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Correction of a Deletion Mutant by Gene Targeting with an Adenovirus Vector

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The usefulness of adenovirus type 5 as a vector for homologous recombination was examined in CHO cells by using the adenine phosphoribosyltransferase (*aprt*) gene. Infection of a hemizygous CHO APRT$^-$ cell line containing a 3-bp deletion in exon 5 of the *aprt* gene with a recombinant adenovirus containing the wild-type gene resulted in restoration of the APRT$^+$ phenotype at a frequency of 10^{-5} to 10^{-6} per infected cell. A relatively high frequency (approximately 6 to 20%) of the transductants appears to result from a homologous recombination event. The mutation on the chromosomal *aprt* gene is corrected in the homologous recombinants, and APRT expression is restored to a normal hemizygous level. Neither adenovirus nor exogenous promoter sequences are detected in the homologous recombinants. The remaining transductants result from random integration of the *aprt* gene with the adenovirus sequence. A number of adenovirus vectors containing different promoter sequences linked to the hamster *aprt* gene were constructed. A possible role for the promoter region in the homologous recombination event was indicated by the lack of homologous recombination in constructs lacking an active promoter.

The use of viral vectors as gene transfer agents is an area of increasing interest. A number of successful viral vectors for use in human gene therapy have been recently developed. These include retrovirus and adenovirus vectors. Retrovirus vectors have been used both in vitro (9) and recently in vivo (11, 40) and are very efficient agents of gene delivery. However, one of the primary objections to the use of retrovirus in human gene therapy is the randomness of the integration event (14). This may result in insertional mutagenesis (20) or in aberrant regulation of the inserted gene. Adenovirus vectors were originally developed (6) and tested for use as recombinant vaccines, and a number of such vectors containing heterologous viral genes such as those encoding hepatitis B virus surface antigen (41), human immunodeficiency virus envelope proteins (30), and pseudorabies virus glycoproteins (12) have been constructed. More recently, it has been shown that replication-defective recombinant adenoviruses can be used as efficient expression vectors for gene therapy. Rosenfeld et al. (31, 32) delivered the cDNAs for the α_1-antitrypsin and cystic fibrosis transmembrane conductance regulator genes to cotton rats by insertion of these exogenous DNAs into the E1A region of adenovirus. Stratford-Perricaudet et al. (34) used the ornithine transcarbamylase cDNA in a similar fashion. In all of these experiments, excellent expression of the inserted gene was obtained.

We have previously reported the construction and efficient expression in mouse L cells of an adenovirus type 5 (Ad5) vector containing the hamster adenine phosphoribosyltransferase (*aprt*) gene (21). Stable transductants of the *aprt* gene occurred in which the *aprt* gene was integrated into the mouse chromosome in a random fashion at a frequency of 10^{-6}. In this paper, we report successful detection of targeted integration of the introduced gene, using this vector in CHO cells. These recombinant adenoviruses are replication competent and contain a wild-type CHO *aprt* gene. Infection of an APRT$^-$ CHO cell line with this vector results in correction of a 3-bp deletion mutation on the chromosomal *aprt* gene. The ratio of targeted recombination to random recombination is 1:5 to 1:14.

MATERIALS AND METHODS

Cell lines. CHO-ATS-49tg (from G. M. Adair, M. D. Anderson Cancer Center, University of Texas) is a spontaneous 6-thioguanine-resistant (HPRT$^-$) and 8-aza-adenine-resistant (APRT$^-$) CHO cell line (1a). It is hemizygotic at the *aprt* locus. Polymerase chain reaction (PCR) amplification and direct sequencing analysis of the remaining CHO-ATS-49tg *aprt* gene revealed a 3-bp deletion within exon 5 (1). CHO-ATS-49tg cells were cultured in alpha minimum essential medium with 10% calf serum or 10% fetal calf serum in the cloning experiments.

Construction of plasmids and recombinant viruses. Standard cloning techniques (3) were used to construct the plasmids. Plasmids pFG-PAR, pFG-PAL, pFG-NPAR, pFG-NPAL, pFG-NPARdE1, and pFG-NPALdE1 were constructed as shown in Fig. 1. Plasmid pHAPRT-2, derived from pHAPRT-1 (24), contains the *Bam*HI fragment of the CHO *aprt* gene. This plasmid was used for construction of Ad5 viruses containing the *aprt* gene with its native promoter (Ad5/PAR and Ad5/PAL; Fig. 2). Plasmid PUC-*aprt* has a *Bam*HI-*Xba*I fragment of the *aprt* gene. This plasmid was used for the construction of recombinant Ad5 viruses with a promoterless *aprt* gene (Ad5/NPAR and Ad5/NPAL; Fig. 2) and viruses carrying a deletion mutant of the *aprt* gene (Ad5/NPARdE1 and Ad5/NPALdE1). The specific fragments of the *aprt* gene were excised by digestion with the restriction enzymes indicated in Fig. 1. The *Tha*I site removes 388 bp from the 5′ end of the *Bam*HI fragment, including the promoter sequence, and is only 5 bp before the ATG start site. The *Eco*RV site deletes a 606-bp segment from the gene which includes the promoter, exon 1, and intron 1 and part of exon 2. These fragments were converted to an *Xba*I fragment by *Xba*I linker addition and cloned into the unique *Xba*I site of pFG-dx1 (19). Plasmid pFG-dx1

