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Gene Targeting with Retroviral Vectors: Recombination by Gene Conversion into Regions of Nonhomology

JAMES ELLIS AND ALAN BERNSTEIN*

Department of Medical Genetics, University of Toronto, and Mount Sinai Hospital Research Institute, Division of Molecular & Developmental Biology, 600 University Avenue, Toronto, Ontario M5G 1X5, Canada

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We have designed and constructed integration-defective retroviral vectors to explore their potential for gene targeting in mammalian cells. Two nonoverlapping deletion mutants of the bacterial neomycin resistance (*neo*) gene were used to detect homologous recombination events between viral and chromosomal sequences. Stable *neo* gene correction events were selected at a frequency of approximately 1 G418^r cell per 3×10^6 infected cells. Analysis of the functional *neo* gene in independent targeted cell clones indicated that unintegrated retroviral linear DNA recombined with the target by gene conversion for variable distances into regions of nonhomology. In addition, transient *neo* gene correction events which were associated with the complete loss of the chromosomal target sequences were observed. These results demonstrated that retroviral vectors can recombine with homologous chromosomal sequences in rodent and human cells.

The genomes of mammalian cells can be predictably modified by homologous recombination with newly introduced DNA. Such gene targeting directs exogenous DNA sequences into specific chromosomal sites (1, 2, 8, 34, 35, 37) or replaces defined segments of a target gene (1, 22, 35, 37, 38). Gene targeting has been widely used in yeast cells to construct mutants carrying defined alleles for genetic analysis (5). Similar procedures could be used to introduce specific mutations into the mouse germ line via homologous recombination in totipotent embryonic stem cells (8, 22, 37). The generation of mice from these mutant embryonic stem cells would make it feasible to construct animal models of human genetic diseases (15, 19) and would facilitate the analysis of cloned genes whose biological functions are unknown.

Retroviruses provide a number of attractive features that might make them useful for gene targeting. They are nontoxic, have a wide host cell range, and infect cells with high efficiency. Under normal circumstances, retroviral DNA integrates into random sites in the host genome (32). This integrative pathway can be abrogated to introduce viral DNA into the cell nucleus, where it fails to integrate efficiently (7, 9, 26, 27, 31). Such a delivery system might be ideal for obtaining homologous recombination events between unintegrated viral DNA and specific chromosomal target sites.

We describe here the stable correction of mutant neomycin resistance (*neo*) genes via homologous recombination between an integration-defective retroviral vector and chromosomally located target genes. Southern blot analysis of the target before and after gene correction suggested that unintegrated viral linear DNA recombined with the target by gene conversion into regions of nonhomology. Unexpectedly, we also observed transient *neo* gene correction events associated with the loss of the chromosomal target sequences.

MATERIALS AND METHODS

Cell culture. All cells, including Rat-2, PA317 (23), and KY21b (35) cells, were grown in alpha minimal essential medium supplemented with 10% fetal bovine serum. DL22 cells were generated by Polybrene transfection (17) of 100 ng of pSV2neoDLSV2gpt into Rat-2 cells and selection with MAX medium (20 μg of mycophenolic acid per ml, 25 μg of adenosine per ml, 250 μg of xanthine per ml [24]).

Bacterial plasmids and viral DNAs. pSV2gpt (24), pSV2neo DLSV2gpt (35), and pMoTN (20) have been described previously. pMoDR was constructed by end filling to destroy the *Hin*dIII site between the TK promoter and *neo* in pTKneo and then by deletion of the 284-base-pair (bp) fragment between the *Nae*I sites in *neo*. The TKneoDR *Bam*HI fragment was then cloned into the *Bam*HI site of pMo593. pMo593 contains a 2-bp deletion in the 5′ long terminal repeat (LTR) and was constructed by ligating the *Cla*I-*Bam*HI fragment from pdl 593 (7) to the *Bam*HI-*Eco*RI fragment from pMoTN.

DNA transfection. PA317 cells (10^5) were transfected with 9 μg of pMoTN or pMoDR by $CaPO_4$ coprecipitation (12) with 1 μg of pSV2gpt and selection with MAX.

Virus infection. For analysis of unintegrated viral DNA, 10^6 Rat-2 cells per 100-mm plate were infected with 5 ml of filtered virus plus 8 μg of Polybrene per ml. At each time point, unintegrated viral DNA was extracted from a single plate as described by Hirt (14), and all the recovered DNA was subjected to Southern blot analysis (36).

