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Recombination Events After Transient Infection and Stable Integration of DNA into Mouse Cells

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To investigate the recombinational machinery of mammalian cells, we have constructed plasmids that can be used as substrates for homologous recombination. These plasmids contain two truncated nontandem, but overlapping, segments of the neomycin resistance gene, separated by the transcription unit for the xanthine guanine phosphoribosyl transferase gene. Recombination between the two nonfunctional neomycin gene sequences generates an intact neomycin resistance gene that is functional in both bacteria and mammalian cells. Using these plasmid substrates, we have characterized the frequencies and products of recombination events that occur in mouse 3T6 cells soon after transfection and also after stable integration of these DNAs. Among the chromosomal recombination events, we have characterized apparent deletion events that can be accounted for by intrachromatid recombination or unequal sister chromatid exchanges. Other recombination events like chromosomal inversions and possible gene conversion events in an amplification unit are also described.

There is accumulating evidence suggesting that mammalian genomes undergo a variety of DNA rearrangements thought to be mediated by the recombinational machinery in these cells. The recombination activities of mitotically dividing mammalian cells span a spectrum of events like sister chromatid exchange (13), DNA sequence amplifications leading to the acquisition of resistance to certain drugs (24) or to overexpression of certain cellular oncogenes during tumorigenesis (1, 5, 12), the excision of small circular DNAs from chromosomes (4, 21), and the generation of deletions or inversions in the chromosome, such as those observed during the rearrangement of immunoglobulin genes (9, 14). If we wish to understand the above phenomena in any detail, it is essential to obtain precise information about the frequencies, mechanisms, and enzymology of these processes. Our hope is that such knowledge will also contribute significantly to improved procedures for targeting exogenous DNA to specific chromosomal sites during DNA-mediated gene transfer experiments in mammalian cells.

In some of our earlier studies (29) we described the construction of substrate molecules that were used to detect and quantitate the products of both homologous and nonhomologous recombination in monkey cells. These assays were based on the recombinational excision of wild-type or defective simian virus 40 (SV40) genomes from pBR322-SV40 hybrid plasmids. However, these substrates could only be used in monkey cells because the recombination products were scored with the SV40 plaque assay.

We have now used a similar general strategy, but with expression vectors carrying dominant selectable marker genes, to extend our studies on homologous recombination to any other cell type in which the dominant selectable markers can function. The vectors contain two truncated (and therefore nonfunctional) nontandem, but overlapping, segments of the neomycin resistance gene (*neo*), separated by a functional transcription unit containing a second dominant selectable marker, the xanthine-guanine phosphoribosyl transferase gene (*gpt*). In cultured mouse cells, these vector DNAs undergo both extrachromosomal and chromosomal homologous recombination between the overlapping segments of the *neo* gene to generate one or more functional copies of the *neo* gene. Measurement of the frequencies of these recombination events indicates clearly that extrachromosomal recombination occurs at a frequency that is substantially higher than the frequency of chromosomal recombination. We have characterized the chromosomal events to show that apparent deletions and inversions can be readily detected. We also present evidence supporting nonreciprocal gene conversion events similar to those that have been detected in *Saccharomyces cerevisiae* (10, 11) and in mammalian cells (16) and that are thought to be important in maintaining the sequence homogeneity between members of multigene families.

MATERIALS AND METHODS

Structure of the recombination substrates. The recombination substrates used in this study contain two truncated nontandem, but overlapping, segments of the *neo* gene (*neo-1* and *neo-2* in Fig. 1) separated by a functional transcription unit for the gpt gene. In these plasmids, whose construction will be described elsewhere (Rubnitz and Subramani, manuscript in preparation), the *neo-1* gene segment contains the SV40 *ori* promoter region linked to the DNA coding for the first 210 amino acids (from the *Hin*dIII-*Nae*I sites) of the 264-amino-acid *neo* gene (3), whereas the *neo-2* segment contains sequences coding for amino acids 71 through 264 (from *Bal*I-*Sal*I in the *neo* gene), thereby providing a 420-base-pair (bp) region of homology for recombination between the *neo-1* and *neo-2* segments. The *neo-2* segment is linked downstream from the *neo* coding region to an intron and polyadenylation signal derived from the SV40 early region. The *gpt* transcription unit has the *gpt*-coding region linked to the herpesvirus thymidine kinase promoter, the rabbit β-globin intron (IVS 2), and the polyadenylation signal from the SV40 late region. The recombination substrates also contain the ampicillin resistance gene and the plasmid origin of DNA replication from pBR322, so that the recombination substrates can be grown in RecA$^-$ *Escherichia coli*. The DR and IR plasmids have the homologous regions arranged as direct and inverted repeats, respectively (Fig. 1).

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DNA transfections of mouse 3T6 cells. Mouse 3T6 cells were grown in Dulbecco modified Eagle medium containing 5% newborn calf serum and antibiotics. About 5×10^5 cells were seeded onto 10-cm plates approximately 12 h before transfection. When the plates were 25 to 50% confluent, 10 μg of the IR or DR DNAs were placed on each plate of cells as calcium phosphate precipitates and shocked for 3 min with 15% glycerol after 4 h as outlined by Parker and Stark (20). The *gpt* or *neo* selections were applied typically after 72 h of nonselective growth. Cells from each plate were trypsinized and replated at a 1:20 ratio in selective medium. Colonies were counted 2 weeks later by staining with crystal violet. Conditions for *gpt* (18) and *neo* selections (28) were as described previously.