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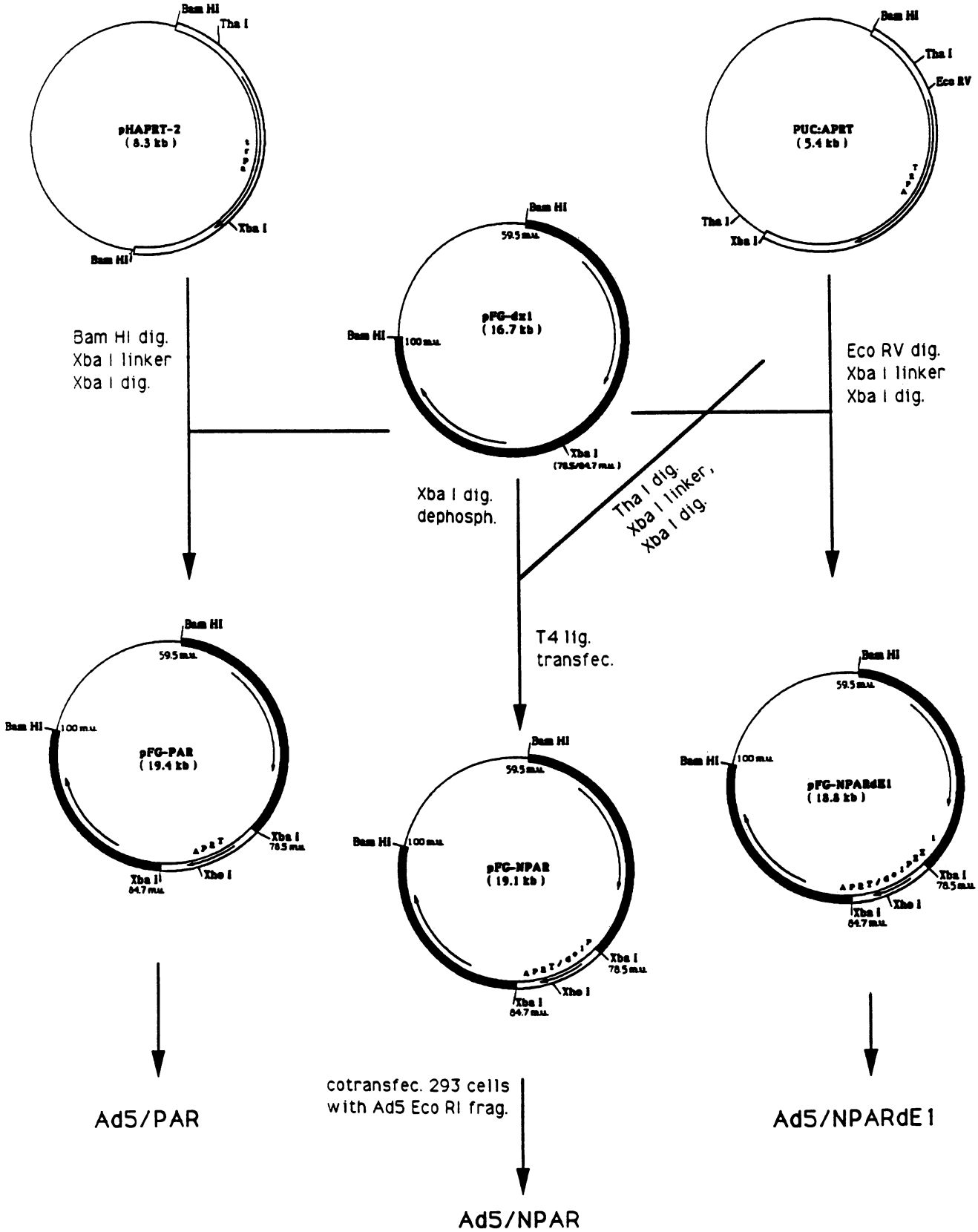

FIG. 1. Construction of Ad5/*aprt* recombinant viruses. The structures of recombinant viruses Ad5/PAR, Ad5/NPAR, and Ad5/NPARdE1 are shown as examples. Ad5/PAL, Ad5/NPAL, and Ad5/NPALdE1 were constructed in the same manner as were Ad5/PAR, Ad5/NPAR, and Ad5/NPARdE1 except that the resulting plasmids, pFG-PAL, pFG-NPAL, and pFG-NPALdE1, contain the *aprt* gene in the opposite orientation relative to pFG-PAR, pFG-NPAR, and pFG-NPARdE1. Recombinant viruses Ad5/PAL, Ad5/NPAL, and Ad5/NPALdE1 have the *aprt* gene in the opposite orientation to the viral E3 promoter. The construction of Ad5/A1 and Ad5/A2 was described previously (21).

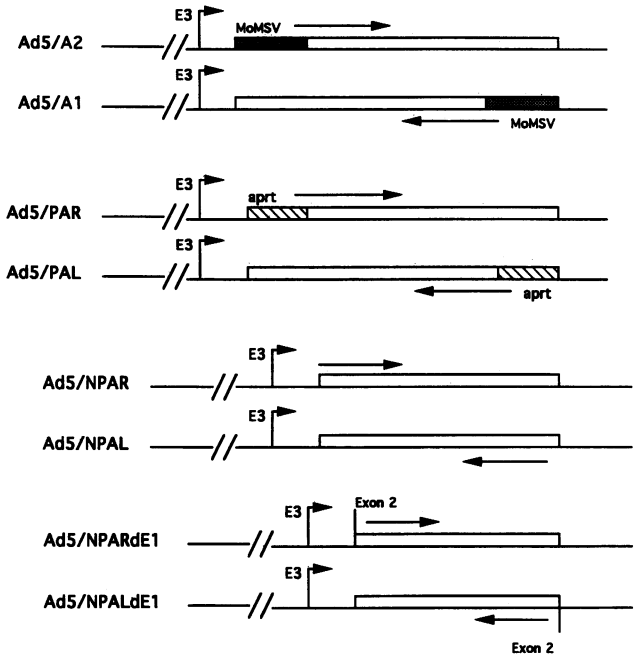

FIG. 2. Recombinant viruses structures. Adenovirus sequences are indicated by thin lines. Diagonal lines stand for segments of the adenoviral genome that are not drawn. The structural *aprt* gene in the adenovirus vectors is represented by an open box. The MoMSV promoter is shown by a cross-hatched box, and the native *aprt* promoter is shown by a shaded box. Arrows indicate the orientations of inserted *aprt* genes relative to that of the adenovirus E3 promoter.

contains 40% of the right part of the adenoviral genome with a deletion from 78.5 to 84.7 map units. This 1.9-kb deletion removes most of the E3 region but leaves the E3 promoter and termination site. 293 cells (16) were cotransfected with each plasmid and *Eco*RI-digested Ad5 DNA to rescue the recombinant viruses. The plaque-purified viruses were analyzed by restriction enzyme digestion. Viruses Ad5/A2 and Ad5/A1, which contain a CHO *aprt* gene with its endogenous promoter replaced by the Moloney murine sarcoma virus (MoMSV) promoter, were constructed as previously described (21).