For gene correction experiments, 5×10^5 recipient cells were plated per 100-mm plate and infected with 5 ml of filtered virus plus 8 μg of Polybrene per ml. At 48 h postinfection, each plate was split onto four plates and G418 selection was applied (400 μg/ml). Medium containing G418 was changed on days 5 and 10, and stable G418^r clones were picked on day 14.

Cocultivations were performed in 100-mm plates in the presence of 8 μg of Polybrene per ml by addition of 5×10^5 recipient cells onto 1×10^6 MoDR-producing PA317 cells pretreated with mitomycin C (10 μg/ml for 2 h). The plates were split and selected with G418 as above.

DNA analysis. Southern blot analysis (36) was performed by digestion of 5 μg of genomic DNA with appropriate restriction enzymes. The DNA was separated on 0.7% agarose gels, blotted onto nitrocellulose filters, and hybridized to nick-translated probes by standard methods (21).

* Corresponding author.

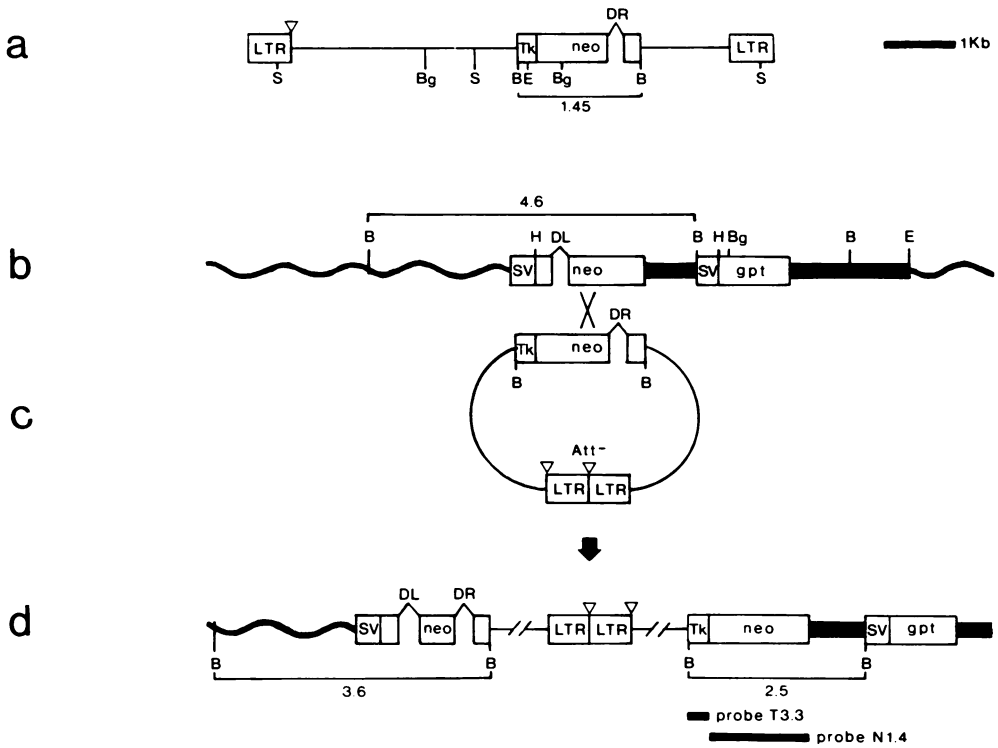

FIG. 1. Planned correction of *neoDL* target sequences. Fragment sizes are given in kilobases. Restriction sites are indicated as follows: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; S, *Sac*I. (a) Map of the MoDR construct. Thin line is murine leukemia virus sequences. ▽, 2-bp deletion cloned from pdl 593 (7). DR, 284-bp *Nae*I deletion. (b) Map of the *neoDL* target in DL22 cells. ∿∿, Host flanking sequence. ▬, simian virus 40 polyadenylation site sequence. DL (deletion left) is a 248-bp *Nar*I deletion. (c) Map of the two-LTR circular form of unintegrated MoDR DNA. ×, Site of predicted reciprocal homologous recombination. (d) Map of the predicted structure of the corrected *neo* sequences. Symbols are as indicated for panels a through c.

Blots were washed to a final concentration of 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 50°C.

