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Human gene targeting by viral vectors

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Abstract

Stable transduction of mammalian cells typically involves random integration of viral vectors by non-homologous recombination. Here we report that vectors based on adeno-associated virus (AAV) can efficiently modify homologous human chromosomal target sequences. Both integrated neomycin phosphotransferase genes and the hypoxanthine phosphoribosyltransferase gene were targeted by AAV vectors. Site-specific genetic modifications could be introduced into approximately 1% of cells, with the highest targeting rates occurring in normal human fibroblasts. These results suggest that AAV vectors could be used to introduce specific genetic changes into the genomic DNA of a wide variety of mammalian cells, including therapeutic gene targeting applications.

The original discovery of transduction in *Salmonella*[1] was ultimately shown to involve recombination of DNA sequences carried by a viral particle with homologous sequences present on the recipient cell's chromosome. In contrast, eukaryotic transducing vectors capable of recombining with host DNA have usually been found to integrate at non-homologous chromosomal locations. While these eukaryotic viral vectors have been effectively used to introduce additional genetic elements at random chromosomal sites, there are many situations where it would be preferable to modify specific chromosomal sequences, thereby eliminating unwanted genotypes and avoiding position effects on gene expression. This is especially true in gene therapy, where mutated genes can have dominant effects and tissue-specific transcriptional controls are often critical.

It is currently possible to introduce defined mutations into mammalian chromosomes by gene targeting using transfection (electroporation or calcium-phosphate precipitation) or microinjection methods[2,3]. Transfection techniques usually produce homologous recombination events in only a small fraction of the total cell population (in the order of 10^{-6} in the case of mouse embryonic stem cells[4,5]). The routine use of these methods requires preselection of transfected cells, making it difficult to apply the technique to normal cells and *in vivo* experiments. Viral transducing vectors have the potential to overcome these limitations, as DNA can be efficiently delivered to large numbers of normal cells. Chromosomal gene-targeting experiments have been performed with retroviral and adenoviral vectors, but the recombination rates were not significantly higher than those obtained by transfection[6–8].

Adeno-associated virus 2 (AAV) is a single-stranded DNA virus of 4.7 kb, which was developed as a transducing vector capable of integrating into mammalian chromosomes[9]. AAV vectors with deletions in the viral *rep* gene have been found to integrate at random chromosomal sites[10–13] by non-homologous recombination[14] These experiments have

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typically used selectable markers with limited homology to chromosomal sequences, and the potential of AAV vectors for gene targeting has not been explored. Here we report our results using AAV vectors to introduce specific genetic modifications into human chromosomes. We find that AAV vectors can target homologous chromosomal genes at high frequencies and introduce defined modifications without additional mutations.

Correction of mutant *neo* genes by AAV vectors

We used the selectable neomycin phosphotransferase gene (*neo*) as a marker to study gene targeting by transduction. The vectors constructed for these experiments were based on the AAV shuttle vector AAV-SNori (Fig. 1*a*), which contains the *neo* gene under the control of both the SV40 and bacterial Tn5 promoters, and the p15A bacterial plasmid replication origin. Mammalian cells transduced by AAV-SNori are resistant to G418, and the integrated proviruses can be recovered as bacterial plasmids conveying kanamycin resistance[14]. Mutations were introduced into the AAV-SNori vector at nucleotide 39 (a 14-bp insertion) and nucleotide 648 (a 3-bp insertion) of the *neo* gene (nucleotide 1 being the translation start), to generate the vectors AAV-SNO39 and AAV-SNO648. Both mutations disrupt *neo* function, but homologous recombination between the two mutated genes can regenerate a functional gene.

HeLa cell lines containing integrated copies of the internal portion of the AAV-SNO39 genome (which lacks the terminal repeats) were created by cotransfection with a hygromycin-resistance gene. One hygromycin-resistant clone, designated HSNO39, contained three intact copies per cell of the *neo* cassette integrated at different locations (Fig. 1*b*, and data not shown) and was chosen for further experiments. HSNO39 cells were infected with the AAV-SNO648 vector, plated at different dilutions and then cultured in the presence or absence of G418. Correction of the mutated chromosomal *neo* genes was measured as the fraction of colonies resistant to G418. Approximately 0.1% of HSNO39 cells were resistant to G418 after infection with AAV-SNO648 (Table 1), and reversion of the chromosomal (nt-39) and vector (nt-648) mutations did not occur at detectable rates. The *neo* gene correction rate was approximately tenfold lower than the random vector integration rate, based on transduction experiments with the AAV-SNori vector, which contains a functional *neo* gene and integrates at random chromosomal locations by non-homologous recombination[14].

Several G418-resistant colonies obtained by transducing HSNO39 cells with AAV-SNO648 were expanded and analysed by Southern blots. After digestion with *Bam*HI, genomic DNA from the parental HSNO39 cells yielded three major bands (of 2.7, 5.0 and greater than 20 kb) that hybridized to *neo*, and a faint 8.0-kb band present at less than one copy per cell that was observed in a subset of cells within the population (Fig. 1*b*). Three transduced clones (3, 4 and 7) were missing bands present in the parental line, which can be explained by rearrangement of a *neo* cassette or targeted modification of a *Bam*HI site, as homology between the vector and chromosomal sequences ends at this site. New bands were observed in four of the G418-resistant clones (5, 6, 8 and 11), suggesting that random vector integration had occurred in addition to gene targeting in these cells. As random integration occurred on average in approximately 1% of the total cell population (Table 1), it must have happened at higher frequencies in the subset of cells undergoing gene targeting.

