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Rapid Assay for Extrachromosomal Homologous Recombination in Monkey Cells

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Most of the recombination assays based on the regeneration of selectable marker genes after transient infection or stable integration of DNA into mammalian cells are time consuming. We have used plasmids containing two truncated but overlapping segments of the neomycin resistance gene to rapidly quantitate and characterize the time course of extrachromosomal homologous recombination of DNA transfected into monkey COS cells. By transiently infecting cells with these recombination substrates, extracting Hirt DNA after 1 to 4 days, and transforming recombination-deficient *Escherichia coli*, we have shown that recombination between direct repeats occurs at frequencies of 1 to 4%. We have also used Southern blot analysis to directly characterize the recombination of this DNA in COS cells and to demonstrate that double-strand breaks in the region of homology increase recombination frequencies 10- to 50-fold.

Mitotically dividing mammalian cells exhibit a wide variety of DNA rearrangements (1–3, 11, 12, 14, 15, 21, 22) that can alter the spatial configuration or expression of genes. However, because of the multiplicity of recombination events and the absence of an easily manipulable genetic system, very little is known about the recombinational machinery that mediates these events. Early attempts to study homologous recombination at the molecular level involved the use of simian virus 40 (SV40) (6, 28), adenovirus (8, 30), and herpes simplex virus genomes (4). Subsequently, we and others (27, 29) demonstrated and quantitated homologous recombination after the transfer of DNA into mammalian cells by using substrates that yielded wild-type viral genomes only after recombination had occurred. Several laboratories, including ours, have also used deletions, insertions, and truncated fragments of selectable marker genes (such as the *CAD* gene [5], herpes thymidine kinase gene [7, 16, 17, 23, 24], and the neomycin resistance (*neo*) gene [13]) to study the mechanism of recombination in mammalian cells. These studies, based on the reconstruction of the selectable marker gene, have demonstrated homologous recombination after the transient infection of DNA into cells and after the stable integration of substrates into the host genome. One disadvantage of these studies, however, is the length of time (often months) needed to select, isolate, propagate, and analyze recombinant clones.

Using pBR322-SV40 shuttle vectors and the *neo* gene, we have devised a system to rapidly analyze extrachromosomal homologous recombination. With this system, we can now measure recombination rates and analyze recombination products in just a few days. The shuttle vectors, capable of replicating in monkey COS cells (9) and in bacteria, contain two truncated, nonfunctional but overlapping segments of the *neo* gene. Homologous recombination between the overlapping *neo* fragments generates a functional gene whose product is capable of conferring resistance to neomycin in bacteria and to the drug G418 in mammalian cells. By transfecting COS cells with these plasmids, extracting plasmid DNA after 1 or more days, and transforming recombination-deficient *Escherichia coli*, we have characterized the time course of extrachromosomal recombination between inverted and direct repeats in COS cells. We have also used Southern blot analysis (25) to directly detect recombination without reintroducing the plasmids into bacteria. Our results indicate that COS cells catalyze extrachromosomal recombination between direct repeats at frequencies of a few percent and these frequencies can be substantially increased by linearization of the recombination substrates within the region of homology. These vectors should therefore permit us to study the properties of extrachromosomal recombination and may allow the extension of these assays, based on transient infections, to other cell types.

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MATERIALS AND METHODS

Structures of the recombination substrates. The recombination substrates used in this study contain two truncated, nontandem but overlapping *neo* gene fragments, designated *neo-1* and *neo-2*. The substrates, IR1 and DR1, were constructed from pSV2neo (26) and ptkgpt-IVS II (Fig. 1). The plasmid pSV2neo contains the neomycin resistance gene from Tn*5* linked to transcription signals (promoter, small t intron, and polyadenylation signal) from the SV40 early region, whereas ptkgpt-IVS II has the bacterial xanthine-guanine phosphoribosyl transferase (*gpt*) gene as part of a transcription unit that contains a promoter from the herpes virus thymidine kinase (*tk*) gene, an intron from the rabbit β-globin gene (IVS II), and a polyadenylation signal from the SV40 late region.

The plasmid pSV2neo was digested with restriction enzymes *Nae*I and *Eco*RI, and the 3.63-kilobase (kb) DNA fragment (containing the ampicillin resistance gene from pBR322, the SV40 promoter and origin of DNA replication, and the 5′ portion of the *neo* gene up to the first *Nae*I site within the *neo* coding sequence [*neo-1*]) was purified (Fig. 1). *neo-1* also contains the promoter for bacterial transcription. The other plasmid, ptkgpt-IVS II, was digested partially with *Eco*RI, and the linear DNA was purified and digested to completion with *Pvu*II. The 2.89-kb *Pvu*II-*Eco*RI DNA fragment containing the complete *gpt* transcription unit was ligated with the *Nae*I-*Eco*RI fragment from pSV2neo, and the ligation mixture was used to transform *E. coli* strain HB101 (*recA*) to ampicillin resistance to obtain the plasmid pneo1-tkgpt-IVS II (Fig. 1).