RESULTS

Design of retroviral targeting vectors. We chose to abrogate the normal integrative pathway in the targeting vectors because retroviruses normally integrate into many possible sites in the host genome by a nonhomologous recombination event (for reviews, see references 25 and 33). Efficient retroviral integration requires two components: (i) the integrase domain of the *pol* gene (9, 27, 31) and (ii) the extreme terminal sequences of the LTRs (7, 26). We have constructed a retroviral vector backbone that has a 2-bp deletion in the U5 region of the 5′ LTR. After virus production and replication, this deletion was transferred to both LTRs and the terminus of the 3′ LTR was altered. Retroviruses bearing this alteration are unable to integrate efficiently (7). We estimate that this mutation reduced integration by 1,000-fold (unpublished data).

Because homologous recombination in mammalian cells is a rare event (18), we utilized a system in which gene-targeting events can be selected directly with the drug G418 (35). A defective *neoDR* (deletion right) gene was inserted into the integration-defective vector backbone to construct the MoDR vector (Fig. 1). MoDR contains over 2 kilobases (kb) of *gag* sequences to increase the titer of infectious virus (3) and an internal promoter from the thymidine kinase (*TK*) gene of herpes simplex virus to drive the expression of the *neoDR* gene.

To generate infectious virus, the MoDR construct was transfected into the PA317 amphotropic packaging cell line (23). A polyclonal packaging cell population (MoDR-A) and a packaging cell clone (MoDR-3) which produce high titers of MoDR virus particles were identified by a viral RNA dot blot assay (manuscript in preparation). The MoDR titers were estimated to be 10^6 infectious particles per ml by comparison with known biological titers of the MoTN vector.

The MoDR titer was confirmed by comparing the levels of unintegrated viral DNA in Rat-2 cells infected with MoDR-3 or MoTN. As illustrated by the MoTN infections in Fig. 2, unintegrated retroviral DNA initially appeared by 8 h postinfection as an abundant linear form with an LTR at each end. By 16 h postinfection, a minor proportion had circularized by blunt-end ligation to form a two-LTR circle or by homologous recombination between the LTRs to form a one-LTR circle. All three forms of unintegrated DNA were detected over the normal time course in MoDR-3-infected Rat-2 cells.

We generated a recipient cell line bearing a defective *neoDL* (deletion left) gene by transfection of Rat-2 cells with pSV2neoDLSV2gpt. The *neoDL* gene is present in a single copy in DL22 cells and can be corrected in several possible ways. First, a single reciprocal recombination between MoDR circular DNA and the *neoDL* target would result in the integration of a permuted provirus into the target gene (Fig. 1). Targeted cells would therefore contain a *neoDLDR* gene and a wild-type *neo* gene which can be selected with G418. Second, the *neoDL* deletion in the target gene could

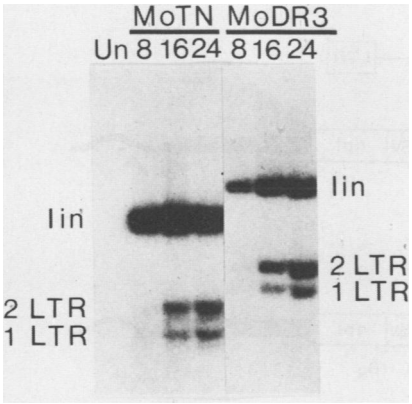

FIG. 2. Characterization of MoDR virus titer and intermediate DNA forms. Unintegrated MoTN and MoDR DNA present at 8, 16, and 24 h after infection of Rat-2 cells was analyzed by Southern transfer. Filter was hybridized to a probe specific for the 1.0-kb *Bgl*II-*Bam*HI fragment of *neo*. MoTN virus has a known titer of 10^6 G418^r CFU/ml. Un, Uninfected; lin, linear.

be repaired by a double crossover or a gene conversion event. Third, the *neoDR* deletion in unintegrated MoDR DNA could be corrected by a double crossover or a gene conversion event followed by integration of the corrected virus elsewhere. Each of these possibilities can be unambiguously identified by Southern blot analysis.

Stable gene correction in rodent cells. The recipient DL22 cell line was infected with the MoDR vector by incubation with filtered MoDR virus or by cocultivation with PA317 cells producing MoDR virus. Infection of DL22 cells with MoDR-A or MoDR-3 virus by either method generated one independent stable G418^r colony per 1×10^6 to 5×10^6 infected cells. In contrast, no G418^r colonies were observed after mock infections of DL22 cells or after infection of normal Rat-2 cells with MoDR virus.