Double digests with *Bam*HI and *Sal*I (Fig. 1*c*) were used to identify the targeted *neo* genes. Gene correction should eliminate the *Sal*I site present at the chromosomal nt-39 insertion mutation; any *Bam*HI fragments not digested by *Sal*I must therefore represent corrected chromosomal targets. In six HSNO39/AAV-SNO648 clones, the corrected *neo* gene could be identified as the 2.7-kb *Bam*HI fragment present in the parental line (clones 1, 2, 5, 6, 8

and 9). Correction of *neo* genes in the larger *Bam*HI fragments presumably occurred in the other clones, but this could not be determined by Southern analysis. Taken together, these data demonstrate that at least six out of eleven G418-resistant clones isolated contained a corrected *neo* gene that had not undergone additional rearrangements.

Corrected *neo* genes were circularized and recovered as bacterial plasmids from seven of eleven HSNO39 clones transduced by AAV-SNO648, including all six cases with corrected *neo* fragments identified by Southern analysis. A 2.7-kb plasmid was recovered from each of the seven clones which, as determined by restriction digestion, appeared to be a circularized version of the corrected *Bam*HI fragment seen on Southern blots. A second 20-kb plasmid was also recovered from clone 11. We sequenced more than 200 base pairs of the regions surrounding the nt-39 and nt-648 mutations of each recovered plasmid, and in all cases the sequence corresponded exactly to that of the wild-type *neo* gene. Thus, the gene correction process led to an accurate deletion of the 14-bp insertion present at the chromosomal nt-39 mutation, without additional genetic changes and without insertion of the nt-648 mutation present in the vector.

Modification of the human *HPRT* gene by AAV vectors

We also studied gene targeting by AAV vectors at the human hypoxanthine phosphoribosyltransferase locus (*HPRT*). HPRT-deficient cells can be selected for because they grow in the presence of 6-thioguanine (6TG), so mutagenesis at the single-copy X-linked locus can be measured in diploid male cells. AAV vectors containing a region of the human *HPRT* locus encompassing exons 2 and 3 were used to introduce a specific mutation into the *HPRT* gene of HT-1080 human fibrosarcoma cells, which have a pseudodiploid male karyotype[15]. The AAV-HPe2/3 vector contains wild-type genomic sequence, while the AAV-HPe2/3X vector contains a 4-bp insertion in exon 3 that causes a frameshift in the *HPRT* coding sequence (Fig. 2*a*). At a multiplicity of infection (MOI) of 50,000 AAV-HPe2/3X vector particles per cell, 0.02–0.05% of HT-1080 cells became 6TG-resistant (Table 2).

Southern analysis of several 6TG-resistant HT-1080 clones isolated after infection with AAV-HPe2/3X confirmed that the vector mutation had been introduced into the chromosomal *HPRT* locus. *Hin*dIII cuts outside of vector sequences and produces a 6.8-kb chromosomal fragment containing exons 2 and 3 in HT-1080 cells. This band was unaltered in all of the clones analysed, demonstrating the absence of major rearrangements in this region (Fig. 2*b*). Five lines contained additional bands, presumably due to random vector integration. Further digestion with *Pvu*I revealed that ten of thirteen clones contained the 2.2-kb band expected from transfer of the vector mutation to the chromosome (Fig. 2*c*). In some lanes, the *Pvu*I site was not completely digested, as confirmed by the lack of wild-type sequence identified during DNA sequencing (see below). We found that this site is resistant to restriction enzyme digestion (data not shown); consequently, there may have been targeted modifications in other clones as well. To date we have analysed 24 independent 6TG-resistant HT-1080 clones transduced by AAV-HPe2/3X, 18 of which had the expected *Pvu*I-site insertion in exon 3 as determined by Southern analysis, and 6 out of 24 contained additional vector insertions.

The *HPRT* genes of three different normal male human fibroblast cultures (MHF1–3) were also efficiently targeted by AAV vectors (Table 2). At an MOI of 15,000 AAV-HPe2/3X vector particles per cell, 0.06–0.42% of the cultured cells became resistant to 6TG. Southern analysis of four 6TG-resistant transduced clones showed that three contained the predicted insertion mutation based on digestion with *Bsp*CI (an isoschizomer of *Pvu*I), and one clone contained an additional vector insertion (Fig. 2*d*). These results with normal human cells

were similar to those obtained with HT-1080 cells, except that targeting occurred at higher frequencies.

We used PCR to amplify and sequence the targeted region of the *HPRT* gene from the genomic DNA of 6TG-resistant HT1080 and normal human fibroblast clones transduced with AAV-HPe2/3X. At least 300 bp of unambiguous sequence was obtained from each clone, including all of exon 3. In each of six HT-1080 clones analysed, the entire sequence was identical to the published *HPRT* sequence[16], except for the predicted 4-bp insertion in exon 3. Eleven of thirteen normal human fibroblast clones also contained the predicted 4-bp insertion in exon 3, without additional mutations. Two other fibroblast clones contained wild-type sequences and presumably had unrelated mutations elsewhere in the *HPRT* locus. Sequence from the parental HT-1080 cell line and the three untransduced normal human fibroblast lines (MHF1, MHF2 and MHF3) did not contain the insertion mutation. Unlike the situation for the *neo* sequences described above, there was no selective disadvantage to additional mutations in the *HPRT* sequences obtained, so we can conclude that the targeting reaction occurred with high fidelity.