Selection of transductants. CHO-ATS-49tg (ATS-49tg) cells were seeded at 5×10^6 cells per 100-mm^2 dish on the day prior to the experiment. The cells were infected with Ad5 or recombinant adenoviruses at a multiplicity of infection of 10 PFU. At 12 h postinfection, the cells were trypsinized, split into 5×10^5 cells per dish, and incubated in alpha minimum essential medium for an additional 12 h, at which time the medium was changed to AAA selective medium (25 μM alanosine, 50 μM azaserine, 100 μM adenine) (33). Cells were refed the next day and subsequently every 3 days for about 2 to 3 weeks with AAA medium. One APRT$^+$ clone per plate was picked by using cloning cylinders. The remainder of the APRT$^+$ colonies were counted after crystal violet staining.

APRT and HPRT activity assays. The APRT and HPRT assays were performed as described previously (4). Monolayers of cells were washed with phosphate-buffered saline and collected. The cell pellet was lyophilized and resuspended in 200 μl of dilution buffer (50 mM Tris-HCl [pH 7.5], 30 mM KCl, 10 mM $MgCl_2$). A 25-μl volume of diluted cell extract was mixed with an equal volume of reaction mixture (100 mM Tris-HCl [pH 7.5], 16 M $MgCl_2$, 1.5 mM phosphoribosylpyrophosphate (PRPP), 25 μM cold adenine, 50 μCi of [^{3}H]adenine per ml, 2 mg of bovine serum albumin per ml) and incubated at 37°C for 10 min. The reaction mixture for the HPRT assay was the same as the APRT reaction mixture except that unlabeled hypoxanthine and ^{3}H-labeled hypoxanthine were used instead of adenine (4). The reaction was terminated by addition of 1 ml of stop buffer (50 mM sodium acetate, 2 mM Na_2HPO_4). Synthesized [^{3}H]AMP and [^{3}H]IMP were precipitated by $LaCl_3$ and collected on Whatman GF/C filters. Radioactivity was detected by scintillation counting, and the protein concentrations of cell extracts were measured by using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, Calif.).

Southern hybridization. High-molecular-weight genomic DNA of the transductants was extracted according to Perbal (27). Genomic DNA (20 μg) was digested with the desired restriction enzyme. The samples were extracted with phenol-chloroform-isoamyl alcohol (CIA). Digested DNA was run on an 0.8 or 1% agarose gel and transferred to a Nylon membrane (Zeta Probe membrane; Bio-Rad). Prehybridization and hybridization to a ^{32}P-labeled probe were done as recommended by Bio-Rad.

PCR and sequencing. PCR was done by a modification of a method described previously (28). The upstream primer (5′-TAC CTG TGT CTT TCC TCG TCC CTT CA-3′) is the CHO *aprt* sequence from positions 2132 to 2158 (26), and the downstream primer (5′-CAG AGT ATG TTA GAT GCC CGT CCC AC-3′) extends from positions 3417 to 3391. The expected PCR product is around 1.3 kb in length. The reaction was carried out in a total volume of 100 μl containing 500 ng of genomic DNA, 1 U of AmpliTaq polymerase (Perkin-Elmer Cetus), 200 ng of each upstream and downstream primer, 50 μM each deoxynucleoside triphosphate, 5% acetamide, 0.05% Nonidet P-40, 1 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), and 50 mM KCl. Amplification was done in a DNA Thermal Cycler (Perkin-Elmer Cetus). The initial denaturation at 94°C for 4 min was followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and elongation at 72°C for 1 min. The final elongation was at 72°C for 7 min. The PCR products were further cloned into pBluescript for sequencing. Double-stranded plasmid was directly used for sequencing according to the protocol provided (United States Biochemical, Cleveland, Ohio).

RESULTS

Structures of the recombinant viruses. Figure 2 presents the structures of a series of recombinant viruses. The viruses initially used in these studies contain the MoMSV promoter juxtapositioned next to the structural *aprt* gene (38, 42). Ad5/A1 is a virus in which the MoMSV-*aprt* gene is in the opposite orientation to the adenovirus E3 promoter. Ad5/A2 has the MoMSV-*aprt* gene in the same orientation as the E3 promoter. Viruses Ad5/PAR and Ad5/PAL contain the CHO *aprt* promoter and structural gene in either the same (PAR) or opposite (PAL) orientation relative to the adenovirus E3 promoter. Viruses Ad5/NPAR and Ad5/NPAL contain a promoterless *aprt* gene, with the structural gene about 320 bp from the viral E3 promoter in NPAR. In the case of Ad5/NPAL, the *aprt* gene is in the opposite orientation to the E3 promoter and thus does not have any functioning promoter sequence. Viral E4 gene transcription terminates before reaching the cloned *aprt* gene (5). In viruses Ad5/NPARdE1 and Ad5/NPALdE1, the inserted gene fragment

TABLE 1. Frequencies of transduction and ratios of homologous to nonhomologous recombinants following infection with recombinant viruses[a]

Virus	No. of cells infected (10^7)	No. of APRT$^+$ colonies	Transduction frequency (10^{-6})	No. of colonies screened	No. of HR identified	HR/NHR ratio	APRT activity
Wild type	5	0	0	0	0	0	−
Ad5/A2	2	112	5.6	37	6	1:5	++
Ad5/A1	2	64	3.4	26	2	1:12	+
Ad5/PAR	2	194	9.7	14	0	ND	+++
Ad5/PAL	2	116	5.8	15	1	1:14	+
Ad5/NPAR	2	104	5.2	33	0	0	++++
Ad5/NPAL	2	0	0	0	0	0	−
Ad5/NPARdE1	5	0	0	0	0	0	−
Ad5/NPALdE1	5	0	0	0	0	0	−

[a] Infections were done at a multiplicity of infection of 10. Data were obtained from two independent experiments per viral infection except that the Ad5/NPARdE1 and Ad5/NPALdE1 infections were done five times. HR and NHR, homologous recombinants and nonhomologous recombinants; ND, not detected.

has a 5′-end deletion which includes the promoter sequence up to several base pairs of exon 2. All virus constructs were assayed for APRT activity in mouse LAT cells and APRT$^-$ CHO cells. There was no detectable APRT activity when either cell line was infected with viruses Ad5/NPAL, Ad5/NPARdE1, and Ad5/NPALdE1.