To determine whether the G418^r clones arose as the result of a targeted recombination event within *neo* sequences, we analyzed the target structure of 10 stable G418^r clones representing five independent correction events. Five independent corrected clones are discussed here. Southern blot hybridization of *Bam*HI-digested DL22 genomic DNA with a probe specific for the *neo* gene (probe N1.4 in Fig. 1) gave rise to a 4.6-kb cell-target junction fragment (Fig. 3a). If *neo* gene correction occurred by the mechanism shown in Fig. 1, then all stably corrected clones should have replaced the 4.6-kb parental *Bam*HI fragment with a 3.6-kb *neoDLDR* gene and a 2.5-kb intact *neo* gene.

All five clones acquired the 2.5-kb *Bam*HI fragment which represented successful *neo* gene reconstruction (Fig. 3a); however, these clones also retained the unmodified parental 4.6-kb *Bam*HI fragment which may have been amplified in

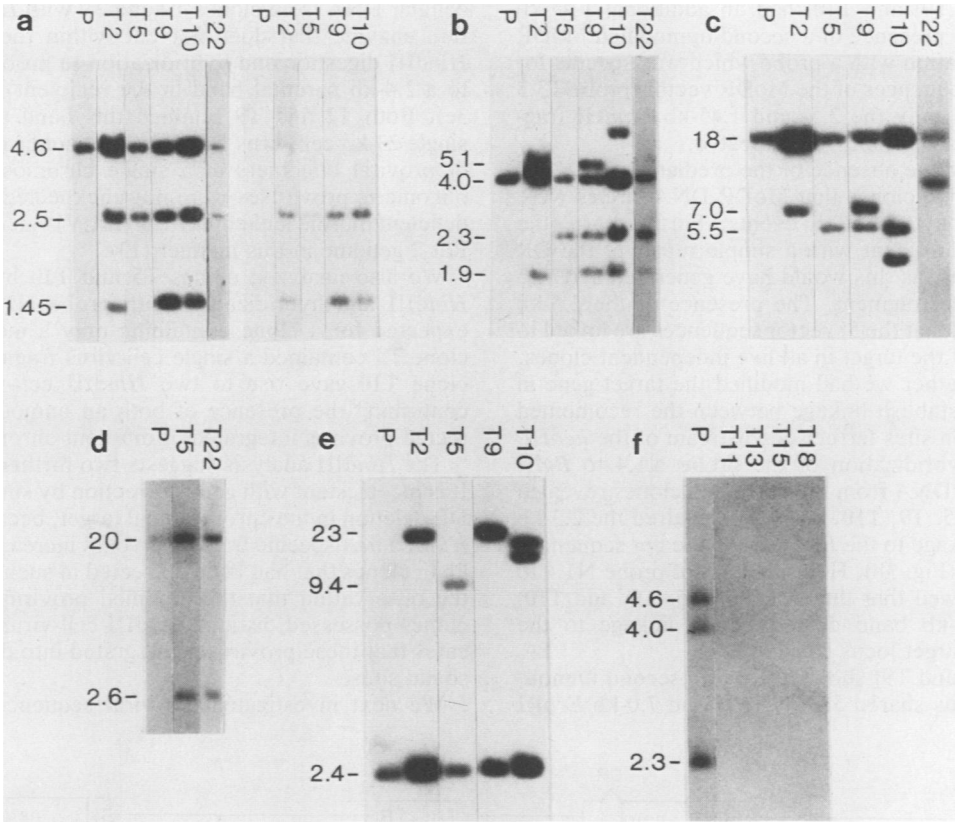

FIG. 3. Southern blot analysis of the target structure in the stable and transient G418^r cell clones. All probes are illustrated in Fig. 4. Fragment sizes are indicated in kilobases on the left of lanes. (a through e) Genomic DNA from stable G418^r clones was analyzed by the following methods: *Bam*HI digestion and hybridization to probe N1.4 (left lanes in panel a) and rehybridization with probe T3.3 (right lanes in panel a); *Bgl*II digestion and hybridization to probe N1.4 (b); *Eco*RI digestion and hybridization to probe N1.4 (c); *Sac*I digestion and hybridization to probe V5.7 (d); and *Hin*dIII digestion and hybridization to probe N1.4 (e). (f) Genomic DNA from transient G418^r clones was analyzed by *Bam*HI digestion and hybridization to pSV2neoDLSV2gpt.

