of the electrotransferring and hybridization procedure described by Church and Gilbert (3) (Fig. 3; Materials and Methods). The *Mbo*I polymorphism present at the 5-8 amber mutation was determined by end-labeling an *Mbo*I digest of each rescued plasmid, using [α-^{32}P]-dATP and the Klenow fragment of DNA polymerase. The ^{32}P-labeled fragments were resolved on polyacrylamide gels (Fig. 4; Materials and Methods). In Tables 2 and 3, a (0) or an (X) denotes that the polymorphism was derived from the 4-14 or the 5-8 parental plasmid, respectively.

If in the mammalian genome the 5′ neighbor of a rescued plasmid was another plasmid, then the rescued plasmid is of unit length (i.e., 5.8 kilobases [kb]). Such plasmids are designated with an L (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′, etc., denote plasmids of a given class containing different junction fragments, that is, plasmids that integrated into different sites in the host genome. Experiments involving injections with supercoiled molecules can yield rescued plasmids of variable length because a single supercoiled molecule integrates into the host genome randomly with respect to plasmid sequences (5). An example of such an event is shown in Table 2 and designated by (int). To ensure that a complete representation of the plasmids present in any given transformant was obtained, the rescue procedure was repeated until multiple examples of most plasmids were identified.

From each G418^r cell line (Tables 2 and 3) we rescued at least one class of recombinant plasmids with the genotype and phenotype expected of a plasmid responsible for conferring G418^r to the mammalian cell line. Bacteria containing such plasmids grew rapidly on kanamycin plates (+++). Analysis of the *Dde*I and *Mbo*I polymorphisms showed that these plasmids did not contain either the 4-14 or the 5-8 amber mutation. From each cell line we also rescued at least one class of plasmid molecules containing a genomic junction fragment. Recombinant plasmids that were not selected for in mammalian cells were also isolated from most of the G418^r cell lines. The number of classes of plasmid molecules rescued from each G418^r cell line was consistent with their Southern transfer pattern. As an example, in Fig. 7 we illustrate Southern transfer analysis of LM37, LM47, LM54, LM88, LM94, and LM98. Genomic DNA from each of the cell lines was digested with the restriction endonuclease *Hin*dIII, which cuts the pRH140/TK plasmids once. From the Southern transfer pattern it is clear that each cell line contains a head-to-tail concatemer of plasmid sequences accounting for the rescue of linear sized plasmids from these cell lines (Tables 2 and 3). It is also clear from the Southern transfer patterns that each of these cell lines contains a very small number of plasmid integration sites (i.e., one, two, or three). This is again consistent with the number of plasmids rescued from these cell lines containing different genomic junction fragments. The number of plasmid copies present in the head-to-tail concatemer is difficult to determine from Southern transfer analysis. However, when we compare the intensity of the hybridization signal of the band comigrating with unit-length plasmid DNA, 9.2 kb, with the hybridization signal of bands corresponding to junction fragments or