Higher vector doses increase gene-targeting rates

We tested the effect of vector copy number on gene targeting in HSNO39 cells and normal human fibroblasts (Fig. 3). In both cases, increasing vector doses led to substantially higher gene-targeting rates. In the case of normal human fibroblasts, up to 0.7% of the total cell population became HPRT-deficient at an MOI of 3×10^5 vector particles per cell. One possible explanation for this dependence on vector copy number is that the large numbers of single-stranded linear vector genome molecules delivered to the cell nucleus could mimic a state of genomic damage and induce additional DNA-repair or recombination functions necessary for the targeting reaction. We tested this hypothesis by infecting HSNO39 cells with mixtures of the targeting vector AAV-SNO648 and the unrelated AAV-HPe2/3X vector. Despite a constant MOI of 2×10^5 total vector particles per cell, the *neo* gene correction rate remained dependent on the dose of the AAV-SNO648 targeting vector (data not shown).

Transduction *versus* transfection methods

In order to compare gene-targeting methods in the same experimental system, we measured *neo* gene correction rates in HSN039 cells transduced with AAV-SNO648 vector stocks or transfected with the plasmid pASNO648, which contains the entire AAV-SNO648 genome (Fig. 4). The targeting rate by transduction was more than 1,000 times higher than that obtained by transfection (and possibly even higher, as control experiments with untransfected cells suggested that the G418-resistant colonies obtained after transfection were actually due to reversion of mutated chromosomal *neo* genes rather than homologous recombination). The low transfection-targeting rates cannot be explained solely by poor DNA uptake, as approximately 5% of HSNO39 cells were transfected by the neo^+ vector pASNori2. We also compared gene-targeting methods at the *HPRT* locus of HT-1080 cells by transfecting plasmids containing the AAV-HPe2/3 and AAV-HPe2/3X vector genomes, but could not detect 6TG-resistance above background mutation rates. This suggests that the *HPRT* gene-targeting rate by transduction was at least 100 times higher than that obtained by transfection (data not shown).

Discussion

We have shown that AAV vectors can be used to introduce defined genetic modifications into homologous human chromosomal loci at high frequencies. Gene targeting was demonstrated by phenotypic changes in the expression of two different targeted genes, by

Southern analysis of targeted chromosomal loci, and by sequencing of the modifications introduced. The majority of transduced cells contained no detectable rearrangements based on Southern analysis, and no secondary mutations were detected by DNA sequencing. Our results expand the number of possible transduction pathways for AAV-based genomes, which can also express transgenes from episomal molecules17, integrate at random locations by non-homologous recombination14 or integrate site-specifically at a non-homologous locus on chromosome 19 (refs 18,19).

Although gene-targeting rates can vary widely, it appears that the AAV-transduction method is particularly efficient. The gene-targeting frequency of greater than 10^{-3} of the total cell population produced by AAV vectors is two to three logs higher than frequencies typically achieved by transfection methods. The difference is even greater if one compares our results with other targeting experiments in the cell types we used. Gene targeting in HeLa cells has been reported at frequencies of approximately 10^{-7} to 10^{-8} (refs 20, 21), in HT-1080 cells at frequencies of 10^{-6} to 10^{-7} (refs 21–23), and in normal human fibroblasts at approximately 10^{-7} (ref. 24). AAV gene-targeting rates are also significantly higher than those obtained by retroviral or adenoviral vectors, which were reported to have homologous recombination rates of 10^{-5} to 10^{-6} in previous experiments[6–8], including *HPRT*-targeting with adenovirus vectors in embryonic stem cells (K. Mitani & C.T. Caskey, pers. comm.).

There are several possible explanations for the high gene-targeting rates we obtained. One critical factor is likely to be the delivery of large numbers of intact vector genomes into the cell nucleus which persist as single-stranded, episomal molecules[13]. This feature of AAV vectors is a disadvantage when gene-addition strategies are employed, as the vast majority of entering vector genomes remain transcriptionally inactive. In the case of gene targeting, these inactive vector genomes are able to participate in homologous pairing with chromosomal sequences, and the large numbers of genomes delivered to the nucleus appear to enhance the targeting process. Gene-targeting frequencies in microinjection experiments can be similar to those obtained with AAV vectors[3,25,26], further highlighting the importance of efficient DNA delivery. The single-stranded nature of the AAV vector genome could also promote homologous pairing, but higher recombination frequencies were not observed in previous gene-targeting experiments with single-stranded substrates[23,27]. Another factor could be the T-shaped hairpins at the AAV vector termini, which might decrease random integration and stabilize vector genomes.