Isolation of APRT$^+$ transductants. ATS-49tg cells were infected with the viruses at a multiplicity of infection of 10. Cells were split to low density 12 h postinfection and maintained in normal medium for an additional 12 h before the addition of selective AAA medium. APRT$^-$ cells die in AAA medium within 3 days as a result of the blockage of de novo purine biosynthesis by azaserine and an inability to utilize free adenine. Uninfected cells and cells infected with wild-type virus were used as controls. Cells infected with Ad5/NPAR, Ad5/PAR, Ad5/PAL, Ad5/A1, and Ad5/A2 survived in AAA medium for about 1 week as a result of transient APRT production from the viral genome. APRT$^+$ colonies were seen after background cells died. APRT$^+$ cells were not found in uninfected cells or cells infected with wild-type virus. They were not detected following infection of 5×10^7 cells with Ad5/NPARdE1, Ad5/NPALdE1, or Ad5/NPAL. Thus, the frequency of APRT$^+$ colonies with these vectors is $<5 \times 10^{-7}$ per cell. The frequencies of APRT$^+$ colonies from Ad5/A1, Ad5/A2, Ad5/PAR, Ad5/PAL, and Ad5/NPAR infections are shown in Table 1.

Gene targeting following Ad5/A1 and Ad5/A2 infection. To determine whether the APRT$^+$ clones arose as the result of homologous recombination or random integration, DNA from a total of 63 APRT$^+$ clones from the Ad5/A1 and Ad5/A2 transductants was examined by Southern analysis. Figure 3A illustrates the restriction map of the mutant *aprt* gene in ATS-49tg cells and the wild-type CHO *aprt* gene introduced by the adenovirus vectors. The APRT$^-$ phenotype results from a 3-bp deletion in the hemizygous *aprt* gene, resulting in a loss of an *Mbo*II restriction site in exon 5 (1a). Figure 3B shows typical *Mbo*II restriction patterns of DNA from ATS-49tg and wild-type CHO cells probed with the full-length CHO *aprt* sequence. A 2.0-kb fragment is found in ATS-49tg DNA instead of the 0.5- and 1.5-kb fragments found in wild-type CHO DNA. As shown in Fig. 4, following *Mbo*II digestion of the transductant DNA from the first 17 clones isolated (10 from Ad5/A2-infected clones and 7 from Ad5/A1-infected clones), two clones, A1-1 and A2-5, show a restriction pattern identical to that of wild-type CHO cells. That the pattern in clones A1-1 and A2-5 was the same as the wild-type pattern was confirmed by *Xba*I, *Kpn*I, *Hin*dIII, and *Eco*RI digestions (Fig. 4B and C; *Hin*dIII and *Eco*RI digestions are not shown). The other 15 clones appear to result from random integration of the *aprt* gene. Following *Xba*I digestion, an extra 2.5-kb fragment can be seen in 12 clones (Fig. 4B). It represents an integrated copy of the gene (a 2.5-kb *Xba*I fragment of MoMSV-*aprt*). Three other clones show a fragment larger than 2.5 kb, indicating that part of the long terminal repeat (LTR)-*aprt* sequence, including at least one of the *Xba*I sites, was deleted during the integration process. Clone A2-8 has two extra *Xba*I fragments, which suggests multiple copies of gene integration. A total of 8 clones were isolated from 63 screened clones that possess the same restriction pattern as do A1-1 and A2-5 (Fig. 5). Six of 37 clones isolated following Ad5/A2 infection and 2 of 26 isolated following Ad5/A1 infection appeared to result from homologous replacement of the endogenous deletion. The other 55 clones showed the presence of both 2.0- and 1.5-kb bands and additional variable flanking fragments. This result indicates that in these cells, transduction occurred via random integration of the introduced gene, with retention of the endogenous mutant *aprt* gene.

HPRT deficiency in the homologous recombinants. Since ATS-49tg cells are also defective in the *hprt* gene, the clones presumed to be due to homologous recombination were examined for HPRT deficiency in order to verify that they did not arise from contamination of the culture with wild-type CHO cells. All eight presumptive homologous recombinants died in hypoxanthine-aminopterin-thymidine medium, and HPRT enzyme activity could not be detected in extracts of these cells (Table 2). Spontaneous revertants have never been detected with ATS-49tg (1a). In agreement, we never detected revertants in uninfected cells or cells infected with wild-type virus, nor were APRT$^+$ colonies found following infection with recombinant viruses carrying a deletion mutant of the *aprt* gene (Ad5/NPARdE1 and Ad5/NPALdE1) or lacking a promoter in the opposite orientation to E3 (Ad5/NPAL) (Table 1).

APRT activity in the transductants. The specific activity of APRT of the transduced clones was measured (Table 2). In the clones resulting from homologous recombination, the level of APRT activity was expressed at a hemizygous level about 50% of the wild-type CHO level. In contrast, the random integrants exhibited APRT specific activity varying from 9 to 160% of the wild-type level.