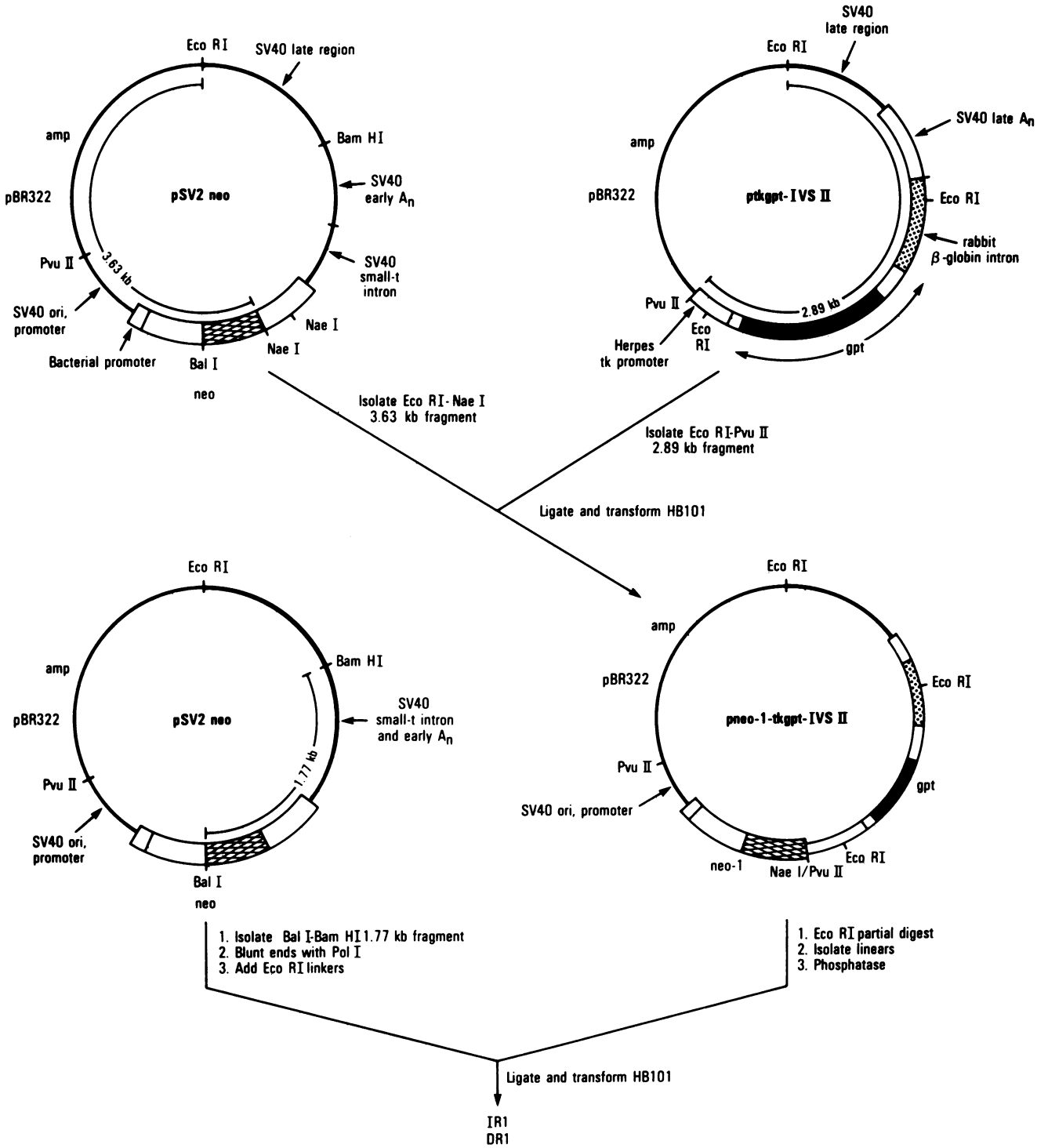

FIG. 1. Construction of the recombination substrates. The 3.63-kb DNA fragment containing the ampicillin resistance gene from pBR322, the SV40 promoter and origin of replication, and *neo-1* was isolated from pSV2neo and ligated to the 2.89-kb DNA fragment containing the *gpt* transcription unit from ptkgpt-IVS II to form pneo1-tkgpt-IVS II. The 1.77-kb DNA segment containing *neo-2*, the SV40 small t intron, and polyadenylation signal (A_n) was isolated from pSV2neo and inserted in either orientation into the *Eco*RI site downstream of the *gpt* transcription unit to create IR1 and DR1.

The second overlapping *neo* gene fragment, *neo-2* (from *Bal*I to *Sal*I in the *neo* gene), along with the small t intron and polyadenylation signal from pSV2neo, was then inserted in either orientation into the *Eco*RI site downstream of the *gpt* transcription unit (Fig. 1). Briefly, the ends of the 1.77-kb *Bal*I-*Bam*HI DNA segment from pSV2neo were made blunt with *E. coli* DNA polymerase I, ligated to synthetic *Eco*RI linkers, and digested with *Eco*RI. The plasmid pneo1-tkgpt-IVS II was digested partially with *Eco*RI, and the linear DNA was isolated and treated with