In previous experiments with adenovirus vectors, the gene-targeting rates were low despite the vector DNA having entered the nucleus[8]. While this could be due to the different structural features of the adenovirus genome, it might simply reflect the lower MOIs used. In this regard, the ability to generate concentrated, purified vector stocks that can be used at high MOIs without cellular toxicity may be an advantage of AAV vectors. This dependence on MOI could, however, also be a disadvantage, as it might limit targeting rates in applications involving large numbers of cells that are difficult to infect. High AAV vector MOIs are also required for standard transduction experiments that do not involve gene targeting (Table 1, ref. 13).

Certain features of the AAV gene-targeting reaction were unexpected. Gene targeting by AAV vectors was dependent on the copy number of the homologous targeting molecule, whereas in previous microinjection and electroporation experiments, targeting frequencies did not increase with higher copy numbers of targeting constructs or targeted loci3,28. The AAV-targeting reaction also exhibited a lower dependence on the extent of sequence homology. The vector genomes we used shared only 2.7 or 3.8 kb of homology with their chromosomal counterparts, which would not have been sufficient for efficient gene targeting by transfection methods4,29. Another distinctive feature of gene targeting by AAV vectors is

the high ratio (one to ten) of targeted to non-targeted integration events. Ratios of about one to one thousand were reported in electroporation experiments using targeting constructs with functional expression cassettes[2,4]. While the reasons for these differences remain unknown, they may be due to distinct recombination or repair pathways involved in the different targeting reactions.

Gene targeting by AAV vectors should prove useful for both scientific and therapeutic applications. The reaction occurs at sufficiently high frequencies that transduced cells can be screened directly by PCR for the desired mutation without selection, which facilitates the introduction of subtle mutations. The wide host range of AAV and its potential for transducing large numbers of cells by a simple viral infection procedure, could allow for gene targeting in cell types that are resistant to DNA delivery by other methods. This is especially true for normal, primary cells, which are often difficult or impossible to transfect, and may not be viable outside the organism. AAV vectors can infect various cell types from several species[9], including primary mammalian cells, both *in vitro* and *in vivo*. As the efficiency of the transduction reaction is improved, therapeutic gene targeting could become a possibility, including *in vivo* correction of mutated genes.

Methods

Cell culture

HeLa cells30, HT-1080 cells15, 293 cells[31] and fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% heat-inactivated fetal bovine serum (HyClone), amphotericin (1.25 μg/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in a 10% CO_2 atmosphere. Human fibroblasts and HT-1080 cells were maintained in HAT medium (DMEM containing 13.61 μg/ml hypoxanthine, 0.176 μg/ml aminopterin and 3.875 μg/ml thymidine) prior to their use in transduction experiments. Normal male human fibroblasts MHF1, MHF2 and MHF3 were obtained from the Coriell Institute for Medical Research (corresponding repository numbers GM04522, GM05387 and GM08333A, respectively). HSNO39 cells were created by cotransfection of HeLa cells with a *Bam*HI fragment containing the SV40 early promoter, mutated *neo* gene and p15A origin (the same fragment is present in pASNO39), and a hygromycin-resistance gene fragment. Transfected cells were selected by growth in hygromycin (0.2 μg/ml, Calbiochem), and HSNO39 cells were cultured in medium containing hygromycin prior to their use in transduction experiments.

Vector production

AAV vector stocks were prepared by cotransfection of helper and vector plasmids, and vector purification on CsCl gradients, as described14. The vector plasmids used were pASNori2 for AAV-SNori, pASNO648 for AAV-SNO648, pASNO39 for AAV-SNO39, pAHPe2/3 for AAV-HPe2/3 and pAHPe2/3X for AAV-HPe2/3X. Vector -stock titres were determined by quantitation of purified, full-length, linear vector genomes on Southern blots of 1.2% alkaline agarose gels probed for vector sequences. Vector particle numbers determined by this method were equal or greater than 10^{11} per ml. The level of contaminating replication-competent AAV was determined by replication center assay (RCA) as described[32], and ranged from 6×10^5 to 3×10^7 per ml. In control experiments, the addition of excess wild-type AAV2 (at MOIs up to 10^4 particles per cell) did not affect *neo* targeting rates (data not shown), so possible coinfection with contaminating wild-type AAV should not have influenced our results.

Plasmids and DNA techniques

Plasmid construction, DNA purification, transfections, Southern-blot analysis and bacterial culture were performed according to standard procedures33. The plasmids pASNori2 (ref. 14) and pTR (ref. 34) have been described. pASNO39 is identical to pASNori2 except for a *Sal*I linker (5'–CGGTCGACCG–3') in the end-filled *Eag*I site. pASNO648 is identical to pASNori2 except for an end-filled and religated *Csp*I site. pAHPe2/3 contains base pairs 14,057–17,809 of the human *HPRT* locus (GenBank HUMHPRTB) in the *Bgl*II site of pTR as determined by DNA sequencing. pAHPe2/3X is identical to pAHPe2/3 except for an end-filled and religated *Xho*I site. Human *HPRT* sequences were from the Huλ3 lambda phage[16].