Detection of vector sequences in homologous recombinants. To further confirm that the homologous recombinants arose from a double-crossover event or gene conversion within the

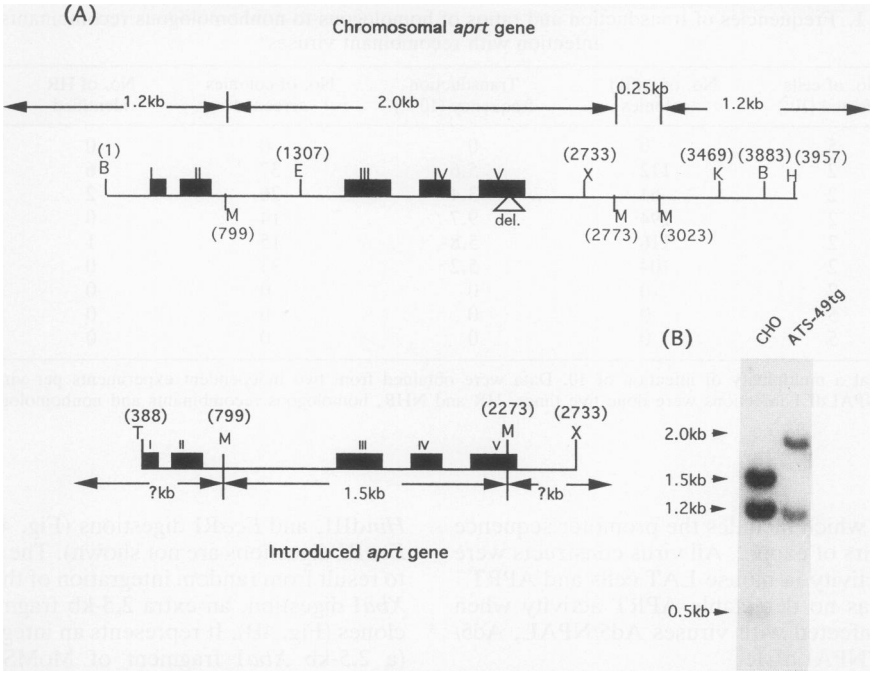

FIG. 3. (A) Restriction map of the CHO *aprt* gene. The solid boxes represent exons of the CHO *aprt* gene. In ATS-49tg cells, there is a 3-bp deletion in exon 5 which results in loss of an *Mbo*II restriction site. The CHO *aprt* gene in virus Ad5/A1 or Ad5/A2 is linked to the MoMSV promoter and is 1.2 kb shorter at its 3′ end than is the chromosomal *aprt* gene. The depicted fragments hybridized to a full-length 3.9-kb *aprt* probe after *Mbo*II digestion are indicated above the chromosomal *aprt* gene and under the introduced *aprt* gene. Restriction enzyme sites are abbreviated as follows: T, *Tha*I; M, *Mbo*II; X, *Xba*I; B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; H, *Hin*dIII. The base numbering is according to Nalbantoglu et al. (26). (B) *Mbo*II restriction patterns of wild-type and mutant CHO *aprt* genes. DNAs were run on a 1% agarose gel, transferred to a nylon membrane, and hybridized to a full-length CHO *aprt* probe. Lane CHO shows normal 1.5- and 0.5-kb fragments. The deletion in exon 5 of the *aprt* gene in ATS-49tg cells generates a 2.0-kb fragment. The 1.2-kb fragment which represents the overlapping flanking sequences of the *aprt* gene is present in both wild-type and mutant cells.

aprt gene, the Southern blots of *Xba*I digests were rehybridized with MoMSV and adenovirus sequences which could detect almost all of the *Xba*I fragments of the adenovirus genome (Fig. 6). Neither adenoviral or MoMSV sequences can be detected in the presumptive homologous recombinants (Fig. 7). In contrast, the other clonal transductants have both adenovirus and MoMSV sequences (Fig. 7).

Sequencing of PCR products of homologous and nonhomologous recombinant clones. As shown in Fig. 8, the 3-bp deletion in the exon 5 of the *aprt* gene is at the site of a direct TTC repeat (1) and within the *Mbo*II recognition site. From the sequence analysis, the restoration of the *Mbo*II site can also be obtained by a single T insertion. However, the frameshift mutation of the *aprt* gene resulting from this single-base insertion should produce a nonfunctional APRT (10). Since the homologous recombinants are $APRT^+$ and have APRT activity at hemizygous levels, it is unlikely that the restoration of the *Mbo*II site in those recombinants results from the single T insertion. To further exclude this possibility, PCR amplification of the *aprt* gene of the homologous recombinants and random integrants was carried out by using two primers. The downstream primer hybridizes only to the sequence which is present in the chromosomal *aprt* gene but not in the introduced *aprt* gene, so that with use of this pair of primers, the randomly integrated *aprt* gene cannot be amplified. In Fig. 9, A1-1 (an example of homologous recombinants) displays a complete wild-type sequence and A2-8 (an example of random integrants) has the same sequence as do ATS-49tg cells.

Role of the promoter region in targeted recombination. Since the constructs originally used for the targeting experiment contain the MoMSV promoter, we investigated whether this promoter played a role in the integration process. A number of viral constructs containing the *aprt* gene either with its native promoter or lacking a promoter (Fig. 2) were used to infect ATS-49tg cells. The results of these experiments are presented in Table 1. Approximately the same overall frequency of transduction to $APRT^+$ occurred for all vectors tested except for Ad5/NPARdE1, Ad5/NPALdE1, and Ad5/NPAL, the vectors which had been shown to lack transient APRT activity (Table 1). These three viruses did not give rise to any $APRT^+$ colonies. Twenty-nine $APRT^+$ colonies were isolated and screened after Ad5/PAR and Ad5/PAL infection. One of fifteen colonies from Ad5/PAL was confirmed as a homologous recombinant. Fourteen individual $APRT^+$ colonies were also picked following Ad5/PAR infection. Homologous recombinants were not detected among this population. However screening of more recombinants would probably have led to the isolation of homologous recombinants, since orientation of the insert (Table 1) does not appear to be important for homologous recombination.