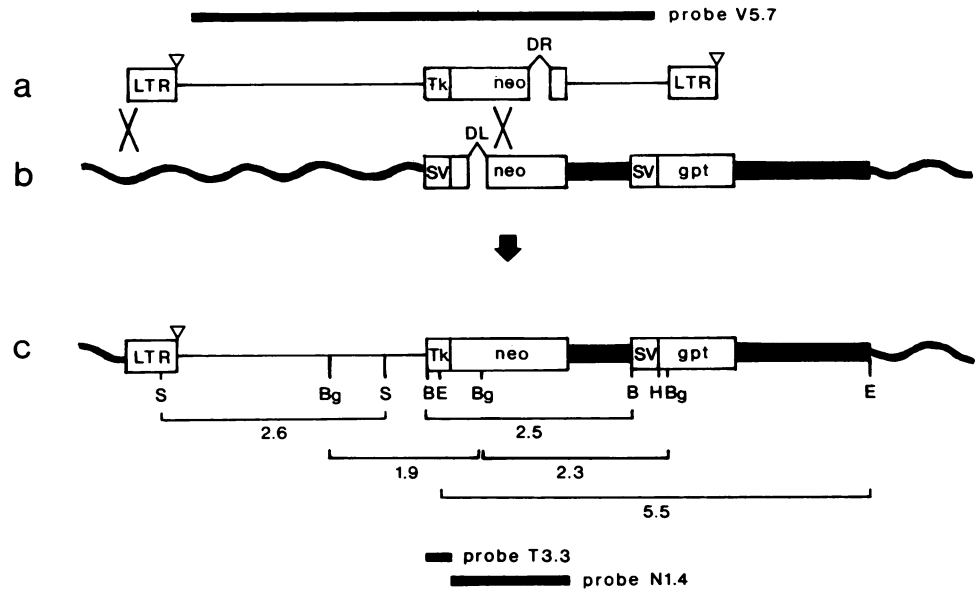

FIG. 4. Possible double-reciprocal recombination capable of generating the observed arrangement of *neo* sequences in the targeted cell clones. (a) Map of unintegrated linear form of MoDR DNA. Probe V5.7 is an *Aat*II-*Cla*I fragment. (b) Map of *neoDL* target in DL22 cells. (c) Map of corrected *neo* gene. Probe T3.3 is a 3.3-kb *Bam*HI fragment specific for the herpes simplex virus *TK* gene. Probe N1.4 is a 1.4-kb *Hin*dIII-*Bam*HI fragment specific for the *neo* gene. Symbols are as in Fig. 1.

some clones (T2, T9, and T10). None of the clones possessed the 3.6-kb *Bam*HI fragment diagnostic of a *neoDLDR* gene. Three clones (T2, T9, and T10) had an additional 1.45-kb band indicating the presence of a second unmodified MoDR provirus. Hybridization with a probe which was specific for the *TK* promoter sequences of the MoDR vector (probe T3.3 in Fig. 1) detected only the 2.5- and 1.45-kb *Bam*HI fragments in the corrected clones, as expected.

We inferred from the absence of the predicted *neoDLDR* fragment from all five clones that MoDR DNA circles were not involved in the correction events. Furthermore, the results were not consistent with a simple repair of the DR deletion in the virus, as this would have generated a 1.7-kb *Bam*HI *neo*-specific fragment. The presence of the 2.5-kb fragment suggested that the 5′ vector sequences are linked to the 3′ sequences of the target in all five independent clones.

To ascertain whether we had modified the target gene in situ, we tried to establish linkage between the recombined virus and restriction sites farther downstream of the *neoDL* target (Fig. 4). Hybridization of the probe N1.4 to *Bgl*II digests of genomic DNA from the corrected clones revealed that four clones (T5, T9, T10, and T22) acquired the 2.3-kb band indicating linkage to the *Bgl*II site in the *gpt* sequences of the target locus (Fig. 3b). Hybridization of probe N1.4 to *Eco*RI digests showed that three clones (T5, T9, and T10) possessed the 5.5-kb band demonstrating linkage to the *Eco*RI site in the target locus (Fig. 3c).