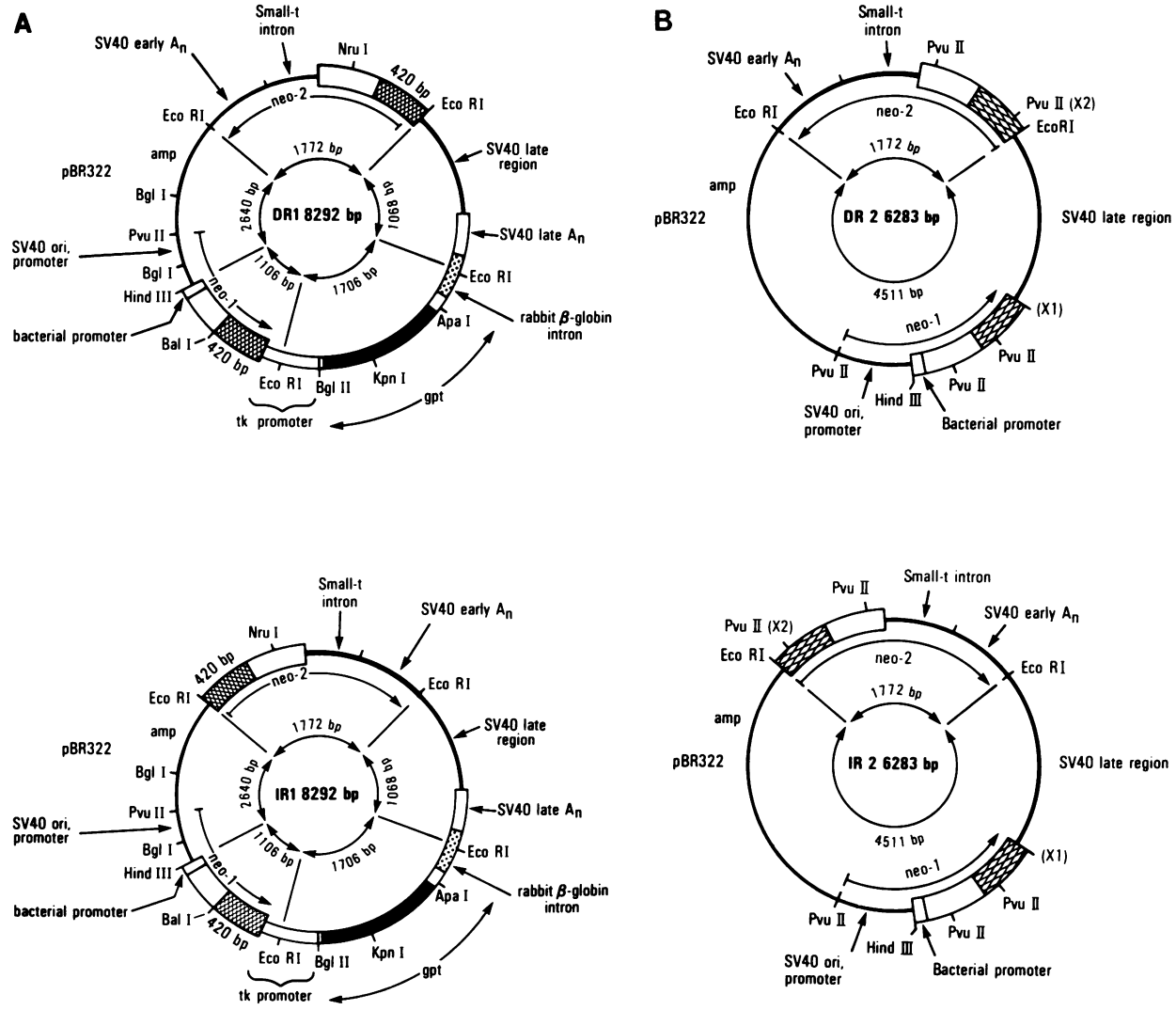

FIG. 2. Structure of the recombination substrates. (A) IR1 and DR1 each contain two truncated, nontandem but overlapping segments of the *neo* gene (*neo-1* and *neo-2*). The homologous segments (hatched) are 420 bp long and are present in either an inverted (IR) or direct (DR) configuration. The two *neo* segments are separated by a *gpt* transcription unit containing the herpes *tk* promoter, the rabbit β-globin intron, and the polyadenylation site from the SV40 late region. The plasmids also contain the ampicillin resistance gene and origin of DNA replication from pBR322, and SV40 early promoter and the viral origin of DNA replication. (B) IR2 and DR2 are similar to IR1 and DR1 but lack the *gpt* transcription unit. The sites of insertion of *Xho*I linkers are shown by (X1) and (X2).

calf alkaline phosphatase. This vector was then ligated to the 1.77-kb *Eco*RI fragment containing *neo-2*, and HB101 was transformed to ampicillin resistance. DNA from several colonies was screened by digestion with restriction endonucleases to identify the plasmids IR1 and DR1 (Fig. 2A). These plasmids each contain the two incomplete but overlapping *neo* gene segments separated by the *gpt* transcription unit.

Related, but smaller, plasmid substrates were then constructed to simplify the analysis of recombination products (Fig. 2B). The plasmids DR2 and IR2 are similar to DR1 and IR1 except for the deletion of the *gpt* transcription unit. Plasmids DR2X1 and IR2X1 are identical to DR2 and IR2 but contain a unique *Xho*I site at the end of the *neo-1* segment. Plasmids DR1X and DR2X2 each contain a unique *Xho*I site inserted at nucleotide 1783 in *neo-2* (Fig. 2B).

Determination of recombination frequency in bacteria. The frequency of recombination events between the overlapping fragments of the neomycin resistance gene to produce an intact gene was determined for both IR1 and DR1 plasmids by the Luria-Delbruck fluctuation test (18). Initially, recombination-deficient *E. coli* HB101 (*recA*) was transformed to ampicillin resistance with either the IR1 or DR1 plasmid. For each plasmid, an Amp^r Neo^s colony was used to inoculate a 5-ml liquid culture which was then grown for 12 to 16 h at 37°C. From each 5-ml culture, 12 to 24 1-ml cultures were inoculated with ca. 100 cells and grown for 6 to 7 h to a density of ca. 10^6 cells per ml. A small sample of each culture was then plated on ampicillin, and the remainder was plated on neomycin. Amp^r and Neo^r colonies were counted after 16 to 20 h at 37°C. Recombination frequencies were calculated as described by Luria and Delbruck (18). Recombination frequencies were determined for *E. coli* C600 (rec^+) in the same manner. Fluctuation tests were performed twice for each plasmid.

As a control, recombination rates during bacterial transformation were also determined for all substrates without passaging these DNAs through COS cells. HB101 was

TABLE 1. Recombination in *E. coli*

Bacterial strain	Plasmid	Recombination frequency/cell generation	Mode of recombination
HB101	IR1	1.8×10^{-7}	Intermolecular
HB101	DR1	3.5×10^{-7}	Intermolecular and intramolecular
C600	IR1	6.3×10^{-6}	Intermolecular
C600	DR1	1.0×10^{-5}	Intermolecular and intramolecular

transformed with each of the plasmids by standard procedures and grown at 37°C for 1 h; samples were then plated on both ampicillin and neomycin. The ratio of Neo^r to Amp^r colonies was taken as the background recombination frequency during transformation.