Genomic DNAs, Southern blots, and DNA probes. Genomic DNAs from various cell lines were isolated by using minor modifications of the procedures of Wigler et al. (32). Cell DNAs were digested with about 3 U of restriction enzyme per μg of DNA for 2 to 3 h. DNA samples (10 μg per slot) were electrophoresed on 0.8% agarose gels and depurinated before they were transferred to nitrocellulose paper (30). The filters were hybridized, washed, and exposed to X-ray film as described previously (30). DNA probes specific for the *neo* gene were made by using the 1,339-bp *Hin*dIII-*Nru*I DNA fragment from pSV2neo (28), whereas *gpt*-specific probes were made by obtaining the *Bgl*II-*Apa*I DNA segment (930 bp) from the *gpt* gene in pSV2gpt (18). In experiments where the blots had to be hybridized with a second DNA, the first DNA probe was removed by washing the nitrocellulose twice for 10 min each with 0.1 N NaOH–0.01 M EDTA, followed by two washes for 15 min each with 0.1% sodium dodecyl sulfate in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). These washed blots were then exposed to X-ray film for 3 to 4 days to confirm that the radioactive DNA probe had been removed before they were hybridized with the second DNA.

Fluctuation tests. Fluctuation tests were done by the method of Luria and Delbruck (17). Approximately 10^4 cells were seeded (after counting) onto about 40 10-cm plates. Half of these plates were placed immediately in G418 medium to determine the number of preexisting G418-resistant colonies, if any. Cells on the other plates were grown in *gpt*-selective medium until there were about 5×10^6 cells per plate, at which time they were switched to G418 medium, after one plate had been used to obtain a cell count. G418-resistant colonies were counted after 2 to 3 weeks. Recombination frequencies were calculated both from the fraction of plates having no G418-resistant colonies and by counting the new G418-resistant colonies that were generated during the test (2, 19). The frequencies determined by the two methods were remarkably similar, showing at most a 2.5-fold difference. The average frequency values obtained with these two methods are presented in the text.

RESULTS

Experimental strategy. The substrates (IR and DR plasmids) used to detect extrachromosomal and chromosomal homologous recombination (Fig. 1) contain truncated and nontandem, but overlapping, fragments of the neomycin resistance gene, which has been used as a dominant selectable marker in mammalian cells (28). For extrachromosomal recombination, the IR and DR plasmids can be introduced separately into mammalian cells, and G418-resistant colonies can be selected directly. This requires an early intra- or intermolecular recombination event (presumably before integration) between the *neo-1* and *neo-2* segments to generate a functional *neo* gene. Alternatively, G418-resistant colonies can be selected after these DNAs are introduced into COS cells (8), where they replicate extrachromosomally, by virtue of the fact that the SV40 large T antigen expressed constitutively by the COS cells can drive DNA replication from the SV40 origin of DNA replication present in the IR and DR plasmids.

Chromosomal recombination can also be studied with the IR and DR plasmids by first identifying *gpt*-positive cells that contain integrated copies of the unrecombined *neo* gene segments. Chromosomal recombination in these cell lines is then detected by the appearance of G418-resistant colonies when these cells are shifted to medium containing the drug G418.

Direct selection for G418-resistant colonies after transfection. In our initial experiments, we asked whether the IR and DR plasmids could recombine in an intra- or intermolecular manner, soon after transfection, to generate an intact *neo* gene. Mouse (3T6) cells were transfected with each of these

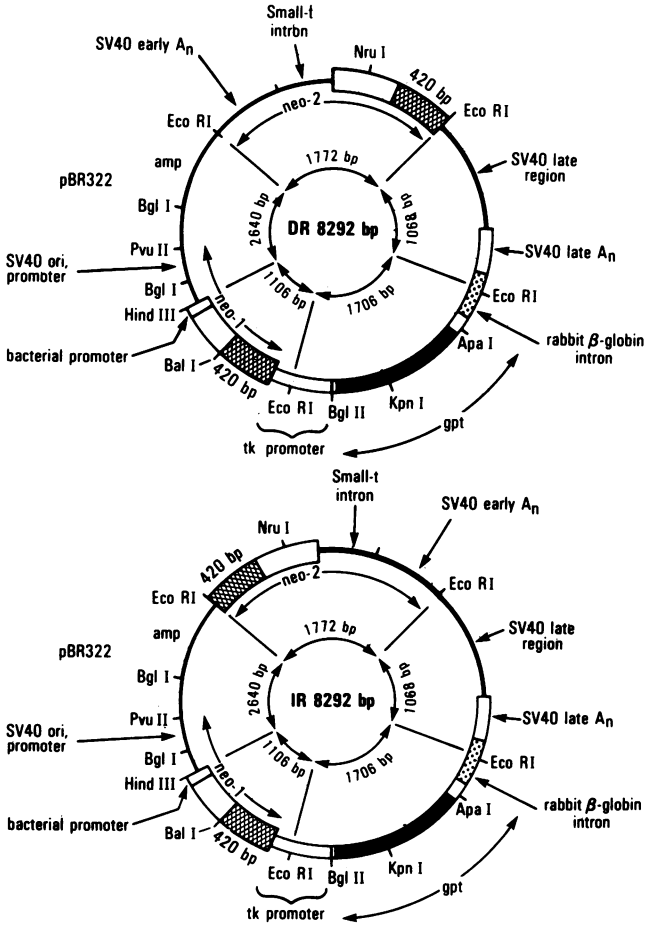

FIG. 1. Structure of the recombination substrates. Each of the plasmids contains two, truncated nontandem, but overlapping, segments of the *neo* gene (*neo-1* and *neo-2*). The homologous segments (hatched region) are 420 bp long and are arranged either as a direct repeat in DR or as an inverted repeat in IR. The two *neo* segments are separated by a complete *gpt* transcription unit containing the herpesvirus *tk* promoter, rabbit β-globin intron, and the polyadenylation site from the SV40 late region. The plasmids also contain the ampicillin resistance gene of pBR322 and the plasmid origin of DNA replication for propagation in bacteria. A_n denotes a polyadenylation signal.