Two clones (T2 and T9) shown to have a second unmodified MoDR provirus shared 5.1-kb *Bgl*II and 7.0-kb *Eco*RI fragments, suggesting that the two proviruses in each clone were linked (Fig. 5). To test this possibility, we digested cellular DNA from clones T2 and T9 with *Hin*dIII, a restriction enzyme that does not cut within the MoDR vector. *Hin*dIII digestion and hybridization to probe N1.4 gave rise to a 2.4-kb parental band in the recipient DL22 cells (Fig. 3e). Both T2 and T9 retained this band and possessed a single 23-kb cell-virus fragment that confirmed the presence of proviral oligomers at a single chromosomal site. Such oligomeric proviruses were not unexpected since integrase-deficient murine leukemia virus (MLV) can integrate into the Rat-2 genome in this manner (13).

We also analyzed clones T5 and T10 by digestion with *Hin*dIII and hybridization with probe N1.4 (Fig. 3e). As expected for a clone containing only a modified provirus, clone T5 contained a single cell-virus fragment. DNA from clone T10 gave rise to two *Hin*dIII cell-virus fragments, confirming the presence of both an unmodified and a corrected provirus integrated at different chromosomal sites.

The *Hin*dIII analysis suggests two further interpretations. It is inconsistent with gene correction by simple repair of the DL deletion in the chromosomal target, because the parental *Hin*dIII *neo*-specific fragment would increase from 2.4 to 2.7 kb in clones that had been corrected in such a manner. Also, the observation that the modified proviruses in the G418^r clones possessed distinct *Hin*dIII cell-virus fragments indicates that these proviruses integrated into different chromosomal sites.

We next investigated the viral sequences linked to the

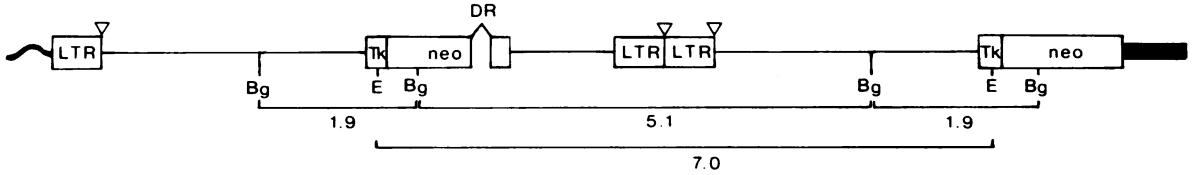

FIG. 5. Structure of MoDR vectors integrated as an oligomer of unmodified and modified proviruses. Symbols are as in Fig. 1.

corrected *neo* gene by digesting genomic DNA from the clones that contained only a modified provirus (T5 and T22) with *Sac*I and hybridizing with a probe specific for the MoDR sequences located between the LTRs (probe V5.7 in Fig. 4a). In both clones this analysis detected the parental 20-kb fragment and a 2.6-kb band indicating the presence of the viral 5′ LTR and *gag* sequences (Fig. 3d). Curiously, we did not observe a 3′ *Sac*I junction fragment in either clone, although it is possible that it comigrated with the 20-kb parental band. Both clones also contained the 1.9-kb *Bgl*II fragment present in the original virus (Fig. 3b). This mapping confirmed that the 5′ sequences of the vector were linked to the corrected *neo* gene, which was in turn linked to the 3′ target sequences. A map of the corrected *neo* gene consistent with the Southern blot analysis of clone T5 is shown in Fig. 4c.

Transient gene correction and target loss. Transient G418^r colonies were also observed in MoDR-infected DL22 cells. Several colonies that were healthy at day 10 of selection but were dying by day 14 were rescued in nonselective medium, isolated, and expanded. Four independent clones (T11, T13, T15, and T18) were subsequently shown to be sensitive to both G418 and MAX. Southern blot analysis of the target structure after *Bam*HI digestion with pSV2neoDLSV2gpt as a probe demonstrated that all four clones had lost the entire target locus, including the *gpt* sequences (Fig. 3f). A fifth clone (T17) had drug resistance and a target structure indistinguishable from those of the parental DL22 cells (data not shown). Since we have not observed transient G418^r colonies in mock-infected DL22 cells, transient resistance to G418 and target loss may be the direct result of a recombination event.