Integrated *neo* genes were rescued from transduced HSNO39 cells by digestion of chromosomal DNA with *Bam*HI, circularization with T4 DNA ligase, transfer to *Escherichia coli*, and selection for kanamycin resistance as described[14]. Sequencing of the nt-39 and nt-648 mutations of corrected *neo* genes recovered as bacterial plasmids was performed with primers 9606D (5′–dATGGCTTTCTTGCCGCCA–3′) and 9607A (5′–ATACGCTTGATCCGGCTAC–3′) respectively. *HPRT* exon-3 sequences were amplified from high-molecular–weight genomic DNA (100 ng) in 20-μl reactions as described[35]. The product (6 μl) was further amplified in a 100-μl volume under the same conditions for 20 cycles, the PCR product was purified using a QIAquick kit (Qiagen), and the purified product (75 ng) was used for DNA sequencing with the primer 5′–ACCTACTGTTGCCACTA–3. Dye terminator cycle sequencing was carried out using the ABI PRISM sequencing kit (Perkin Elmer) and analysed on an Applied Biosystems sequencer.

Transduction assays

Standard transduction experiments were performed by plating 5×10^3 or 1×10^4 HSNO39 cells per well into 96-(Nunc) or 48- (Costar) well plates, respectively, or 2×10^4 HT-1080 cells into 48-well plates, or 5×10^4 normal human fibroblasts into 24-well plates (Nunc) on day 1. On day 2, the medium was changed and vector stock was added to the well. The MOI was calculated by assuming one doubling of the cell population subsequent to the original plating. On day 3, each well was treated with trypsin, and the cells were plated into 10-cm dishes. On day 4, the assays differed for each cell type.

For *neo* gene correction experiments, 90%, 9.5% and 0.5% of the cells from each well were plated into different dishes on day 3. On day 4, G418 (1 mg/ml active compound) was added to the 90% and 9.5% dishes and selection was continued for 10–12 days with medium changes every 3–4 days. G418 was not added to the 0.5% dishes to determine the total number of colony-forming units (CFU) from each original well. The colonies were counted after staining with Coomassie brilliant blue G, and the *neo* gene correction rate was calculated as the number of G418-resistant CFU per total CFU for each original well. The plating efficiency of HSNO39 cells (total CFU per infected cell counts) was 88 ± 15% in these experiments, with no decrease in total CFU due to vector infection.

For HPRT experiments, all the cells from each well were cultured without selection for 10–14 days after being plated into 10-cm dishes on day 3, to allow for elimination of existing HPRT protein in HPRT-deficient cells. No significant differences were noted in *HPRT* mutation rates after 10-day or 14-day culture periods. The medium was changed every 3–4 days and when dishes became too dense the cells were treated with trypsin and dilutions were plated into new dishes. After this phenotypic expression period, 10^5, 10^4 and 10^2 cells from each culture were plated into new 10-cm dishes, and the following day 6TG (5 μg/ml) was added to the 10^5- and 10^4-cell dishes. 6TG selection was not applied to the 10^2-cell dishes as these were used to calculate plating efficiencies. The cells were cultured for 10–13

additional days, and the surviving colonies were counted. The percentage of 6TG-resistant CFU was determined after correcting for plating efficiencies.

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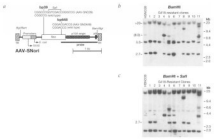

Fig. 1.

Chromosomal *neo* gene correction by AAV vectors, ***a***, Map of the AAV vector genomes used. AAV-SNori is the parent vector containing a functional *neo* gene. AAV-SNO39 and AAV-SNO648 are identical to AAV-SNori but contain the nt-39 or nt-648 mutations (sequence differences shown), respectively. The locations of the AAV terminal repeats (TR), promoters with transcription start sites (arrows), *neo* gene, p15A replication origin, polyadenylation site (pA), *Sal*I and fused *Bam*HI-*Bgl*II restriction sites, and the probe used in Southern analysis are indicated. ***b***, Southern blot of genomic DNA from untransduced HSNO39 cells and 11 G418-resistant clones of HSNO39 cells transduced with AAV-SNO648, digested with *Bam*HI and probed for *neo* sequences. The sizes of fragments hybridizing to *neo* in the parental HSNO39 line are indicated on the left. The positions of size standards are shown on the right. ***c***, Southern analysis as in a but digested with *Bam*HI and *Sal*I. The corrected 2.7-kb fragments are indicated.