DNA transfections into and DNA recovery from COS cells. Monkey COS cells (9), which support the replication of plasmids containing the SV40 origin of replication, were grown in Dulbecco modified Eagle medium containing 10% newborn calf serum and antibiotics. When plates were 25 to 50% confluent, 10 μg of plasmid DNA was added as a calcium phosphate precipitate, and the cells were shocked for 2 to 3 min with 15% glycerol after 4 h as outlined by Parker and Stark (20). Low-molecular-weight DNA was extracted from the infected COS cells after 1 to 4 days by the method of Hirt (10). To rapidly determine recombination frequencies, this low-molecular-weight DNA was used to transform HB101 by standard procedures. After 1 h at 37°C, bacterial cells were plated on ampicillin (50 μg/ml) or neomycin (30 μg/ml), and colonies were counted after 16 h. The ratios of Neo^r to Amp^r colonies were used to calculate recombination frequencies.

Southern blots and DNA probes. Low-molecular-weight DNAs extracted from COS cells were digested with appropriate restriction enzymes for 12 to 16 h, electrophoresed on 0.8% agarose gels, and transferred to nitrocellulose paper (25). The filters were hybridized, washed, and exposed to X-ray film as previously described (25). All filters were hybridized with a DNA probe specific for the neo gene which was made by nick translation of the 1,339-base-pair (bp) *Hin*dIII-*Nru*I DNA fragment from pSV2neo (26).

RESULTS

Expected recombination products. In the recombination substrates described above, the homology between the two overlapping *neo* segments is 420 bp (from *Bal*I to *Nae*I). Intra- or intermolecular reciprocal recombination between *neo-1* and *neo-2* generates a functional *neo* gene capable of conferring resistance to either G418 in mammalian cells or to neomycin in bacteria—the latter being possible because the *neo-1* segment contains the bacterial promoter for the *neo* gene. For both the IR and DR plasmids, intermolecular reciprocal recombination yields a dimer molecule containing the complete *neo* gene. Intramolecular recombination in the IR clones inverts the part of the plasmid between the homologous sequences (e.g., the *gpt* transcription unit in IR1) relative to the rest of the plasmid. In the DR plasmids, intramolecular recombination deletes the part of the plasmid between the homologous sequences (e.g., the *gpt* transcription unit in DR1), resulting in a Neo^r plasmid smaller than the starting plasmid. All of the reciprocal recombination products, however, from all substrates should contain the complete *neo* gene on a diagnostic 4,977-bp *Eco*RI fragment.

Besides reciprocal recombination, intermolecular gene conversion between either the *neo-1* or *neo-2* segment on one molecule and appropriate homologous segments of another molecule can also result in a Neo^r product identical to the intramolecular reciprocal recombination products described above.

Recombination frequencies in bacteria. Because we intended to use bacterial transformations to quantitate recombination in COS cells, we first characterized recombination for the IR and DR plasmids in bacteria. To measure the frequencies of recombination in *E. coli*, strains HB101 (*recA*) and C600 (*rec*$^+$) were transformed to ampicillin resistance with either IR1 or DR1. For each plasmid and each bacterial strain, Amp^r Neo^s colonies were chosen for further study. Luria-Delbruck fluctuation tests (18) were then performed to determine recombination frequencies. Recombination frequencies per cell generation for IR1 and DR1 were quite low and ca. 30 times higher in C600 than in HB101 (Table 1). Frequencies for DR1 were generally about twice the frequency for IR1.

Recombination rates during transformation were also determined for the seven substrates. These rates (Table 2) were used as the background rates in the COS cell recombination studies described below.

Products of recombination in bacteria. The products of recombination were analyzed by isolating plasmid DNA from neomycin-resistant colonies that arose from independent cultures. Because the cultures were inoculated with a small enough number of cells so that each culture initially contained no Neo^r cells (i.e., no recombinants), Neo^r colo-

TABLE 2. Recombination frequencies in COS cells[a]

Plasmid	Background rate in HB101	Days post-infection	Recombination frequency (%)
IR1	0.01%	1	0.09
		2	0.5
		3	0.6
IR2	0.01%	1	0.1
		2	0.1
		3	0.1
DR1	0.02%	1	1.5
		2	2.6
		3	3.8
DR1X	0.01%	1	3.5
		2	4.0
		3	1.3
DR2	0.02%	1	0.1
		2	0.6
		3	0.6
DR2X1	0.02%	1	0.3
		2	0.3
		3	0.9
DR2X2	0.02%	1	0.3
		2	0.8
		3	2.0

[a] Recombination frequency was measured by the ratio of Neo^r to Amp^r colonies (expressed as percent) obtained after transformation of HB101 with Hirt DNAs from COS cells infected with various supercoiled plasmids. Background frequencies in HB101 were obtained by direct transformation of the DNA substrates into bacteria, without passage through COS cells.

nies from independent cultures represent independent recombination events. Restriction enzyme analysis of the plasmid DNAs in the recombinant colonies often allowed easy determination of the mode of recombination. For IR1, intramolecular recombination inverts part of the plasmid with respect to the other part, resulting in a plasmid of the same size, but with different restriction patterns from IR1. Intramolecular recombination in DR1, however, deletes part of the plasmid, yielding a plasmid of 4.98 kb, ca. 3.3 kb smaller than DR1. For both IR1 and DR1, intermolecular recombination produces a dimer, although the dimers are different for the two plasmids. Dimers could also arise from intramolecular recombination events involving dimeric substrates containing inverted repeats. Southern blot analysis (25), however, confirmed that our starting substrates contained only monomers, indicating that dimers would be formed only by intermolecular recombination.