Stable gene correction in human cells. To determine whether genes in human cells could also be corrected with retroviral vectors, we infected the human KY21b cell line with MoDR. KY21b cells contain about five copies of *neoDL*, which can be corrected by gene conversion or targeted integration of *neoDR*-bearing plasmids (35). MoDR-A infection of 3×10^6 KY21b cells generated one stable G418^r colony (T1).

Southern analysis of T1 by *Bam*HI digestion and hybridization with probe N1.4 detected several fragments in the parental KY21b cells (Fig. 6a). The 2.0-kb parental fragment was replaced by a 2.5-kb fragment in T1, indicating that the 5′ vector sequences were linked via the corrected *neo* gene to the 3′ target sequences. T1 had no additional *Bam*HI fragments, indicating that neither a *neoDLDR* gene nor an unmodified MoDR provirus was present.

To confirm that the structure of the corrected *neo* gene was identical to that illustrated in Fig. 4c, we performed additional Southern blot analyses on T1. The corrected *neo* gene was linked to the 3′ target sequences by the 2.5-kb *Bam*HI and 2.3-kb *Bgl*II fragments and to the 5′ vector sequences by the 2.6-kb *Sac*I and 1.9-kb *Bgl*II fragments. The modified vector does not have a wild-type proviral structure because *Xba*I digestion, which cuts only in the LTRs, did not reveal a viral fragment migrating at 6.4 kb, and the *Sac*I digestion detected only the viral 2.6-kb band.

DISCUSSION

In this paper, we have shown that unintegrated retroviral vector DNA can recombine with single-copy homologous sequences to stably correct a selectable gene. We also observed transient *neo* gene correction events associated with the loss of the entire target sequence. The target

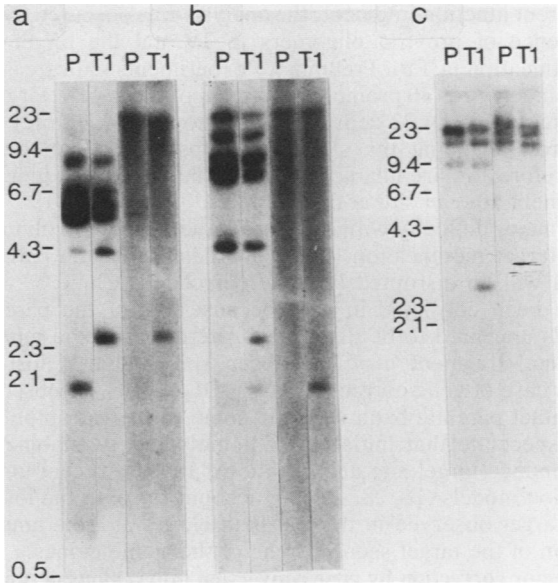

FIG. 6. Southern blot analysis of the target structure in the stably corrected clone T1. P, parental KY21b cell DNA. Genomic DNA was analyzed by the following methods: *Bam*HI digestion and hybridization to probe N1.4 (left lanes in panel a) and rehybridization with probe T3.3 (right lanes in panel a); *Bgl*II digestion and hybridization to probe N1.4 (left lanes in panel b) and probe T3.3 (right lanes in panel b); and hybridization to probe V5.7 after digestion with *Sac*I (left lanes in panel c) and *Xba*I (right lanes in panel c).

structure of the stably corrected clones suggests that neither the recombination event predicted in Fig. 1 nor simple gene conversions took place.

The corrected *neo* genes are flanked on the 5′ side by the 5′ LTR, *gag* sequences, and the *TK* promoter from the virus and on the 3′ side by the sequences downstream of the *neo* gene in the target. This structure and the failure to detect a *neoDLDR* gene indicate that unintegrated viral linear DNA participated in the gene correction events. This interpretation is reinforced by the observation that linear DNA was the earliest form detected after MoDR infection and was more abundant than the circular forms at later time points. Moreover, MLV linear DNA has been shown to integrate into DNA substrates in vitro (6, 10), and we have recently obtained evidence that unintegrated viral circular DNA molecules containing tandem LTRs are not precursors to normal MLV integration events in vivo (submitted for publication).