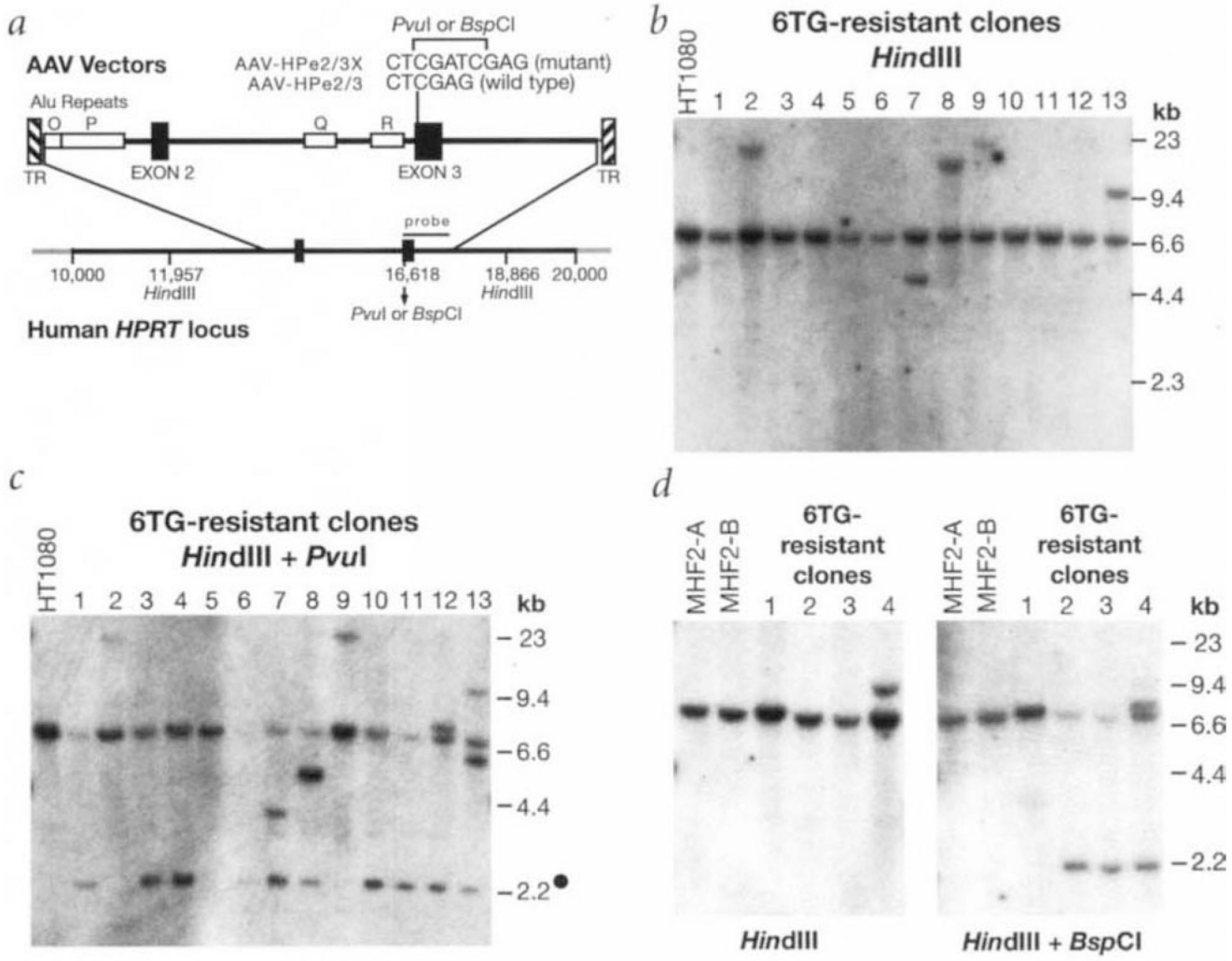

Fig. 2.

Targeting of the human *HPRT* locus by AAV vectors, ***a***. Structure of the AAV vectors used and the human locus. The locations of the AAV terminal repeats (TR), exons 2 and 3 of the *HPRT*gene, Alu repeats O, P, Q and R, and the probe used in Southern analysis are shown. The sequence differences between the wild-type AAV-HPe2/3 and mutant AAV-HPe2/3X vectors are indicated. The positions of relevant restriction sites are depicted in the chromosomal locus. The AAV-HPe2/3X mutation introduces a *Pvu*I or *Bsp*CI site, ***b***, Southern blot of genomic DNA from HT-1080 cells and 13 6TG-resistant clones derived from HT-1080 cells transduced with AAV-HPe2/3X, digested with *Hin*dIII and probed for *HPRT* sequences. The positions of size standards are indicated on the right. ***c***, Southern analysis of genomic DNA as in *b*, but digested with *Hin*dIII and *Pvu*I. The mark at 2.2 kb indicates the position of fragments containing the predicted *HPRT* modification. ***d***. Southern blot of genomic DNA from MHF2 normal fibroblast clones digested with *Hin*dIII or *Hin*dIII plus *Bsp*CI and probed as in *b*. Two untransduced (MHF2-A and MHF2-B) and four 6TG-resistant clones transduced by AAV-HPe2/3X were analysed.