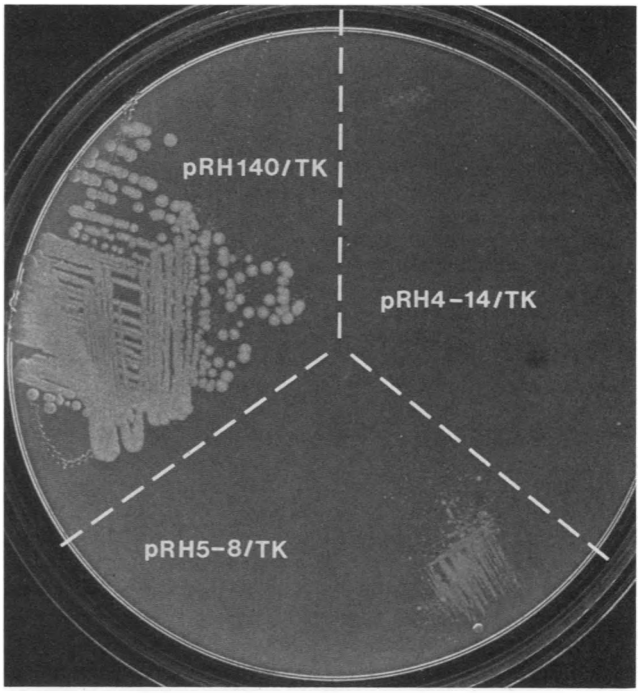

FIG. 6. Kanamycin resistance phenotype induced by plasmids carrying the different Neo^r genes. *E. coli* strain MH1 was transformed with pRH140/TK and the derivative plasmids pRH4-14/TK and pRH5-8/TK and selected for resistance to ampicillin. A single colony from each transformant was streaked on an M63 minimal plate containing 100 μg of kanamycin per ml as described in the text. Note that the bacteria containing pRH5-8/TK grow slowly at the origin of the streak and therefore can be distinguished from bacteria containing pRH4-14/TK. In Tables 2 and 3, +++, ±, and − denote the growth characteristics on kanamycin plates of bacteria equivalent to those carrying pRH140/TK, pRH5-8/TK, and pRH4-14/TK, respectively.

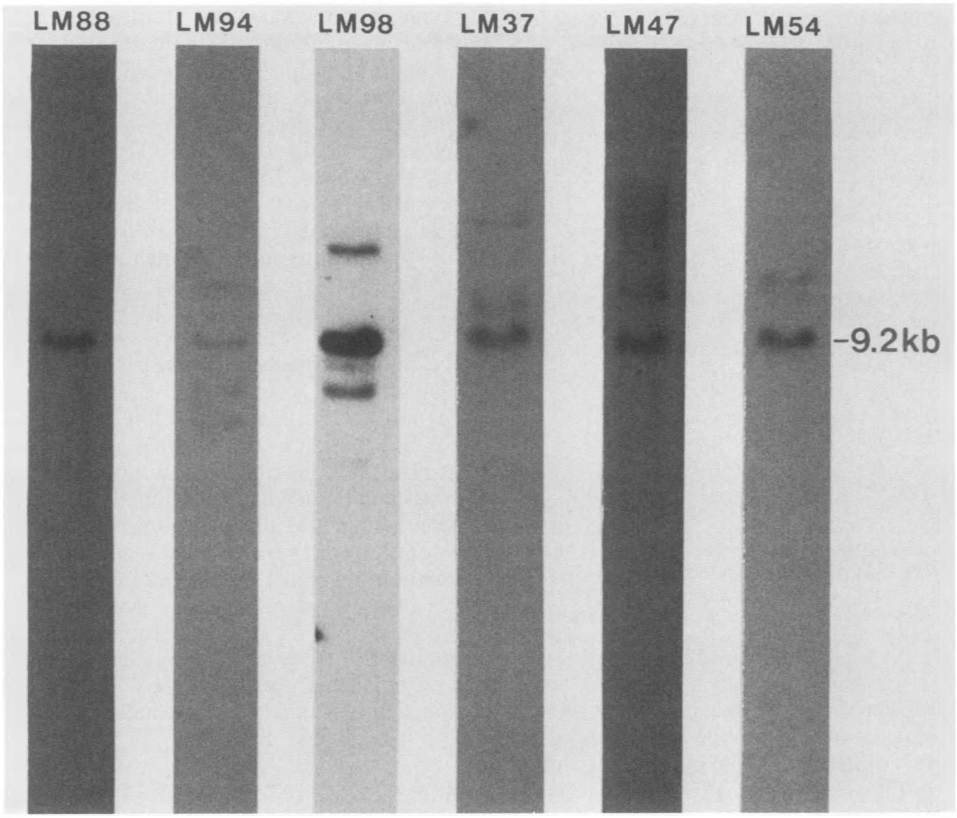

FIG. 7. Southern transfer analysis of G418^r cell lines obtained by coinjecting pH4-14/TK and pRH5-8/TK DNA. LM88, LM94, and LM98 were obtained by coinjecting supercoiled molecules, whereas LM37, LM47, and LM54 were obtained by coinjecting linear molecules (see Tables 2 and 3). Genomic DNAs from the above cell lines were digested with *Hin*dIII and processed for Southern transfer analysis as previously described (5). Nick-translated pRH140/TK DNA was used as a hybridization probe. Note that each transformant contains a hybridizing band that comigrates with unit-length plasmid DNA, 9.2 kb, indicating that each cell line contains a head-to-tail concatemer. Hybridizing bands of variable length, presumably representing junction fragments, are also apparent. This pattern is consistent with the plasmid sequences having integrated into the host genome at one or a very few sites.

to the hybridization signals of bands observed in cell lines known to contain only a single copy of the plasmid sequence, our best estimate is that these cell lines contain 3 to 15 copies of the plasmid sequence per cell.