DISCUSSION

We have demonstrated that a recombinant adenovirus vector can be used as an in vitro gene-targeting vector. A functionally and structurally normal *aprt* gene was regenerated in these homologous recombinants, as revealed by

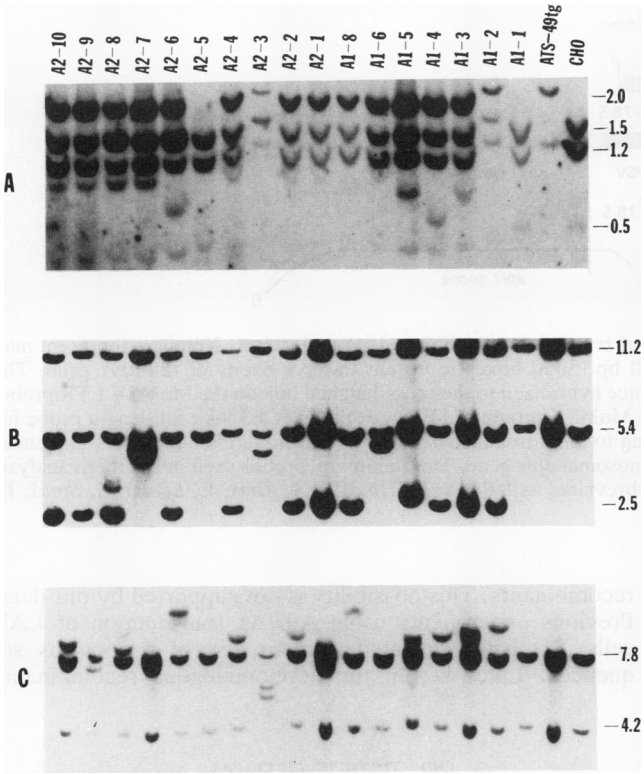

FIG. 4. Southern blot of APRT$^+$ colonies following Ad5/A1 and Ad5/A2 infection. DNAs from APRT$^+$ cultures derived from individual colonies were digested with *Mbo*II (A), *Xba*I (B), and *Kpn*I (C). Digested samples were run on a 0.8 to 1% agarose gel, transferred to a nylon (Zeta Probe) membrane, and then hybridized to the 3.9-kb *Bam*HI fragment of the CHO *aprt* fragment. Clones A1-1 and A2-5 show wild-type restriction patterns.

Southern analysis, by direct DNA sequencing, and by the levels of APRT. The ratio of gene-targeted events (homologous recombination to random integration) is higher by a factor of 400 than that reported with use of an introduced *aprt* gene on a plasmid in the same cell line (1a), although the absolute frequency of homologous recombination does not show improvement over the plasmid transfection system.

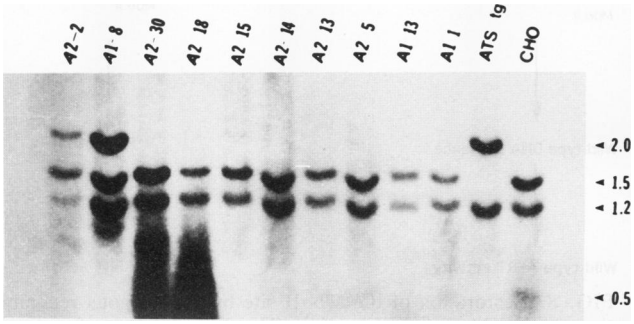

FIG. 5. Southern blot of eight clones of homologous recombinants identified following Ad5/A1 and Ad5/A2 infection. DNAs isolated from eight identified homologous recombinants were digested with *Mbo*II and run on a 1% agarose gel. After transfer, the DNAs were hybridized to a CHO *aprt* probe. DNAs extracted from CHO cells, ATS-49tg cells, and another two random integrants were used as controls.

TABLE 2. Specific APRT and HPRT activities of transductants[a]

Cells	HR	% of SAA	% of SHA
CHO	−	100	100
ATS-49tg	−	0	0
A1-1	+	50	0
A1-13	+	43	0
A2-5	+	36	0
A2-13	+	41	0
A2-14	+	45	0
A2-15	+	55	0
A2-18	+	45	0
A2-30	+	52	0
A1-2	−	84	NA
A1-3	−	160	NA
A1-4	−	15	NA
A1-5	−	38	NA
A1-6	−	9	NA
A1-8	−	33	NA
A2-1	−	27	NA
A2-2	−	95	NA
A2-3	−	38	NA
A2-4	−	15	NA
A2-6	−	33	NA
A2-7	−	70	NA
A2-8	−	52	NA
A2-9	−	79	NA
A2-10	−	134	NA

[a] Data are averages of two experiments of duplicated samples. HR, homologous recombinants; SAA, specific APRT enzyme activity; SHA, specific HPRT enzyme activity; NA, not assayed. Specific activities were determined as nanomoles per minute per milligram.

For the possible application of gene targeting in human gene therapy, random integration is often undesirable because of the danger of inactivation of an essential gene or activation of an oncogene. The adenovirus/*aprt* vector reduces the frequency of random integration but appears to retain the same or a higher frequency of homologous recombination by increasing the ratio of homologous recombination to random integration by about 2 to 3 orders of magnitude.

The adenovirus vector and the MoMSV sequences are not detectable in the homologous recombinants, suggesting that the homologous recombination event is a double crossover or gene conversion. In the random integrants, the various levels of APRT activity might reflect the impact of the site of integration or the integration of multiple copies of the *aprt* gene.

The factors that influence homologous recombination or random integration are basically unknown in mammalian systems. It has been shown that homologous recombination between the adenoviruses does not require E1A gene expression (13). It is also known that the E1A gene is required for adenovirus transformation of rodent cells (17, 18). However, a reduced level of E1A gene expression results in an increase in transformation efficiency (2). It is possible that expression of the E1A gene in Ad5/*aprt* recombinant viruses keeps the frequency of random integration relatively low (similar to the frequency of adenovirus transformation in rodent cells) (15) and therefore enriches for homologous recombination when the adenovirus sequence contains a cellular gene.