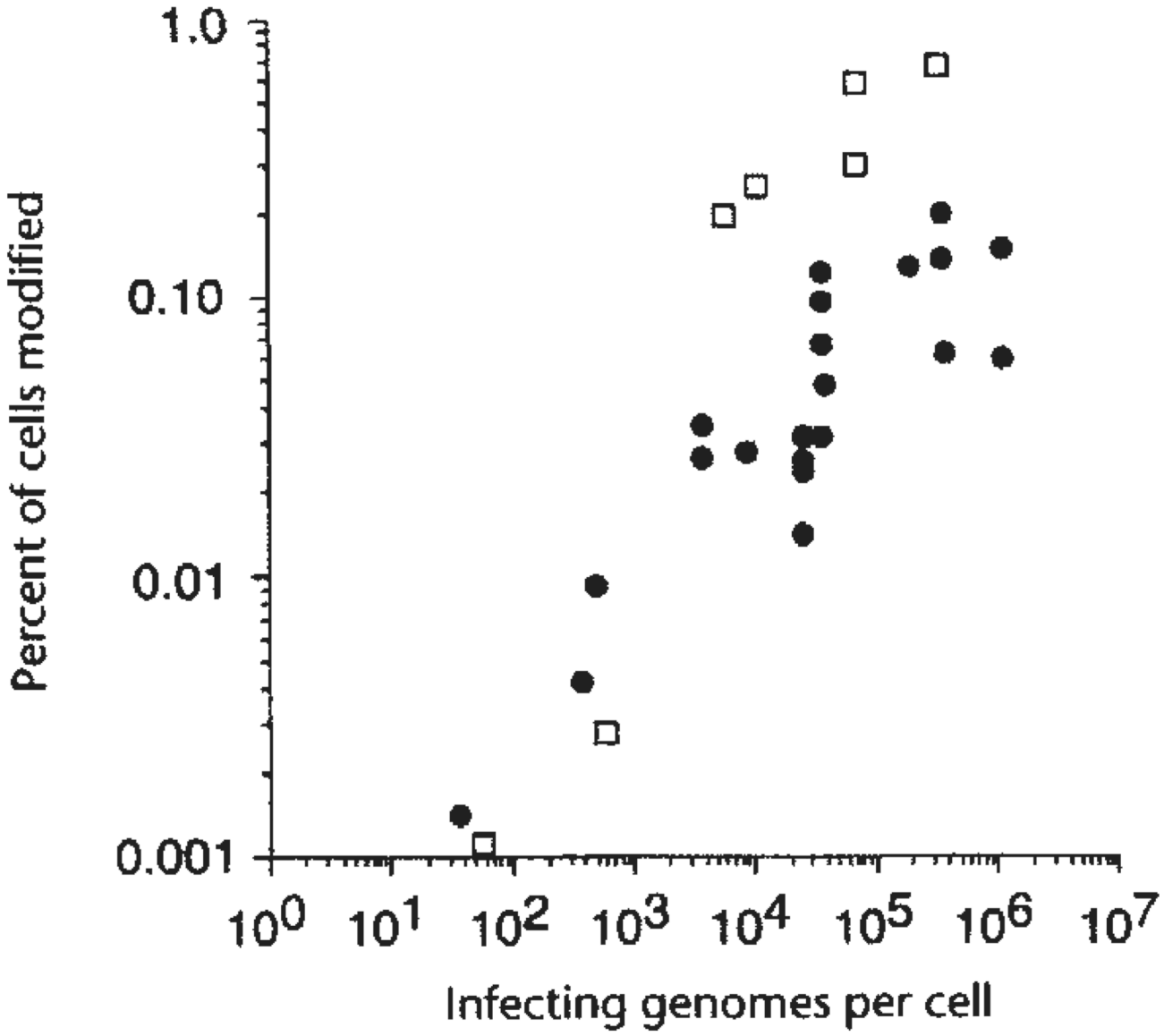

Fig. 3.

Gene targeting is dependent on vector dose. HSNO39 (filled circles) or MHF2 (open squares) cells were infected with AAV-SNO648 and AAV-HPe2/3X, respectively at the indicated MOI (vector genomes per cell), and the percentage of cells with modified *neo* or *HPRT* genes was determined (as in Tables 1 and 2). The results of several experiments are combined with each symbol representing a single result.