Independent recombination products were analyzed for IR1 and DR1 in HB101 (Table 1). Every recombinant arising from IR1 in HB101 was a dimer produced by intermolecular recombination. No products of intramolecular recombination were detected in 50 Neo^r colonies analyzed. For DR1, however, both intra- and intermolecular recombination products were found. Approximately 50% of the Neo^r colonies arising from DR1 contained dimers or larger species formed by intermolecular recombination. Likewise, ca. 50% contained the 4.98-kb product of intramolecular recombination but still retained substantial amounts of the unrecombined plasmid. After these cultures grew in neomycin-containing medium for several days, most of the unrecombined DR1 was lost, leaving predominantly the 4.98-kb plasmid. The dimers formed from both IR1 and DR1 were stable for many generations in selective medium.

The IR1 intramolecular recombination product was never detected in HB101 or C600 (Table 1). Two explanations for this observation are possible. First, the product may be unable to replicate or confer neomycin resistance. Alternatively, intramolecular recombination may not be able to occur at any appreciable frequency between inverted repeats in *E. coli*. To distinguish between these possibilities, a plasmid that is identical to the product of intramolecular recombination between the inverted repeats of IR1 was constructed in vitro and used to transform HB101. All resulting colonies were both Amp^r and Neo^r and contained plasmids identical to the input plasmid. Therefore, the plasmid in question clearly can replicate and confer neomycin resistance, suggesting that intramolecular recombination does not occur between inverted repeats in *E. coli*.

Overall, three major products of recombination were seen in HB101 and C600: the dimers formed by intermolecular recombination for both IR1 and DR1 and the 4.98-kb plasmid formed by intramolecular recombination within DR1. These three plasmids were prepared in large quantity and analyzed in detail. Digestion with several restriction enzymes that cut within the neomycin resistance gene showed that all three plasmids contain neomycin genes that are identical to the wild-type gene in pSV2neo. It thus appears that the three plasmids studied are, as expected, produced by homologous recombination.

Recombination frequencies in COS cells. Extrachromosomal recombination frequencies for COS cells were determined by transfecting these cells with each of the IR and DR plasmids, allowing the plasmids to replicate for 1 to 4 days, and extracting the plasmid DNA by the method of Hirt (10). The extracted DNA was then used to transform *E. coli* HB101, and cell samples were plated on ampicillin (50 μg/ml) or neomycin (30 μg/ml). Since Neo^r colonies arise only if recombination has occurred between *neo-1* and *neo-2* to produce an intact gene, the ratio of Neo^r to Amp^r colonies reflects the frequency at which recombination occurred in the COS cells. Recombination frequencies were highest for DR1 (and DR1X), ranging from ca. 1 to 4% during the first 3 days after transfection (Table 2). Frequencies for the smaller plasmid DR2 (and its derivatives DR2X1 and DR2X2) were ca. 5 to 10 times lower, despite its similarity to DR1 in length (420 bp) and direction of homology. Recombination frequencies were lowest for IR1, in the range of 0.1 to 0.6%.

To insure that the Neo^r colonies did not arise from recombinant molecules that might already be present in our plasmid preparations, HB101 was transformed directly with each of the plasmids, and recombination frequencies were determined as described above. For DR plasmids, recombination frequencies were ca. 0.02% (Table 2), or 30- to 200-fold lower than after passage through COS cells. Also, Hirt extracts from mock-infected and uninfected COS cells gave rise to no Amp^r or Neo^r colonies, indicating that all bacterial colonies arose from plasmid DNA substrates that had been passaged through the COS cells.

Recombination products generated in COS cells were analyzed by isolating plasmid DNA from Neo^r bacterial colonies that arose from transformations with DNA from independent plates of COS cells. Although an extensive study was not conducted, our results indicate that of 54 recombination products, more than 80% of the recombination products from the DR plasmids are the 4,977-bp product of intramolecular recombination. For IR1, none of the 18 recombinants analyzed could be explained by simple intra- or intermolecular recombination. Because recombination frequencies were ca. 10-fold lower for IR1 than for DR1 (which undergoes primarily intramolecular recombination), we believe that the rare IR1 Neo^r recombinants may arise by multiple events involving both intermolecular recombination in the substrates and intramolecular recombination between direct repeats in the intermediates. Although the pathway of recombination for IR1 is still under investigation, all Neo^r recombinants, including those of complex structure, contain the intact *neo* gene.

Southern blot analysis of recombination. Although the bacterial transformations described above provided very rapid quantitation of recombination frequencies, we also wished to directly determine the fate of our recombination substrates in COS cells without reintroducing the substrates into bacteria. This would ensure that the events we had been measuring and analyzing were indeed occurring in the COS cells. Accordingly, we isolated transfected plasmid DNA from COS cells after 1 to 4 days, electrophoresed it on agarose gels, transferred it to nitrocellulose, and hybridized it with DNA probes specific for the *neo* gene. Because the IR plasmids undergo recombination at lower rates, this analysis was done only for DR plasmids. Figure 3A shows an autoradiogram of DR1 DNA extracted from COS cells after 1 to 4 days and run uncut. Although the majority of DNA appears to be supercoiled DR1, the putative supercoiled recombination product is seen as a faint band at 4,977 bp. To confirm that this was the recombinant plasmid, the DR1 DNA was digested with *Eco*RI (Fig. 3B). DR1 gives rise to intensely hybridizing bands at 1,772 and 3,746 bp, corresponding to *neo-2* and *neo-1*, and the recombined intact *neo* gene is clearly visible as the 4,977-bp *Eco*RI fragment. The intensity of the 4,977-bp fragment relative to the unrecombined fragments gives a rough estimate of the recombination frequency. The 4,977-bp band (Fig. 3C, lane c) is roughly