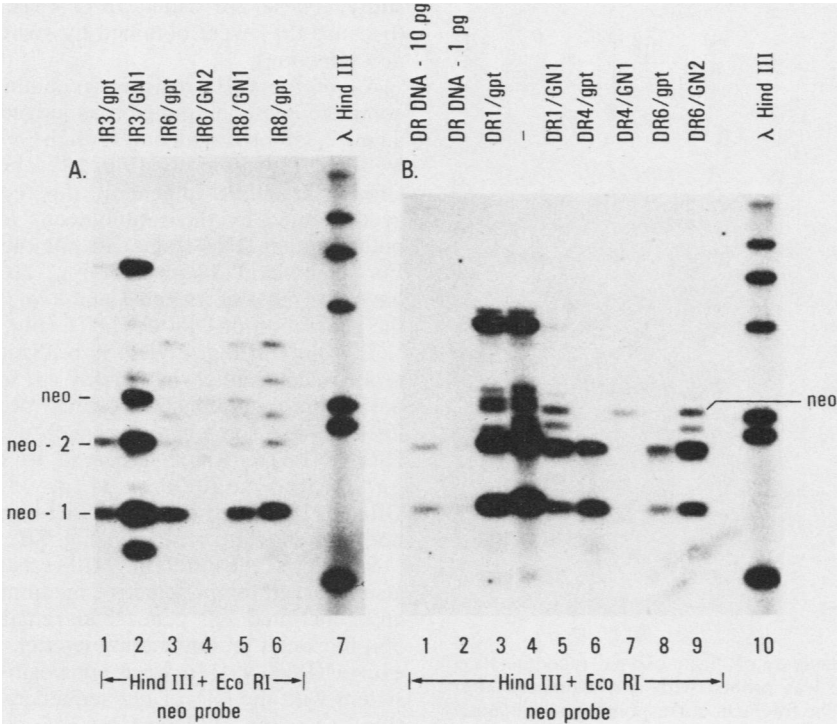

FIG. 2. Southern blot analysis of chromosomal recombination events. Genomic DNAs from the IR/gpt/3T6 and DR/gpt/3T6 lines and their G418-resistant derivatives were digested with *Hin*dIII and *Eco*RI and hybridized with a *neo* probe. The recombined *neo* gene should generate a 2,338-bp DNA fragment. (A) Blots of IR/gpt/3T6 lines and their G418-resistant derivatives. (B) Blots of DR/gpt/3T6 lines and their G418-resistant derivatives. Track 4 shows DNA not relevant to this paper. λ markers: 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.56 kb.

DNAs along with the control plasmids, pSV2neo (28) and pSV2gpt (18). The IR and DR plasmids were just as effective as pSV2gpt in generating colonies on *gpt*-selective medium (transformation frequency of 3×10^{-5} to 5×10^{-5}). These plasmids (which must recombine to acquire a functional *neo* gene) also generated G418-resistant colonies at a frequency of about 4×10^{-5}, only six- to sevenfold lower than the frequency with which pSV2neo generated such colonies. This indicates that about 15% of the cells that were potentially capable of integrating and expressing an intact *neo* gene (as in pSV2neo) had already recombined the two *neo* segments and were expressing the *neo* gene. Southern blot analysis (27) of the genomic DNA in 11 independent G418-resistant recombinants indicated that they all had an intact *neo* gene, as expected (data not shown). Since these data are similar to those published earlier (25, 26), they are not described in detail.

To determine whether the above frequencies were generally consistent with extrachromosomal recombination occurring before integration, we repeated the same experiment in COS cells in which the IR and DR plasmids should replicate extrachromosomally. In this line also, about 5 to 10% of the cells that were capable of growing up as G418-resistant colonies with pSV2neo had recombined the *neo* segments in the IR and DR plasmids and were expressing the *neo* gene. Therefore we believe that the frequency of appearance of G418-resistant colonies directly after transfection must be a reflection of extrachromosomal recombination events.

Chromosomal homologous recombination. The recombination events that generated intact *neo* genes soon after transfection of 3T6 or COS cells with the IR and DR plasmids were presumed to be extrachromosomal events because of their timing and frequency. To measure chromosomal recombination frequencies, we transfected 3T6 cells with the IR and DR plasmids and selected initially for expression of *gpt* by requiring the cells to grow on *gpt*-selective medium. These colonies appeared at frequencies of 3×10^{-5} to 5×10^{-5}. Three IR/gpt/3T6 clones (IR3/gpt/3T6, IR6/gpt/3T6, and IR8/gpt/3T6) and three DR/gpt/3T6 clones (DR1/gpt/3T6, DR4/gpt/3T6, and DR6/gpt/3T6) were grown up for further study. Though these lines were propagated in *gpt*-selective medium, all of them (except DR4/gpt/3T6) remained *gpt* positive even after growth in nonselective medium for at least 40 generations, and the pattern of hybridizing DNA segments remained unchanged when genomic DNA from these lines was analyzed by Southern blots. Thus these lines (except DR4/gpt/3T6) have stably integrated copies of IR or DR DNAs. The DR4/gpt/3T6 line contained amplified integrated DNA as shown later, but maintained its *gpt*-positive phenotype only when propagated in *gpt*-selective medium. After growth for 2 months in nonselective medium it showed only a 0.1% plating efficiency in *gpt*-selective medium.