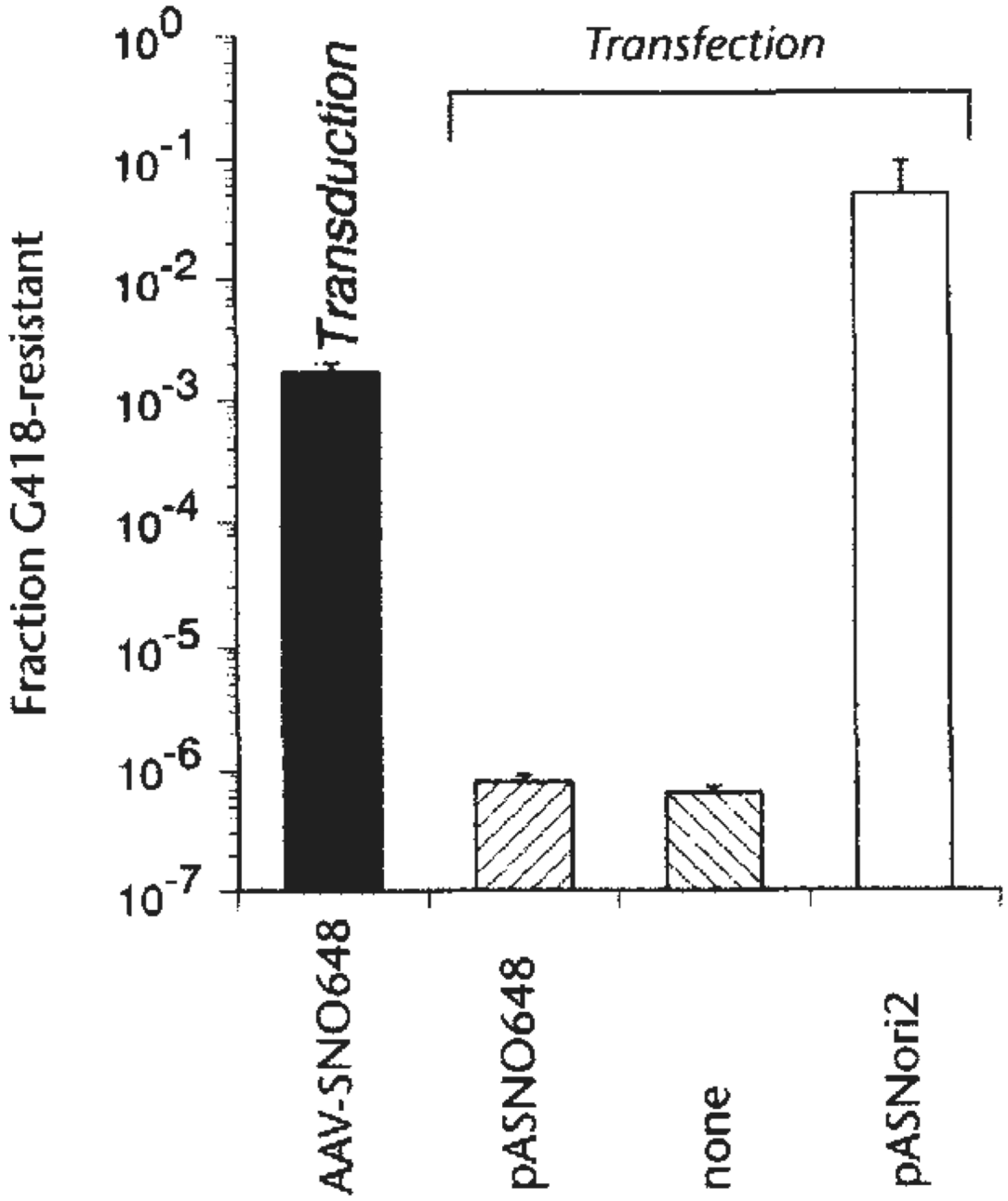

Fig. 4.

Gene targeting by transduction and transfection. HSNO39 cells were either transduced with the AAV-SNO648 vector at a MOI of 4×10^5 vector genomes per cell or transfected with pASNO648 or pASNori2 plasmids, and the fraction of G418-resistant CFU was determined. Transfections were performed by plating 4×10^6 cells per 15-cm dish on day 1, changing the media and transfecting with 20 μg of the indicated plasmid as a calcium-phosphate precipitate on day 2, treating with trypsin and replating dilutions of cells on day 3, and selecting with G418 on day 4 (as in the transduction experiments). pASNO648 was linearized by digestion with *Sac*II before transfection. Mean values with standard errors are plotted.

Table 1

Chromosomal *neo* gene correction rates

Cell line	Vector	MOI[a]	Fraction G418^R
HSNO39	none	-	$<5.3 \times 10^{-5}$
"	"	-	$< 4.3 \times 10^{-5}$
"	"	-	$< 4.3 \times 10^{-5}$
"	"	-	$< 1.4 \times 10^{-5}$
HSNO39	AAV-SNO648	40,000	9.6×10^{-4}
"	"	40,000	6.7×10^{-5}
"	"	400,000	2.0×10^{-3}
"	"	400,000	1.4×10^{-3}
HeLa	AAV-SNO648	40,000	$<6.0 \times 10^{-5}$
"	"	40,000	$< 5.7 \times 10^{-5}$
"	"	400,000	$< 6.8 \times 10^{-5}$
"	"	400,000	$< 6.6 \times 10^{-5}$
HSNO39	AAV-SNO39	375,000	$< 6.3 \times 10^{-5}$
"	"	1,500,000	$<6.6 \times 10^{-5}$
HSNO39	AAV-SNori	40,000	7.3×10^{-3}
"	"	40,000	7.9×10^{-3}
"	"	400,000	1.7×10^{-2}
"	"	400,000	2.7×10^{-2}
HeLa	AAV-SNori	100,000	7.3×10^{-3}
"	"	100,000	5.3×10^{-3}

[a] vector genomes per cell.

Table 2

HPRT gene modification in human cells

Cell Type	Vector	MOI*	Fraction $6TG^R$
HT-1080	AAV-HPe2/3X	50,000	5.3×10^{-4}
"	"	"	2.4×10^{-5}
"	AAV-HPe2/3	"	$<1.0 \times 10^{-5}$
"	"	"	2.2×10^{-5}
"	none	-	$<1.4 \times 10^{-5}$
"	"	-	1.0×10^{-5}
MHF1	AAV-HPe2/3X	15,000	3.2×10^{-5}
"	"	"	4.2×10^{-5}
"	AAV-HPe2/3	"	6.3×10^{-5}
"	"	"	$<2.4 \times 10^{-5}$
"	none	-	$< 1.9 \times 10^{-5}$
"	"	-	$< 2.0 \times 10^{-5}$
MHF2	AAV-HPe2/3X	15,000	2.6×10^{-3}
"	"	"	8.8×10^{-5}
"	AAV-HPe2/3	"	$<2.2 \times 10^{-5}$
"	"	"	2.4×10^{-5}
"	none	-	2.2×10^{-5}
"	"	-	$<1.9 \times 10^{-5}$
MHF3	AAV-HPe2/3X	15,000	7.3×10^{-4}
"	"	"	6.4×10^{-4}
"	AAV-HPe2/3	"	$<2.8 \times 10^{-5}$
"	"	"	$<2.4 \times 10^{-5}$
"	none	-	$<2.6 \times 10^{-5}$
"	"	-	$<2.3 \times 10^{-5}$

* vector genomes per cell.