Does a localized high concentration account for the high recombination frequency between coinjected plasmids? It could be argued that, using either calcium phosphate coprecipitation or microinjection to introduce plasmid sequences into mammalian cells, the high homologous recombination frequency observed between cotransferred plasmids results from a localized high concentration of the newly introduced molecules. Such a localized high concentration of plasmid sequences would facilitate homologous pairing of the exogenous DNA sequences in a sea of chromosomal DNA sequences. We have tested this hypothesis by introducing the two plasmids, pRH4-14/TK and pRH5-8/TK, using separate glass micropipettes directed to opposite ends of the nucleus. The diameter of the micropipette tips, 0.1 μm, is small relative to the diameter of the nucleus, approximately 5 μm. The efficiency of obtaining G418^r colonies was not markedly reduced when the two plasmids were introduced into the nucleus with separate micropipettes (Table 4) compared with coinjecting the plasmids with a single micropipette (Table 1). Even though we injected on average only five molecules of each plasmid at opposite ends of the nucleus, these molecules had no difficulty in finding each other and participating in homologous recombination in the presence of a vast excess of host DNA sequences.

TABLE 4. DNA-mediated transformation frequencies to G418^r obtained by injecting LM*tk*$^-$ cells with pRH4-14/TK and pRH5-8/TK plasmids, using separate micropipettes[a]

Time interval between successive injections	No. transformants/10^3 cells receiving injections	No. cells injected
<5 s	10.5	2×10^3
15 min	12	2×10^3
30 min	12	2×10^3
60 min	2	2×10^3
90 min	0	2×10^3

[a] LM*tk*$^-$ cells were injected with five copies of pRH4-14/TK and pRH5-8/TK linearized at their unique *Hin*dIII sites, using separate micropipettes. The time interval between successive injections was increased as indicated. After injections of both plasmids the cells were subjected to G418^r selection as described in footnote *a*, Table 1.

Kinetics of homologous recombination. As discussed above the frequency of homologous recombination between newly introduced plasmids is five to six orders of magnitude greater than between the same plasmid sequences when present as a contiguous array in the host genome. This enormous difference in frequencies may result from DNA being highly refractory to the host recombination machinery when incorporated into chromatin. If this were the case, we might anticipate that a time interval exists after which newly