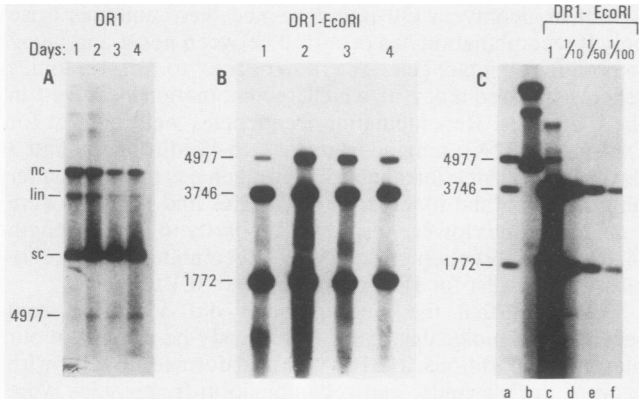

FIG. 3. Southern blot analysis of extrachromosomal recombination. DR1 was transfected into COS cells and extracted by the method of Hirt (10) after 1 to 4 days. All blots were hybridized with a *neo* probe. (A) DR1 DNA extracted from COS cells and run uncut. Blot shows supercoiled (sc), linear (lin), and nicked circular (nc) forms of DR1 as well as the 4,977-bp supercoiled product of recombination. (B) DR1 DNA extracted from COS cells and digested with *Eco*RI. The bands correspond to *neo-1* (3,746 bp), *neo-2* (1,772 bp), and the intact *neo* gene (4,977 bp). (C) Hirt DNA extracted from COS cells 1 day after infection with DR1 was digested with *Eco*RI and run in lane c. Lanes d to f have 1/10, 1/50, and 1/100 dilutions of this sample. Lanes a and b show DNA markers and undigested DR1 DNA extracted from COS cells 1 day after infection.

equal in intensity to 1/100 of the 3,746-bp band, indicating a recombination frequency of ca. 1%, consistent with the transformation data (Table 2). Control experiments with Southern blots of the recombination substrates prepared in bacteria did not reveal the 4,977-bp recombination product. Similarly, blots of DR1 DNA extracted from COS cells immediately after transfection did not show the 4,977-bp recombination product but showed primarily the supercoiled and nicked circular forms of DR1 (data not shown).

Double-strand breaks in the region of homology stimulate recombination. Several reports have indicated that DNA ends may be recombinogenic both in yeast (19) and in mammalian cells (13, 16). To study the effects of free DNA ends on extrachromosomal recombination in COS cells, we constructed derivatives of DR1 and DR2 that contained unique *Xho*I sites at which the plasmids could be linearized. DR1X was created by inserting an *Xho*I linker in the *neo-2* segment of DR1 at nucleotide 1783, originally a *Pvu*II site. *Xho*I sites were introduced into DR2 either at the end of *neo-1* (DR2X1) or in *neo-2* at nucleotide 1783 (DR2X2) (Fig. 2B).

We then transfected COS cells with DR1X, DR2X1, or DR2X2, either as supercoiled DNA or after linearization with *Xho*I. For the supercoiled DNAs, bacterial transformations were used to calculate recombination frequencies, which ranged from 0.3 to 4.0% (Table 2). The linearized plasmids, however, gave very high background frequencies, in the range of 10 to 50% when the linearized plasmids were used to directly transform HB101 without prior passage through COS cells. Furthermore, transformation results with linearized substrates would be complicated by the fact that some of these molecules might become circularized or supercoiled in the COS cells, as has been suggested by Wake and Wilson (29). Since supercoiled DNA transforms *E. coli* several orders of magnitude more efficiently than does linear DNA, the ratio of Neo^r to Amp^r colonies might only reflect the proportion of recombinant molecules in the population of supercoiled molecules rather than in the total population of transfected DNA.

To overcome these problems, we decided to directly visualize the fate of the linearized recombination substrates in COS cells by Southern blot analysis. Figure 4A shows the plasmid DR1X extracted 1 to 3 days after transfection into COS cells either as supercoiled or linear DNA. The faintly hybridizing bands running at 4,977 bp (the size of the recombination product) in lanes b to d indicate a level of recombination of ca. 1% for supercoiled DR1X. Linearized DR1X, however, undergoes a much higher frequency of recombination, shown by the more intense bands at 4,977 bp in lanes e to g. After 2 and 3 days, the recombinant species appears to be at least as abundant as the starting linear DNA. A small amount of DR1X supercoiled DNA is also seen, suggesting that some end-to-end ligation of the linear DNA has occurred. To confirm the identity of the 4,977-bp recombination product, the DNA corresponding to this region of the gel was isolated after agarose gel electrophoresis of linearized DR1X extracted from COS cells after 2 days, and used to transform HB101. All resulting colonies were *neo* resistant and contained the 4,977-bp product of intramolecular recombination.

The extracted DR1X supercoiled and linear DNAs were also digested with *Eco*RI and hybridized as above. As expected, DR1X supercoiled DNA gives rise to very intense bands corresponding to *neo-1* (3,746 bp) and *neo-2* (1,772 bp) as well as the 4,977-bp *Eco*RI fragment containing the intact *neo* gene (Fig. 4B, lanes a to c). Linearized DR1X yields much higher levels of the 4,977-bp band relative to the unrecombined fragments (Fig. 4B, lanes d to f) or the 4,977-bp band in lanes a to c of Fig. 4B.