The *gpt*-positive clones have no intact *neo* gene. The genomic DNAs from the above clones were digested with *Hin*dIII and *Eco*RI, and analyzed by Southern blots with the *neo* probe. With these enzymes (Figure 1), the unrecombined *neo* segments should be 1,772 bp (*neo-2*) and 1,106 bp (*neo-1*) in length, whereas the recombined *neo* gene should be 2,338 bp long. None of the IR/gpt/3T6 (Fig. 2A, lanes 1, 3, and 6) or DR/gpt/3T6 (Fig. 2B, lanes 6 and 8) lines (except DR1/gpt/3T6) had a band corresponding exactly to the intact, recombined *neo* gene. Instead, all of these lines had the two unrecombined *neo* gene segments, *neo-1* and *neo-2*.

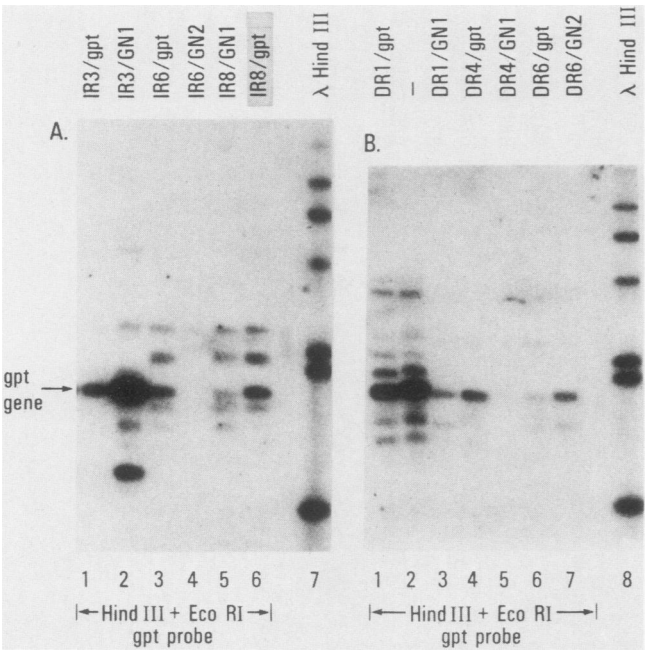

FIG. 3. Southern blot analysis of chromosomal recombination events. The blot in Fig. 2 was probed with *gpt* sequences after melting off the *neo* probe. The fragment corresponding to the intact *gpt*-coding sequence is shown.

The *Hin*dIII-*Eco*RI digest of DR1/gpt/3T6 (Fig. 2B, lane 3) appeared to have a band of the same size as the intact *neo* gene. However, this was shown not to have a complete *neo* coding sequence by other digests (data not shown).

All *gpt*-positive clones have intact *gpt* genes. The blots in Fig. 2 were used to determine whether the *gpt*-positive lines did indeed have intact *gpt*-coding regions. The *neo* probe was removed from the blots in Fig. 2, and the blots were reprobed with a *gpt*-specific sequence as described above. All three IR/gpt/3T6 lines (Fig. 3A, lanes 1, 3, and 6) and the three DR/gpt/3T6 lines (Fig. 3B, lanes 1, 4, and 6) had the expected 1,706-bp hybridizing fragment diagnostic of an intact *gpt*-coding region.

Chromosomal recombination in the *gpt*-positive lines generates complete *neo* genes. The IR/gpt/3T6 and DR/gpt/3T6 lines, which were propagated in *gpt* selective medium, were transferred at low cell densities to G418 medium to see whether any chromosomal homologous recombination would occur to generate a functional *neo* gene. Indeed, all of the lines were found to generate G418-resistant colonies at a low frequency. Fluctuation tests were therefore done to get better estimates of the frequency of chromosomal homologous recombination (Table 1). The recombination frequency varied over 2 orders of magnitude from a value as low as 2.3×10^{-8} per cell generation for IR6/gpt/3T6 to 1×10^{-6} per cell generation for DR4/gpt/3T6. Subclones of some lines were tested again and produced G418-resistant colonies at the same frequency, indicating that the recombination frequency was an inherent property of that cell line. For example, as shown below, IR6/gpt/3T6 and IR8/gpt/3T6 were subclones, and their recombination frequencies are therefore very similar.

Isolation and characterization of G418-resistant chromosomal recombination products. Several G418-resistant colonies that arose by recombination from the IR/gpt/3T6 and DR/gpt/3T6 cell lines were grown up and used for further study. These are called IR/GN/3T6 or DR/GN/3T6 lines (because they were obtained by switching cells from *gpt* to *neo* selection).

All of the G418-resistant recombinants had acquired a complete *neo* coding region as judged by the appearance of a new 2,338-bp fragment (*Hin*dIII-*Eco*RI digest) that hybridized with the *neo* probe (Fig. 2A, lanes 2, 4, and 5; Fig. 2B, lanes 5, 7, and 9). In general, this recombination event was accompanied by the simultaneous loss or appearance (or both) of other DNA fragments homologous to the *neo* probe. For example, DR4/gpt/3T6 (Fig. 2B, lane 6) appeared to have lost most of its *neo-1* and *neo-2* gene segments during the generation of DR4/GN1/3T6 (Fig. 2B, lane 7).