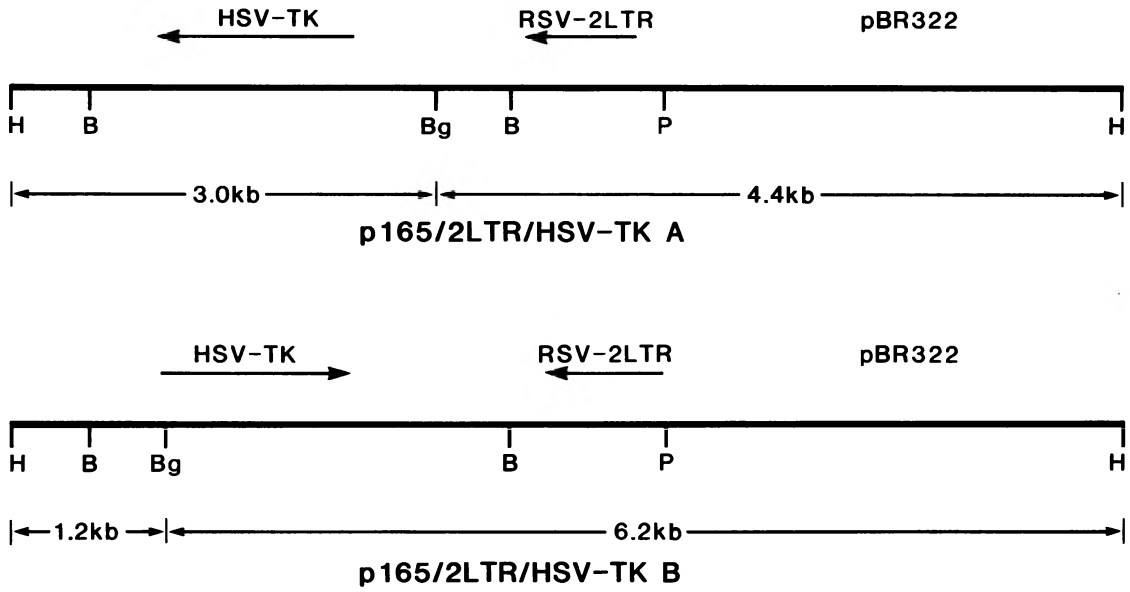

FIG. 8. Maps of the p165/2LTR/TK A and B plasmids. These two vectors have been previously described (5, 11) and contain the RSV-LTR region, pBR322 sequences, and the HSV TK gene. The two vectors are identical except with respect to the orientation of the HSV TK gene. Each vector is represented in linear form from the unique *Hin*dIII site. The restriction sites are as designated: H = *Hin*dIII; Bg = *Bgl*II; B = *Bam*HI; P = *Pvu*II.

introduced plasmid molecules would no longer participate in homologous recombination. This time interval would correspond to the time period required to assemble naked exogenous DNA into nucleosomes.

The existence of such a time interval, after which newly introduced plasmid molecules no longer participated in homologous recombination, was tested by introducing plasmids pRH4-14/TK and pRH5-8/TK into the nucleus, using separate micropipettes and increasing the time interval between successive injections. G418^r colonies are not obtained if the time interval between successive injections exceeds 60 min (Table 4). This result also suggests that most of the homologous recombination events that we observed with coinjected plasmids occurred within 1 h after their introduction into the nucleus. Alternative explanations include cell death after the second injection or that the cells' homologous recombination machinery is impaired if the time interval separating injections exceeds 1 h. We do not believe that the cells are dying as a result of the injections because we have followed cells receiving two injections under the microscope and found that these cells enter cell division with equal efficiency as cells receiving one injection or those receiving no injections. To rule out the second possibility, we have asked the same question, using a different system in which cell survival did not depend on the execution of a homologous recombination event.

Previously we demonstrated that when two marked plasmids, A and B, are coinjected, they are randomly interspersed into a head-to-tail concatemer as a result of homologous recombination (5). We used this system to again ask if a time interval exists after which the two vectors, A and B, would no longer recombine with each other. The A and B plasmids were introduced into the cell with separate micropipettes. The prediction is that if the time interval between injecting the A and B plasmids is small, then the A and B vectors should be randomly interspersed within a given head-to-tail concatemer. However, if the time interval is greater than 60 min, we should obtain head-to-tail concatemers which contain only A and only B plasmids due to homologous recombination among themselves.

The vectors used for these experiments were two plasmids containing the HSV TK gene which were identical except that the HSV TK genes were in opposite orientation with respect to the pBR322- and RSV-2LTR sequences (Fig. 8). The arrangement of the vectors within the head-to-tail concatemer could be identified by hydrolyzing genomic DNA with *Bgl*II, a restriction endonuclease that cleaves the plasmid once, asymmetrically within the inverted HSV TK gene sequence. If the A vectors were next to A vectors and the B vectors were next to B vectors, then the resulting fragment that hybridizes to plasmid DNA would be of unit length (7.4 kb). If the A and B vectors were interspersed, then two additional fragments that hybridize to vector DNA are predicted, one longer (9.2 kb) and one shorter (5.6 kb) than the unit length (Fig. 8 and 9).

In Fig. 9 we illustrate Southern transfer analysis of six cell lines, three obtained by coinjecting the A and B vectors (lanes a to c) and three obtained by injecting the A and B vectors successively separated by a 90 minute interval (lanes d to f). It is observed that in the first three cell lines the A and B vectors were interspersed within the head-to-tail concatemer, whereas in the last three cell lines A vectors are next to A and B vectors are next to B. Thus in the latter case the A and B vectors were capable of homologous recombination with themselves to generate head-to-tail concatemers but not with each other to generate a concatemer in which the A and B vectors were interspersed. The presence of both the A and B vectors in the latter cell lines was demonstrated by Southern transfer analysis of genomic DNA hydrolyzed with both *Hin*dIII and *Bgl*II which yielded the predicted 3.0-, 4.4-, 1.2-, and 6.2-kb bands diagnostic of the A and B vectors (Fig. 8).