Target correction by in situ modification. In three independent stably corrected clones (T5, T9, and T10), the 5′ vector sequences were contiguous with the entire length of the 3′ target sequence up to the *Eco*RI site. A possible mechanism for the correction of these clones is that the target *neoDL* gene was modified in situ. A double-reciprocal recombination (shown schematically in Fig. 4) could replace sequences upstream of the target with the 5′ sequences of the virus. In this model the observed *neo* gene structures would be produced by a homologous recombination between the shared *neo* sequences and a nonhomologous recombination event farther upstream (perhaps mediated by the viral integrase acting on the wild-type terminus of the 5′ LTR).

A *Hin*dIII 5′ junction fragment common to T5, T9, and T10 would prove that the target gene was modified in situ in these clones. Although each of these clones possessed a

different junction fragment, the analysis was obscured by the presence of proviral oligomers in T9 and the target site amplification in T10. Preliminary experiments indicated that the simian virus 40 promoter upstream of the *neoDL* target is incomplete in DL22 cells (data not shown), which prevents us from mapping the site of the upstream *Hin*dIII site. Therefore, we are unable to predict the size of the junction fragment after in situ correction.

A major difficulty with a recombination model involving in situ target modification is that it predicts that the parental band will be disrupted by gene correction. Clone T1 may have been corrected in situ, because one of the parental bands appeared to be disrupted. Alternatively, the missing parental fragment may have been spontaneously lost. In each case of gene correction in the DL22 cells we observed an intact parental band which in some cases was amplified. We speculate that initiation of homologous recombination can induce target site amplifications. Indeed, recent amplification models (16, 28, 29) can account for both the loss of the target observed in the transient clones and the amplification of the target seen in some of the stable clones.

Vector correction by gene conversion into regions of nonhomology. In situ modification cannot adequately account for the structure of the corrected *neo* genes in the stable clones T2 and T22. In these clones, the 5′ vector sequences were contiguous with variable but incomplete lengths of the 3′ target sequences. These events may derive from a nonreciprocal transfer of *neo* gene sequences from the target to viral linear DNA molecules before integration of the now modified virus into some other site. We emphasize that these gene conversions were not simply a repair of the DR deletion in an otherwise unmodified provirus. Rather, the conversion tract appears to extend past the *neo* sequences and through at least a 1-kb region of nonhomology to transfer the *Bam*HI site from the target to the vector in clone T2. Likewise, the tract would need to be 1.5 kb in size to transfer both the *Bam*HI and *Bgl*II sites from the target to the vector in the clone T22.

Gene conversion through nonhomology has been proposed to account for unexpected recombination events in mammalian, yeast, and bacterial cells (4, 11, 30, 39). In our case, the tract would have to extend from a single region of homology into at least a 1.5-kb region of DNA nonhomology. Similar but larger tracts can fully explain the structure of the corrected genes in the clones in which the 5′ vector sequences were contiguous with the entire length of the 3′ target sequences (T5, T9, and T10). In these clones the conversion tract would have to extend through a 4-kb region of nonhomology to transfer the *Bam*HI, *Bgl*II, and *Eco*RI sites from the target to the vector prior to integration of the virus elsewhere. These clones would therefore have unique *Hin*dIII fragments and retain the original parental band as observed.

Gene replacement strategies. Our targeting retroviruses may correct defective *neo* genes by gene conversion into regions of nonhomology because there is only a 500-bp region of homology between the DL and DR mutations in which to initiate recombination and a less than 300-bp region of shared homology outside the deletions in which to resolve it. Events that successfully initiate recombination but fail to resolve within the region of homology may be aborted and generate transient G418^r clones or, alternatively, may generate stable G418^r clones by the mechanism described above. It may be possible, therefore, to increase the frequency of gene correction with retroviral vectors by increasing the lengths of homology for both initiation and resolution of recombination. If resolution can be directed to regions of homology in this way, then gene replacement would be the result.

A successful strategy for the replacement of cellular genes involves homologous recombination with substrates that include two regions of homology interrupted by a large region of nonhomology, such as a *neo* transcriptional unit (22, 37). In these instances, gene targeting after DNA-mediated gene transfer may utilize the same recombination pathway as our vectors do. If so, then gene replacement occurs by: (i) initiation in one region of homology, (ii) gene conversion through an internal stretch of nonhomology, and (iii) resolution within the second region of homology. The polarity that we have observed suggests that higher frequencies of gene replacement may be possible by gene transfer of retroviral vectors or plasmids containing only small regions of internal nonhomology, such as oligonucleotide linkers.

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