The blots in Fig. 2 were hybridized with the *gpt*-specific probe to determine whether any *gpt* sequences had been lost during the recombination events. Most of the recombined lines still had some *gpt* sequences, except IR6/GN2/3T6 and DR4/GN1/3T6, which appeared to have lost most or all *gpt*-related sequences (Fig. 3). As shown below DR4/GN1/3T6 still has one intact copy of the *gpt* gene that does not show up clearly in Fig. 3B.

Samples of the different G418-resistant recombinants were also returned to *gpt*-selective medium to determine whether any functional *gpt* genes had remained intact during the chromosomal recombination events. All of the lines tested except IR6/GN2/3T6 were *gpt* positive. This result is consistent with the loss of *gpt* sequences observed for this line (Fig. 3A, lane 4). DR4/GN1/3T6 showed a lower plating efficiency on *gpt*-selective medium than did the other *gpt*-positive lines. This is consistent with the requirement for amplified *gpt* sequences in DR4/gpt/3T6 for growth in *gpt*-selective medium.

Chromosomal recombination events that generate deletions. In two cell lines, DR4/GN1/3T6 and IR6/GN2/3T6, the recombination caused apparent deletion events. This was determined by comparing Southern blots of DNAs from these lines and their *gpt*-positive progenitors. When the DR4/gpt/3T6 DNA was digested with *Sst*I (an enzyme that does not cut the DR plasmid) and hybridized with a *neo* probe, a single chromosomal integration site was seen (Fig. 4, lane 1). Similar results were obtained with other enzymes that do not cut the plasmid. The intensity of the band in Fig 4 (lane 1) indicated quite clearly that either many copies (8 to 10) of the DR DNA had integrated at a single site, or alternatively DNA amplification of the integrated DNA had occurred at this site. We support the latter interpretation because if multiple copies of the DR DNA, which lacks an *Sst*I site, had integrated at a single chromosomal location, then the size of the hybridizing fragment from DR4/gpt/3T6 should be much larger than the size of the DR plasmid (8.3 kilobases [kb]). However, the band in Fig. 4 (lane 1) is about 9.7 kb. Thus, the amplification unit must have at least two

TABLE 1. Fluctuation tests to determine chromosomal recombination frequencies[a]

Cell line	Avg recombination frequency
IR3/gpt/3T6	4.3×10^{-7}
IR6/gpt/3T6	2.3×10^{-8}
IR8/gpt/3T6	2.8×10^{-8}
DR1/gpt/3T6	2.2×10^{-7}
DR4/gpt/3T6	1.0×10^{-6}
DR6/gpt/3T6	1.3×10^{-7}

[a] Fluctuation tests were performed as described in the text.

*Sst*I sites within it (Fig. 5). In addition, data from other blots indicate that each amplification unit containing DR DNA is at least 35 kb long (data not shown).

Interestingly, when the *Sst*I digest of DR4/GN1/3T6 was hybridized with the *neo* probe, a new prominent hybridizing fragment of lower molecular weight (6.4 kb) appeared, and a faint band of the original size (9.7 kb) remained. We interpret the new, 6.4 kb fragment (Fig. 4, lane 2) as having arisen by intrachromosomal recombination or unequal sister chromatid exchange between the *neo-1* segment in one amplification unit and the *neo-2* segment in another nearby unit, followed by recombination or gene conversion in all but one of the original amplification units. This accounts not only for the fact that the new (6.4-kb) band is smaller than the *Sst*I fragment of each unrecombined unit (9.7 kb) by the predicted size of 3.3 kb (Fig. 5), but also that the new band is of lower intensity than the band in Fig. 4, lane 1 (because it arises from the deletion of a few amplification units during recombination). The fainter 9.7-kb band in Fig. 4 (lane 2) corresponds to the single amplification unit in which no recombination had occurred. This unit still maintained the *gpt* gene (see below); hence DR4/GN1/3T6 could still grow on *gpt*-selective medium, albeit poorly.

If the above interpretation was correct, then one would predict that if the blot in Fig. 4 (lane 2) was rehybridized with a *gpt*-specific probe, then only the upper band in Fig. 4 (lane 2), for DR4/GN1/3T6, should hybridize. This is indeed the case (Fig. 4, lane 6).

Figure 5 illustrates schematically the arrangement of the amplification unit in DR4/gpt/3T6 that is consistent with the data in Fig. 4 and Fig. 2B and other unpublished data. When DNA from DR4/gpt/3T6 was digested with *Kpn*I, two bands of approximately equal intensity were seen (Fig. 4, lane 3); thus each amplification unit has at least three *Kpn*I sites. When this same digest was hybridized to the *gpt* probe (Fig. 4, lane 7), the same two bands were seen. However, the intensity of the lower band was about 4 times higher than that of the upper band. This could be explained by the fact that the upper band has only 200 bp of DNA homologous to the *gpt* probe, whereas the lower band has 736 bp homologous to the *gpt* probe.

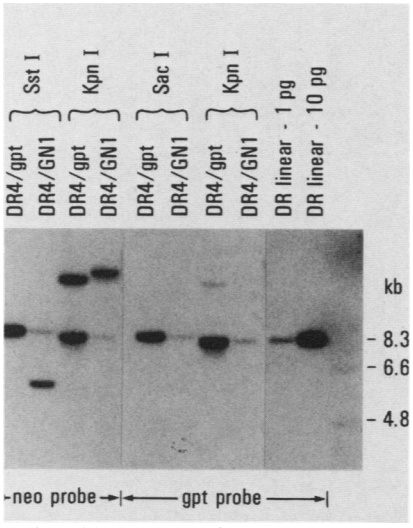

FIG. 4. Southern blot analysis of DR4/gpt/3T6 and DR4/GN1/3T6 with *neo* and *gpt* probes. Genomic DNAs from these cell lines were digested with the indicated enzymes and hybridized to the probes shown. Note that *Sst*I and *Sac*I are isoschizomers.