A similar analysis was carried out for DR2X1 and DR2X2. *Xho*I linearizes DR2X1 at the end of *neo-1* and DR2X2 within the region of homology in *neo-2* (Fig. 2B). Nevertheless, linearized DR2X2 (and DR1X) still retains 396 bp of homology, compared with 420 bp of homology in linearized DR2X1. Figure 5A shows both DR2X1 and DR2X2 after transfection into COS cells as supercoiled or linear DNA. For both supercoiled plasmids, faint recombination products are seen at 4,977 bp after 2 and 3 days (lanes b, c, h, and i). The bacterial transformation assay, which is 10- to 100-fold more sensitive than Southern blot analysis, indicated that recombination also occurred after 1 day, even though the

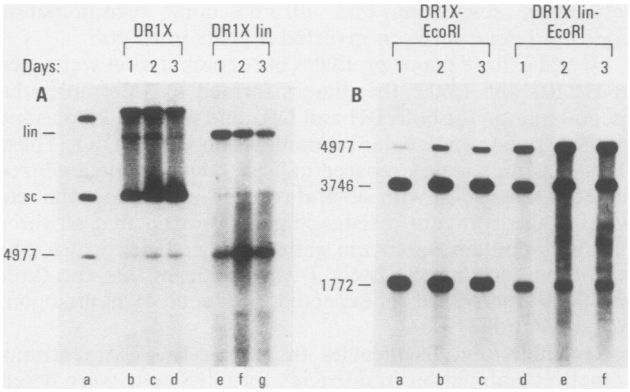

FIG. 4. Southern blots of DR1X DNA after passage through COS cells. (A) DR1X DNA extracted 1 to 3 days after transfection as supercoiled DNA (lanes b to d) or linear DNA (lanes e to g) and run uncut. Lane a has markers. (B) DNAs as in (A), but digested with *Eco*RI. Both blots were hybridized with a *neo* probe.

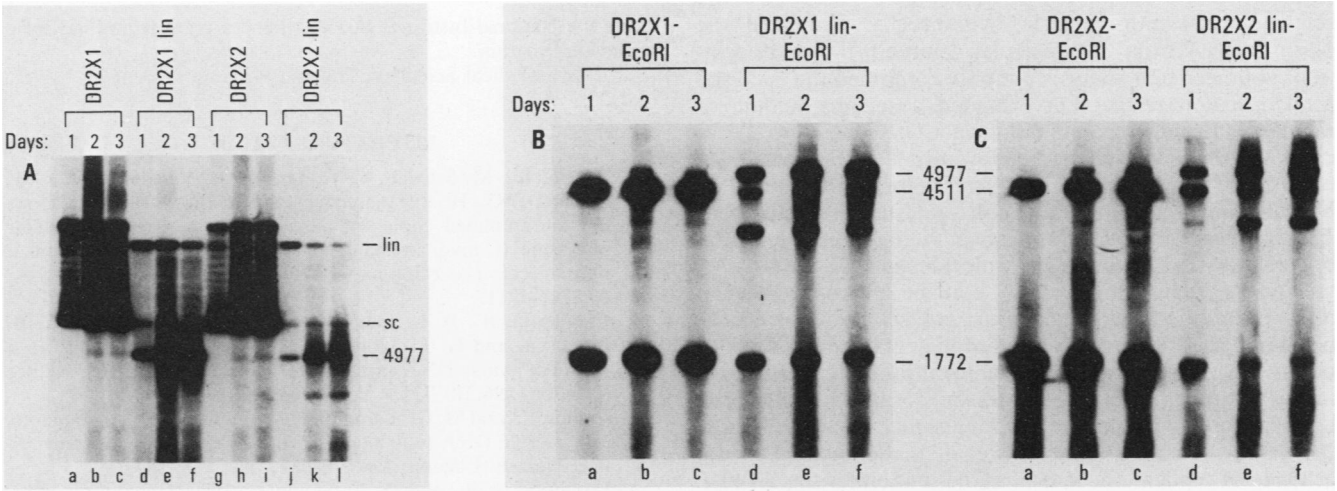

FIG. 5. Southern blots of DR2X1 and DR2X2 after passage through COS cells. (A) DNAs extracted 1 to 3 days after transfection with supercoiled or linear DNA. The bands are identified on the right. (B) DR2X1 DNA extracted 1 to 3 days after transfection with supercoiled DNA (lanes a to c) or linear DNA (lanes d to f) and digested with *Eco*RI. The bands correspond to *neo-1* (4,511 bp), *neo-2* (1,772 bp), and the intact *neo* gene (4,977 bp). The bands at 3,625 bp (lanes d to f) correspond to *neo-1* after digestion with *Xho*I and *Eco*RI. (C) DR2X2 DNA extracted 1 to 3 days after transfection with supercoiled DNA (lanes a to c) or linear DNA (lanes d to f) and digested with *Eco*RI.

4,977-bp band was not seen on the autoradiogram. Much more intense recombinant bands are seen for both linearized plasmids, even after 1 day (lanes d to f and j to l). In several cases (e.g., linear DR2X2 after 3 days [lane l]) the recombinant species is actually more abundant than the unrecombined linear DNA. Figure 5A shows a series, or ladder, of hybridizing species different from the input and recombinant molecules. These ladders were also generated by transfecting the DR substrates into cell lines that do not allow plasmid replication (data not shown), indicating that they are not replicative intermediates. We are currently investigating whether these molecules are topoisomers of the starting substrates.

The above results were confirmed by digesting the 12 DNAs in Fig. 5A with *Eco*RI (Fig. 5B and 5C). In the DR2 plasmids, *neo-2* is present on a 1,772-bp *Eco*RI fragment, and *neo-1* is on a 4,511-bp *Eco*RI fragment. The intact *neo* gene, however, again occurs on a 4,977-bp *Eco*RI fragment, clearly visible in Fig. 5B and C. For both linearized DR2 plasmids, the recombinant band is of equal or greater intensity than the *neo-1* and *neo-2* fragments and of much greater intensity than the recombination product generated by transfection of COS cells with supercoiled DNA.