One other possibility to be considered is that some of the Ad5 gene may be lethal to the cell and act in negative selection. This would result in positive selection for APRT$^+$ cells and negative selection for adenovirus, thus enhancing the frequency of homologous recombinants to heterologous

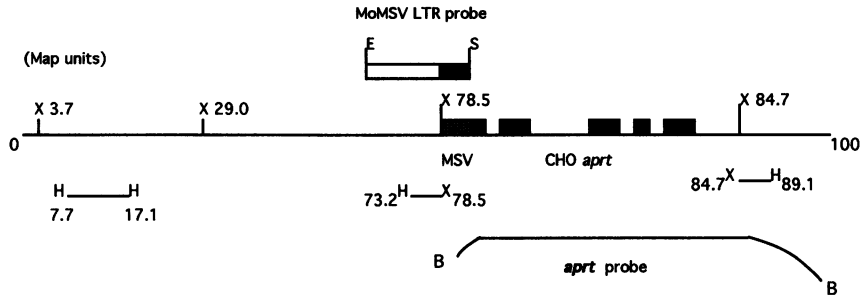

FIG. 6. The *aprt* probe and viral probes. The solid line shows the *Xba*I restriction map of the adenoviral vector. Numbers represent map units. One map unit of adenoviral sequence equals approximately 360 bp. Solid boxes represent the five exons of the *aprt* gene. The cross-hatched box on the vector represents the MoMSV promoter sequence hybridized to the cross-hatched box on the MoMSV LTR probe. The open box in the LTR probe indicates the upstream sequence of the MoMSV promoter. The *aprt* probe is a 3.9-kb full-length probe for the CHO *aprt* gene. The straight line represents the sequence hybridizing to the introduced *aprt* gene. The curved line indicates sequences which do not hybridize to the introduced gene but hybridize to the chromosomal *aprt* gene. The adenoviral probes used in Southern analysis are indicated by the bars under the vector. Restriction enzymes are abbreviated as follows: H, *Hin*dIII; X, *Xba*I; E, *Eco*RI; S, *Sma*I; B, *Bam*HI.

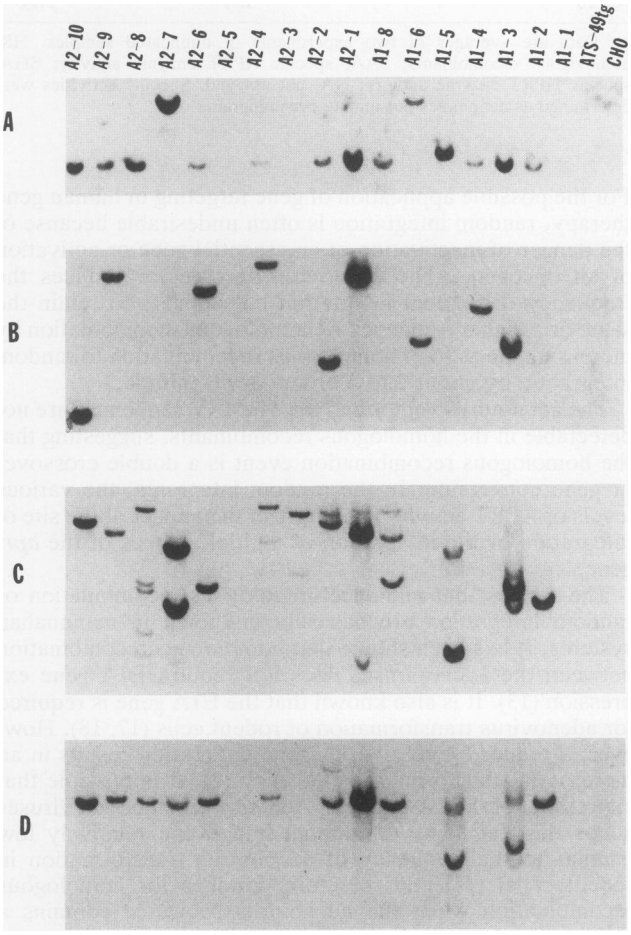

FIG. 7. Detection of MoMSV and adenoviral sequences by Southern hybridization. The Southern blot of *Xba*I digestion from Fig. 5 was rehybridized with an *Eco*RI-*Sma*I fragment of the MoMSV LTR (A), adenoviral sequence from 73.2 to 78.5 map units (B), adenoviral sequence from 84.7 to 89.1 map units (C), and adenoviral sequence from 7.7 to 17.1 map units (D).

recombinants. This possibility is not supported by our data. Previous experiments using Ad5/A1 transduction of LAT cells (21) have indicated random loss of adenovirus sequences. Likewise, in the nonhomologous recombinants

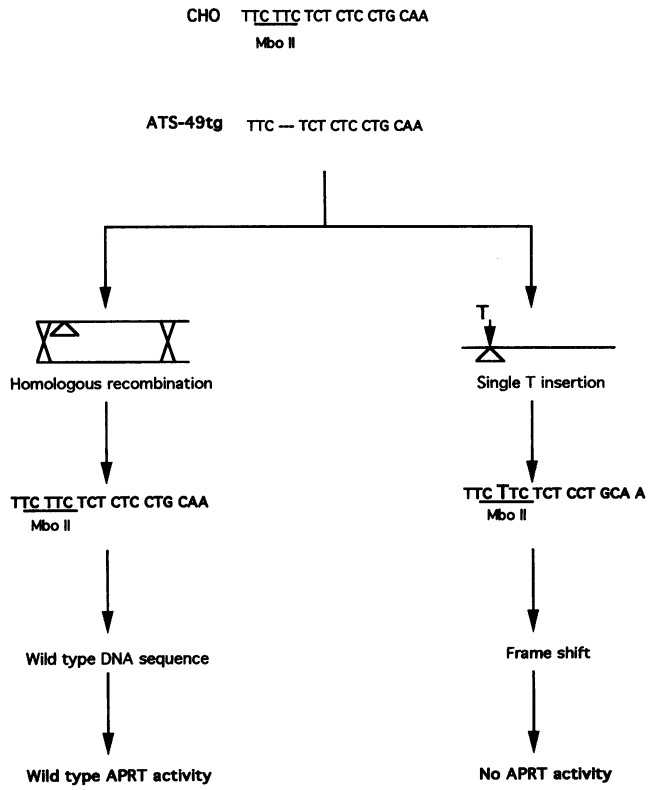

FIG. 8. Restoration of the *Mbo*II site by homologous recombination or single-base-pair insertion. The 3-bp deletion of a direct TTC repeat in exon 5 of the *aprt* gene in ATS-49tg cells destroys the *Mbo*II recognition site (TCTTC). Restoration of the *Mbo*II site can result from two possibilities. The first is homologous recombination between the mutant chromosomal *aprt* gene and the introduced *aprt* gene. The second possibility is a T insertion which results in the restoration of the *Mbo*II site, a frameshift mutation, and a functionally defective *aprt* gene.