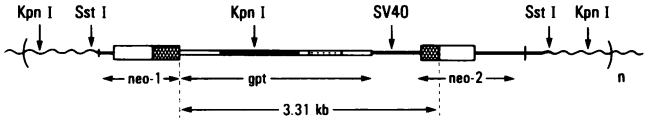

FIG. 5. Schematic arrangement of integrated DR DNA in DR4/gpt/3T6. The DNA is arranged as an amplified unit where *n* is 8 to 10. The relative locations of the *Kpn*I and *Sst*I sites within the amplification unit and outside the input DR DNA are schematic and based on data in Fig. 4 and other unpublished data, which indicate that each amplification unit has two *Sst*I sites and three *Kpn*I sites. Recombination between *neo-1* and *neo-2* within an amplification unit or between such units would appear as a deletion in a *Sst*I digest of a fragment of 3.31 kb containing the *gpt* transcription unit.

The *Kpn*I digest of DR4/GN1/3T6 was also hybridized with *neo* and *gpt* probes. Recombination between *neo-1* and *neo-2* in Fig. 5 would generate a larger *Kpn*I fragment containing the *neo* genes. Since this same event happened in several amplification units, the intensity of this band (Fig. 4, lane 4) was high when hybridized with the *neo* probe. However, there were also two other, fainter bands in this track corresponding to the *Kpn*I fragments from the amplification unit in which recombination had not occurred. When this same *Kpn*I digest of DR4/GN1/3T6 is probed with *gpt*, only the two bands from the unrecombined amplification unit should hybridize. This is indeed the case, even though the exposure in Fig. 4 (lane 8) shows only one band. (Note that, as mentioned above, the missing band has only 200 bp of homology with the probe.)

Thus, the evidence for DR4/gpt/3T6 is that it has several amplification units containing the unrecombined *neo* gene segments (Fig. 5). In DR4/GN1/3T6 recombination seems to have occurred between the *neo-1* and *neo-2* gene segments, deleting in the process a few amplification units and the *gpt* transcription unit. Interestingly, this recombination event appears to have occurred in all but one amplification unit.

Sequential recombination or gene conversion in amplification units of DR4/gpt/3T6. Since DR4/gpt/3T6 had 8 to 10 amplification units, one can consider two formal pathways, for the generation of DR4/GN1/3T6. In the first mechanism, homologous recombination could have occurred simultaneously or sequentially in each of the recombined amplification units. We consider this highly unlikely, because the frequency of such multiple recombination events would be very low based on the numbers in Table 1. Alternatively, after homologous recombination had occurred between the *neo-1* and *neo-2* genes in two adjacent or nearby units, the other amplification units could have been sequentially gene converted relative to the recombined amplification unit. Though we favor the latter model, our data does not distinguish between these two possibilities.

We decided to look at other independent recombinants generated from DR4/gpt/3T6, to see whether we could find any evidence for sequential recombination or conversion of the amplification units. Genomic DNAs from three other recombinants, DR4/GN2/3T6, DR4/GN3/3T6, and DR4/GN4/3T6, were digested with either *Eco*RI and *Hin*dIII, *Sst*I, or *Kpn*I, and these DNAs were hybridized to the *neo* probe. The *Eco*RI-*Hin*dIII digests indicated clearly that the amounts of the unrecombined *neo-1* and *neo-2* segments were the lowest in DR4/GN4/3T6 and the highest in DR4/GN2/3T6 (Fig. 6). The corresponding increase in the amount of the intact *neo* gene was not as obvious in the *Eco*RI-*Hin*dIII digests as it was in other digests.

All three G418-resistant recombinants in Fig. 6 showed

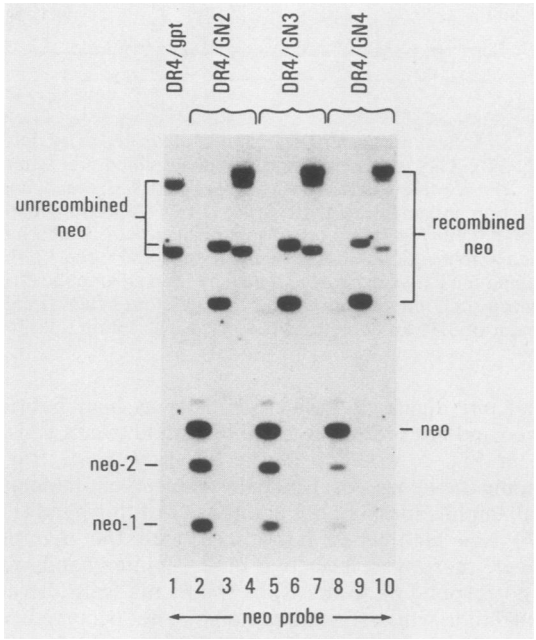

FIG. 6. Analysis of recombination in DR4/gpt/3T6 and several of its G418-resistant derivatives. DNAs in lanes 1 through 10 were digested as follows: 1, 4, 7, and 10 with *Kpn*I; 2, 5, and 8 with *Eco*RI and *Hin*dIII; 3, 6, and 9 with *Sst*I. The blot was hybridized to a *neo* probe. The origins of the various bands are indicated on the left and right.