DISCUSSION

Homologous recombination between coinjected plasmids. The recombination frequency between coinjected plasmid

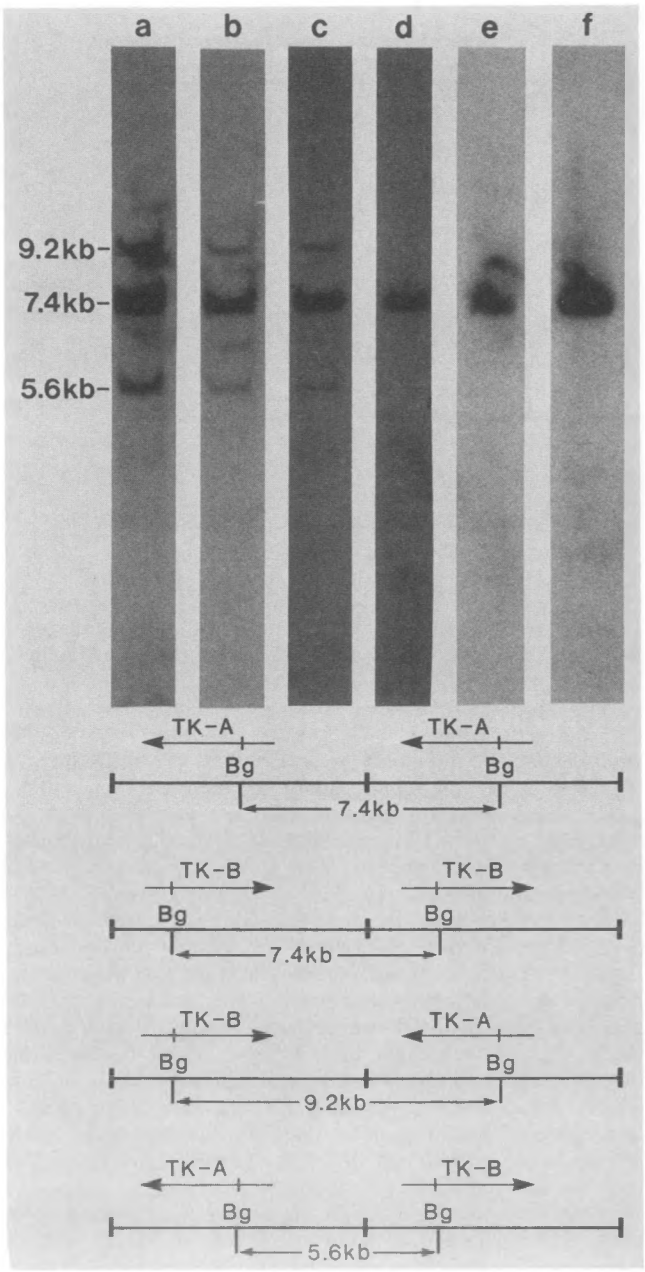

FIG. 9. Southern transfer analysis of cell lines obtained by injecting p165/2LTR/TK A and B plasmids either simultaneously (a to c) or separated by an interval of 90 min (d to f). Genomic DNA from the above cell lines was digested with *Bgl*II (Bg) and processed for Southern transfer analysis as previously described (5). When the A and B vectors were coinjected we obtained the Southern transfer pattern predicted for the interspersion of A and B vectors in a head-to-tail concatemer. When the injections of the A and B vectors were separated by 90 min, the concatemers contained only A or only B vectors.

molecules is very high. By coinjecting two plasmids containing two different mutations in the Neo^r gene, we obtained a transformation efficiency that was only 10-fold lower than that obtained by injecting the wild-type gene (Table 1). In both experiments we injected approximately five molecules per cell. The distance between the 4-14 and 5-8 amber mutations represents only 5% of the total plasmid length. If the homologous recombination frequency between coinjected plasmids is equal along the length of the plasmid, the above results imply that each injected plasmid DNA molecule participates in at least one recombination event (i.e., the recombination frequency between coinjected plasmid DNAs approaches unity).

The high homologous recombination frequency between coinjected plasmid molecules cannot be attributed to a localized high concentration of plasmid sequences since we can obtain comparable recombination frequencies if the two plasmids are injected separately into opposite ends of the nucleus.This result also implies that plasmid sequences have no difficulty in seeking themselves out and participating in homologous recombination in the presence of a vast excess of host DNA sequences.

We have analyzed recombination between coinjected plasmids by selecting for the reconstruction of a functional Neo^r gene from two plasmids that bear different amber mutations in the Neo^r gene. In addition, these plasmids contain restriction-length polymorphisms within and near the Neo^r gene. These polymorphisms do not confer a selectable phenotype but were used as markers to identify and categorize selected and nonselected recombinant molecules.