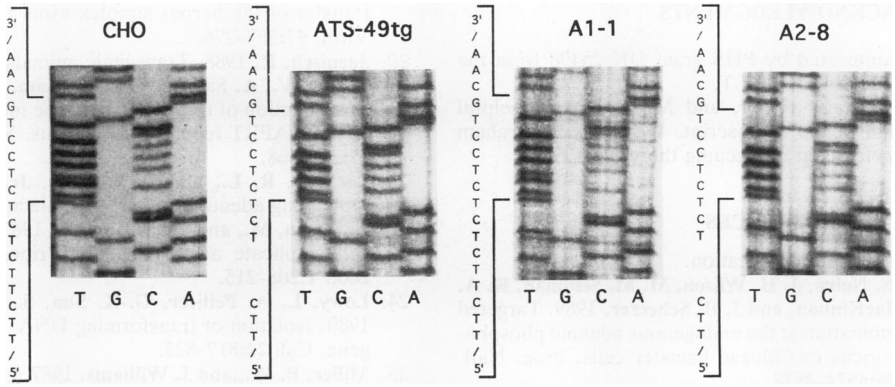

FIG. 9. Sequence analysis of homologous recombinants and random integrants. The 1.3-kb PCR products of wild-type CHO, ATS-49tg, A1-1 (homologous recombinant), and A2-8 (random integrant) cells were further cloned into pBluescript. Double-stranded DNA sequencing was done as described in Materials and Methods.

examined in the current experiments, there was no obvious loss of any one specific sequence (Fig. 7), but rather there appeared to be random loss of the viral DNA. Moreover, since the cells used in these experiments were not permissive for virus production, only early adenovirus gene functions are expressed. Thus, proteins normally associated with cytopathic effect (coat proteins or adenovirus fiber proteins) are not synthesized under these conditions.

We also propose that adenoviral replication might play a role in the gene targeting observed in the adenovirus/*aprt* system. The recipient CHO cells are semipermissive for adenovirus replication, leading to a blockage in a late adenovirus function (23). However, the viral DNA replicates as efficiently as in a permissive HeLa cell, resulting in the production of thousands of copies of the introduced gene. The large numbers of DNA molecules may facilitate the finding of homologous sequences in the cellular genome. However, this hypothesis is not in agreement with the work of Zheng and Wilson (43). Their experimental evidence, based on nonreplicative plasmid transfection, has shown that homologous recombination is independent of the copy number of the introduced DNA or targeted gene. However, the instability of transfected plasmid should be taken into account in this suggestion. The replication of adenovirus in CHO cells is delayed and prolonged after infection (23). The viral genome is protected by the viral core proteins so that it is not readily accessible to DNase. These features might influence the time course of homologous recombination events and create more opportunities for the introduced DNA molecules to find their counterpart chromosomal genes.

One possibility that should be considered in considering the mechanism of homologous recombination in the adenovirus/*aprt* system is the involvement of the single-stranded DNA substrate. During adenovirus replication, single-stranded DNA is generated by the displacement of parental strands during DNA replication (22, 36, 37). Studies with *Escherichia coli* and mammalian cells using plasmid vectors indicate that single-stranded DNA is an intermediate in homologous recombination (8, 29).

Our initial strategy for studying the absolute frequency of homologous recombination in the adenovirus vector system was to use Ad5/NPARdE1 and Ad5/NPALdE1 for the targeting experiments. These viruses carry a mutant *aprt* gene which is nonfunctional if integrated in a random fashion. $APRT^+$ colonies formed following infection by either of these virus constructs would arise only as a result of homologous recombination. Infection with the promoterless Ad5/NPAR and Ad5/NPAL viruses should permit direct analysis of the ratio of homologous recombination to nonhomologous recombination. Both homologous and nonhomologous recombinants could arise following infection with Ad5/NPAR, and only homologous recombinants could arise following Ad5/NPAL infection. Thus, the number of $APRT^+$ colonies derived from Ad5/NPAL infection versus the number of $APRT^+$ colonies derived from Ad5/NPAR infection in selective medium is the ratio of nonhomologous to homologous recombination.

The failure to detect homologous recombination after infection with viruses having either the 5′-end deletion of the *aprt* gene (Ad5/NPARdE1 and Ad5/NPALdE1) or the promoterless *aprt* genes (Ad5/NPAR and Ad5/NPAL) implies a role for promoter sequences in initiating or enhancing the homologous recombination. The MoMSV LTR sequence might contain specific sequences which are recognized by cellular recombinase-like activities (25) or might serve as a transcriptionally active region which enhances homologous recombination. The latter has been suggested for both mammalian and yeast systems (7, 35, 39) except that the transcriptionally active region is on the recipient homologous DNA sequences. The ratios of homologous to nonhomologous recombination obtained in Ad5/A1 and Ad5/PAL infections are at similar levels, indicating that promoter sequence specificity is unlikely since the MoMSV promoter and the *aprt* promoter do not share sequence similarity.

The proximity of a promoter sequence to the targeting gene on the vector may also play a role in homologous recombination. Following infection of Ad5/NPAR, in which the adenoviral E3 promoter is located about 320 bp upstream of the *aprt* gene, no homologous recombinants are found, although the level of *aprt* gene transcription initiated from the adenovirus promoter is high (data not shown). The 320 bp of heterologous sequence (adenovirus sequence) residing between the promoter and the structural gene might interfere with the homologous recombination process initiated from an active region.

We show in vitro that an adenovirus vector may have potential in human gene therapy and that it offers one feature that is not available in the retrovirus vector system, namely, targeted integration by homologous recombination. It may also be an excellent model for studying the mechanism of homologous recombination in mammalian cells.

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