two *Sst*I fragments (lanes 3, 6, and 9). The upper one (9.7 kb) corresponded to the unrecombined amplification unit, and the lower one (6.4 kb) was diagnostic of the recombined amplification unit. The band corresponding to the unrecombined amplification unit decreased in intensity, whereas the fragment for the recombined amplification unit increased in intensity, in going from DR4/GN2/3T6 to DR4/GN4/3T6. The *Kpn*I digests confirmed this same trend. Thus of the recombinants derived from DR4/gpt/3T6, DR4/GN4/3T6 had the maximum number of recombined amplification units and the minimum number of unrecombined amplification units, whereas in DR4/GN2/3T6 the converse was true. This suggests that the recombination or gene conversion can occur sequentially. However, it is not possible to say whether any of the recombinants containing more than one recombined amplification unit were generated sequentially or in a single step.

Recombination events in cell lines derived from IR DNA. The IR6/gpt/3T6 line recombined to generate the IR6/GN2/3T6 line that was G418 resistant, but was unable to grow on *gpt*-selective medium (see above). The *Sst*I digests of these two DNAs were hybridized with *neo* and *gpt* probes. With a *neo* probe there was a single integration site in IR6/gpt/3T6 where multiple copies were present (Fig. 7A). This integrated DNA hybridized to both *neo* probes (Fig. 7A, lane 1) and *gpt* probes (Fig. 7B, lane 1). In IR6/GN2/3T6, a deletion had occurred (Fig. 7A, lanes 6 and 7) so that the DNA fragment that hybridized with the *neo* probe was smaller than that in IR6/gpt/3T6. This smaller DNA fragment, however, did not hybridize to *gpt* (Fig. 6B, lanes 6 and 7). This result is consistent with data from Fig. 3A, that IR6/GN2/3T6 was unable to grow on *gpt*-selective medium and it had no *gpt* sequences.

Chromosomal inversions. In IR6/gpt/3T6, the input DNA had integrated at a single chromosomal location, because when the *Sst*I digest of this DNA was hybridized to the *neo* or *gpt* probes, only one band hybridized (Fig. 7A and B, lanes 1). With both probes, the intensity of the band suggested that multiple copies of the DNA had integrated at this site. In IR6/GN1/3T6, which was generated from IR6/gpt/3T6

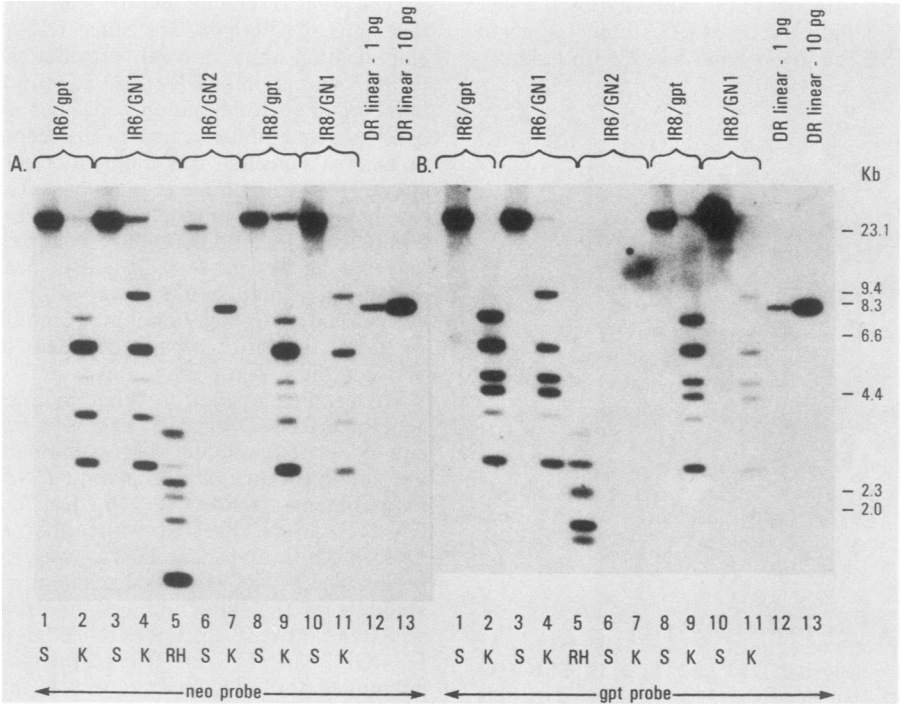

FIG. 7. Analysis of chromosomal recombination events of IR/gpt/3T6 lines. The indicated DNAs were digested with *Sac*I or *Sst*I (S), *Kpn*I (K), or *Eco*RI plus *Hin*dIII (RH). (A) Blot hybridized to *neo* probe. (B) Blot hybridized to *gpt* probe. DNA size markers are on the right.

by chromosomal recombination, no deletions occurred. Instead the size and intensity of the DNA fragments in the *Sst*I digest of the IR6/GN1/3T6 remained the same as that in its *gpt*-positive progenitor, when either the *gpt* or the *neo* probe was used for hybridization (Fig. 7A and B, lanes 1 and 3). The simplest explanation for this is an intrachromosomal inversion event.