To ensure that our results were not complicated by the presence of input plasmid DNA that had not been taken up by the COS cells, we treated the extracted DR1 and DR2 DNAs with the restriction enzyme *Dpn*I. Because *Dpn*I cleaves only methylated DNA, it will not cleave DNA that has replicated in COS cells but will cleave all contaminating input DNA. Results of both bacterial transformations and Southern transfers were unchanged after *Dpn*I treatment of Hirt DNAs (data not shown), indicating no contamination by input DNA.

DISCUSSION

We have used substrates containing two truncated but overlapping segments of the neomycin resistance gene (*neo-1* and *neo-2*) to quantitate and characterize extrachromosomal homologous recombination in monkey COS cells. Recombination between the *neo-1* and *neo-2* segments, present in an inverted (IR) or direct (DR) configuration, generates a functional *neo* gene capable of conferring resistance to neomycin in bacteria and to the drug G418 in mammalian cells. Although we and others (5, 7, 13, 16, 17, 23, 24) have previously used selectable markers to detect recombination in mammalian cells, those studies were somewhat limited by the length of time needed to conduct them and the difficulty in isolating and analyzing large numbers of recombinants. We have now used the IR and DR plasmids to rapidly detect homologous recombination by introducing the plasmids transiently into COS cells, extracting low-molecular-weight DNA after 1 to 4 days, and reintroducing the DNA into recombination-deficient *E. coli*.

Because we intended to use *E. coli* in our analysis of recombination in COS cells, we first characterized recombination for IR1 and DR1 in *E. coli* HB101 (*recA*). Our results show that recombination between *neo-1* and *neo-2* to generate an intact *neo* gene occurs at a low but detectable rate in HB101 (Table 1). Recombination frequencies were similar for IR1 and DR1, but the types of recombination products were different for the two plasmids. IR1 was seen to undergo only intermolecular recombination, whereas DR1 underwent inter- and intramolecular recombination at about equal rates.

In COS cells we measured extrachromosomal recombination frequencies for IR1 and DR1 as well as for the related, but smaller, plasmids IR2 and DR2. The IR plasmids undergo recombination at the lowest frequencies (Table 2), and DR1 gives the highest recombination frequency (1 to 4%). DR2, which differs from DR1 primarily in the distance between the repeats—886 bp in DR2 compared with 2,895 bp in DR1—recombines at a frequency ca. 10-fold lower than DR1. We are not sure, however, if the distance between the repeats alone accounts for the difference in recombination frequency between DR1 and DR2. In our quantitation of recombination frequencies for supercoiled plasmids, we have assumed that all molecules, input and recombinant, replicate with equal efficiencies in COS cells. The 4,977-bp recombinant, DR2, and DR1 transform *E. coli* with approximately equal efficiencies (data not shown).

To confirm that the recombination events we were detecting were actually occurring in the COS cells rather than after

reintroduction into bacteria, we directly visualized the plasmid DNA from COS cells by Southern blot hybridization. Southern blot analysis not only confirmed the bacterial transformation results but provided a second method of rapidly analyzing recombination in COS cells (Fig. 3). In cases in which recombination occurred at frequencies of 0.5% or higher, recombinant species were clearly visible on hybridizations of uncut and *Eco*RI-digested plasmid DNA. The Southern blot analysis was facilitated by the replication of substrate and recombinant molecules in COS cells which yielded larger quantities of DNA in the Hirt extracts than might otherwise be expected. Our analysis, however, would be complicated if the replication-proficient conditions (SV40 origin-containing plasmids in T-antigen-producing cells) also influenced recombination. Preliminary results with other cell lines, however, that do not allow plasmid replication (e.g., 293 cells) indicate a similar level of recombination in the absence of replication. It is possible that either the SV40 T antigen or the transformed phenotype of the COS cells could account for the unusually high recombination frequencies described in this study.

Recent reports indicate that double-strand breaks in the region of homology stimulate recombination in mammalian cells (13, 16). We thus compared recombination frequencies for supercoiled versus linear DNA by introducing unique *Xho*I sites into the DR plasmids either at the end of *neo-1* (DR2X1) or within the region of homology in *neo-2* (DR1X and DR2X2). We then transfected COS cells with these plasmids either as supercoiled DNA or after linearization by *Xho*I digestion. Because background recombination frequencies for linear DNAs in *recA* bacteria were very high, it was necessary to rely solely on Southern blot analysis. Although exact recombination frequencies could not be calculated, it was clear from a comparison of the abundance of the recombination product generated in these experiments (Fig. 4 and 5) that all three plasmids underwent 10- to 50-fold increases in recombination frequency after linearization. In several cases, the recombinant species were actually more abundant after 48 h than the unrecombined linear DNA. However, since the linear DNA does not replicate (unpublished data), its abundance would be expected to be low. Similar double-strand breaks in IR2X1 also stimulated recombination (data not shown).

We are now using the IR and DR plasmids, along with the rapid methods to detect recombination, to investigate factors other than double-strand breaks that may stimulate homologous recombination in mammalian cells. If the same enzymatic machinery is involved in both extrachromosomal and chromosomal recombination, then parameters that stimulate extrachromosomal recombination may also enhance chromosomal recombination or the targeting of genes into specific chromosomal locations. It is also important to understand why the extrachromosomal recombination frequency (10^{-2} to 10^{-3}) in COS cells is several orders of magnitude more predominant than the chromosomal recombination frequencies (10^{-6} to 10^{-8}) that we and others have measured in mouse cells. Finally, if this rapid recombination assay, based on transient infection of cells with appropriate recombination substrates, can be extended to other cells, it may provide a way of studying recombination in primary cells, including those derived from patients aberrant in recombination and repair functions. Experiments are now in progress to answer these questions.

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