To associate a specific set of polymorphic markers with a specific recombinant molecule, the plasmids in the G418^r transformants were rescued from the host genome. By selecting the resulting bacterial colonies for ampicillin resistance, rather than for kanamycin resistance, we rescued plasmid molecules from the mammalian genome irrespective of whether it had participated in a recombination event or, if a recombinant, whether or not it was the recombinant which conferred G418^r to the mammalian cells.

From each transformant (Tables 2 and 3) we isolated three to five classes of molecules. In each case one class of rescued plasmids included the recombinant molecule which conferred G418^r to the mammalian cell lines. In addition, unselected recombinants and parental molecules were isolated. As expected, at least one class of rescued plasmids contained a chromosomal junction fragment. It is safe to assume that we isolated from each transformant most, if not all, plasmids containing a bacterial origin of DNA replication and an Amp^r gene. Thus, multiple examples of plasmids in each class were frequently rescued. Further, these transformants were obtained by injecting low numbers of plasmids per cell. Southern transfer analyses of these transformants indicated that they contained more than 3 and less than 15 plasmid copies. Each transformant contained a head-to-tail concatemer. The number of classes of plasmids rescued from these transformants is consistent with the number and organization of these plasmids in the genome as deduced from Southern transfer analysis.

On analyzing the data in Tables 2 and 3, the one striking feature is the apparent lack of reciprocal recombinants. Only in one cell line (LM95) are there recombinant plasmids which could have arisen from reciprocal recombination (class 3 and 4). Both classes arose from an unselected recombinational event(s). We isolated 28 plasmids of the selected genotype (i.e., confer G418^r in mammalian cells), yet we did not rescue a single plasmid containing the reciprocal double-mutant genotype. This apparent lack of reciprocal recombination products indicates to us that exchange of information between the coinjected plasmids proceeded primarily by nonreciprocal mechanisms (i.e., gene conversion).

We previously demonstrated a proportionality between the number of plasmid DNA molecules injected into the cell

and the number of plasmid DNA molecules present, integrated, in the resulting transformants (5). With supercoiled molecules the proportionality constant is approximately 0.5, whereas with linear molecules it is closer to 1. Thus, with supercoiled plasmids it could be argued that our lack of detecting reciprocal recombinants resulted from the unselected reciprocal product not integrating into the host genome and therefore escaping detection. However, after a reciprocal homologous recombination event between two supercoiled molecules, the products of recombination should be contiguous. It is difficult to envision a mechanism of reciprocal homologous recombination whereby if we detect one of the products of recombination, the selected product, we should not also detect the reciprocal product with nearly equal frequency.

The above results lead us to an apparent paradox. We just argued that our inability to detect reciprocal recombinant products indicated that exchanges of information between coinjected plasmid molecules proceeded primarily by nonreciprocal mechanisms. On the other hand, we previously showed that when a cell in injected with multiple copies of either supercoiled or linear plasmid molecules, these molecules are integrated into one or a few sites as head-to-tail concatemers. Most important, the head-to-tail concatemers were shown to be generated by homologous recombination and presumably, since the product is a head-to-tail concatemer, reciprocal homologous recombination. This apparent paradox is resolved if we postulate that recombination between two homologous plasmids proceeds by reciprocal mechanisms, but that between closely linked markers, <1 kb, nonreciprocal events occur with greater frequency than the reciprocal events.

Similar classes of recombinant molecules were isolated from transformants obtained by coinjecting supercoiled and linear molecules, indicating that by this criterion recombination between supercoiled and between linear molecules proceeds by a similar mechanism.

If the time interval between successive injections of two plasmids into the cell nucleus exceeds 1 h, they are refractory to recombination. This implies that the homologous recombination events between coinjected plasmid molecules occur within 1 h. This result also raises the question as to why the newly introduced DNA sequences become refractory to recombination after a 1-h period. An attractive hypothesis is that during this period the exogenous DNA is assembled into nucleosomes. Once in a chromatin structure the newly introduced DNA could be less susceptible to the cellular homologous recombination machinery. A similar hypothesis could explain the enormous difference in the recombination frequencies observed between newly introduced plasmid DNA sequences and the same sequences after integration into the host genome.

ACKNOWLEDGMENTS

We thank Laurie Fraser and Melanie Culver for their excellent technical assistance with experiments involving mammalian tissue culture.

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