Subclones of a *gpt*-positive clone can generate identical G418-resistant recombination products. IR6/gpt/3T6 and IR8/gpt/3T6 showed very similar recombination frequencies (Table 1) (see above). Although these had been believed to be independent colonies, Southern blots of their genomic DNAs (Fig. 7A and B, lanes 1, 2, 8, and 9) showed unequivocally that they were identical. We therefore asked whether these related clones could generate identical recombination products. The recombinants IR6/GN1/3T6 and IR8/GN1/3T6 were also identical (Fig. 7A and B, lanes 3, 4, 10, and 11). Thus both of these recombinants appeared to have arisen from chromosomal inversions.

DISCUSSION

In this study, we have used substrates containing two truncated nontandem, but overlapping, segments (*neo-1* and *neo-2*) of the neomycin resistance gene to detect recombination events. Intra- or intermolecular reciprocal recombination between the *neo-1* and *neo-2* segments or intermolecular gene conversion generates a functional *neo* gene whose gene product is capable of conferring resistance to the drug G418.

While this work was in progress, other groups have also used expression vectors carrying selectable markers genes to detect recombination in mammalian cells (15, 16, 22, 26). Because most of these studies (15, 16, 26) have looked at recombination events between *tk* genes, and since *tk* is not a dominant selectable marker, these substrates have all been used only in *tk*-deficient cells (specifically Ltk$^-$ cells). The substrates used in this study can be used in any cell in which the *neo* gene functions as a dominant selectable marker (28), including human cells.

Another important advantage of the IR and DR plasmids arises from the fact that the recombination between the *neo-1* and *neo-2* gene segments generates a functional *neo* gene that works in bacteria (unpublished data) and mammalian cells. Thus these plasmids could also be used to study recombination in transiently infected mammalian cells, followed by the introduction of Hirt supernatant DNA obtained from the mammalian cells, into recombination-deficient *E. coli* for the analysis of recombination frequencies and products.

High extrachromosomal recombination frequencies of transfected DNAs. The experiments in which G418-resistant colonies were selected directly after transfection of 3T6 cells with the IR and DR plasmids showed that about 15% of the cells that were capable of integrating and expressing an intact *neo* gene had also mediated at least one recombination event. This frequency was the same for the IR and DR plasmids. Consistent with this are the observations that in experiments with the *tk* gene in Ltk$^-$ cells (25, 26) and in our studies with the IR and DR plasmids in COS cells (in which these plasmids do replicate extrachromosomally), approximately 5 to 20% of the cells that were capable of stably expressing the selectable marker gene had also mediated a recombination event. This high frequency of extrachromosomal recombination may be a property only of newly transfected DNA. We are currently testing whether this phenomenon is due to an induction of the host recombination enzymes or whether it is related specifically to some aspect of the structure of newly transfected DNA.

Chromosomal recombination events are rare. To determine chromosomal recombination frequencies, we first introduced the IR and DR plasmids into the mouse chromosome (generally at a single site) by selecting for *gpt* expression. Several such *gpt*-positive lines that had only unrecombined *neo* segments (by Southern blots) were analyzed by fluctuation tests for the frequency of chromosomal recombination. In contrast to the high frequency of extrachromosomal recombination, the chromosomal recombination frequencies (10^{-6} to 10^{-8}) were low. This frequency is similar to that observed for recombination between retroviral long terminal repeats (6, 31). The 100-fold range in chromosomal recombination frequencies may be a reflection of either the site or the structure of the integrated DNA.

Chromosomal inversions. In IR6/GN1/3T6 and IR8/GN1/3T6, recombination was not accompanied by the loss or gain of any DNA. This is therefore consistent with chromosomal inversion events generated by recombination between the *neo-1* and *neo-2* segments arranged in an inverted repeat configuration. This kind of inversion is analogous to those that have been detected during rearrangement of immunoglobulin genes (9, 14), phase variation in *Salmonella* sp. (33), or FLP-mediated recombination in *S. cerevisiae* (7), although these processes are mediated by completely different enzymes. At the present time we do not know why IR6/gpt/3T6 exhibits recombination events consistent with both deletions (IR6/GN2/3T6) and inversions (IR6/GN1/3T6). The answer to this probably lies in the exact structure of the integrated IR DNA, which is currently under investigation.

Sequential recombination and gene conversion in amplification units of DR4/gpt/3T6. The line DR4/gpt/3T6 had 8 to 10 amplification units with the structure shown in Fig. 5. In DR4/GN1/3T6, several amplification units appeared to have deleted the *gpt* gene in an identical fashion. Since the frequency of chromosomal recombination is so low, it seems unlikely that all the amplification units could have undergone homologous recombination, either sequentially or simultaneously. It seems more likely that recombination occurred in one amplification unit and that sequential gene conversion occurred in the other units, a process that would be formally analogous to homogenization among various members of multigene families.

The unusual recombination events exhibited by the amplification units in DR4/gpt/3T6 may well be a function of the detailed arrangement of the amplification units relative to each other. We do not know yet whether the events depend in any way on the fact that the amplification units in DR6/gpt/3T6 are unstable in the absence of selection. Further work is in progress to address this issue.

Different independent recombination products isolated from DR4/gpt/3T6 showed varied numbers of recombined versus unrecombined amplification units (Fig. 6), suggesting that gene conversion or recombination (or both) can occur sequentially. The gene conversion or recombination process must be quite efficient, although it is difficult to measure the exact frequency. Similar events have been observed in amplification units by Roberts and Axel (23), where the frequency of mutation and conversion was about 10^{-4}.

Since both the *gpt* and the *neo* genes function as dominant selectable markers in a variety of mammalian cells, the IR and DR plasmids should now make it possible to study recombination events in other cell lines of interest, including those of human origin.

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