Adeno-Associated Virus Type 2-Mediated Transfer of Ecotropic Retrovirus Receptor cDNA Allows Ecotropic Retroviral Transduction of Established and Primary Human Cells

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The cellular receptors that mediate binding and internalization of retroviruses have recently been identified. The concentration and accessibility of these receptors are critical determinants in accomplishing successful gene transfer with retrovirus-based vectors. Murine retroviruses containing ecotropic glycoproteins do not infect human cells since human cells do not express the receptor that binds the ecotropic glycoproteins. To enable human cells to become permissive for ecotropic retrovirus-mediated gene transfer, we have developed a recombinant adeno-associated virus type 2 (AAV) vector containing ecotropic retroviral receptor (*ecoR*) cDNA under the control of the Rous sarcoma virus (RSV) long terminal repeat (LTR) promoter (*vRSVp-ecoR*). Established human cell lines, such as HeLa and KB, known to be nonpermissive for murine ecotropic retroviruses, became permissive for infection by a retroviral vector containing a bacterial gene for resistance to neomycin (RV-Neo^r), with a transduction efficiency of up to 47%, following transduction with *vRSVp-ecoR*, as determined by the development of colonies that were resistant to the drug G418, a neomycin analog. No G418-resistant colonies were present in cultures infected with either *vRSVp-ecoR* or RV-Neo^r alone. Southern and Northern blot analyses revealed stable integration and long-term expression, respectively, of the transduced murine *ecoR* gene in clonal isolates of HeLa and KB cells. Similarly, ecotropic retrovirus-mediated Neo^r transduction of primary human CD34⁺ hematopoietic progenitor cells from normal bone marrow was also documented, but only following infection with *vRSVp-ecoR*. The retroviral transduction efficiency was approximately 7% without prestimulation and approximately 14% with prestimulation of CD34⁺ cells with cytokines, as determined by hematopoietic clonogenic assays. No G418-resistant progenitor cell colonies were present in cultures infected with either *vRSVp-ecoR* or RV-Neo^r alone. These results suggest that sequential transduction of primary human cells with two different viral vectors may overcome limitations encountered with a single vector. Thus, the combined use of AAV- and retrovirus-based vectors may have important clinical implications for ex vivo and in vivo human gene therapy.

The majority of human gene therapy clinical protocols have been conducted with retroviral vectors (6, 7, 11, 31). However, these vectors may have certain limitations, since retroviruses require a specific receptor to gain entry into cells as well as active cell division for successful proviral integration into target cell DNA (3, 14, 23, 30). A retrovirus enters the host cell following interaction of a viral envelope glycoprotein (a product of the viral *env* gene) with a host cell receptor (3, 12–14). Murine retroviruses express mainly two classes of envelope glycoproteins that interact with different host cell receptors. The ecotropic type of murine retroviral glycoprotein binds to the ecotropic receptor (*ecoR*) found only on mouse and rat cells (3, 12). The envelope (*env*) genes of the amphotropic class of viral glycoproteins, on the other hand, endow a murine retrovirus with a broad host range, including the mouse, rat, chicken, dog, cat, gibbon, and human, since the amphotropic

receptor is expressed in cells of these species (3, 9, 14, 16). The *ecoR* has been cloned and characterized in the mouse and rat (1, 15, 22, 42). It has been demonstrated to function as the basic amino acid and phosphate transporter, and it has a wide but variable tissue distribution (15, 18, 40, 42, 43).

The adeno-associated virus type 2 (AAV)-based vector system appears to be promising in overcoming some of the limitations of retrovirus vectors (4, 8, 36). For example, in contrast to retroviruses, AAV is a nonpathogenic, replication-defective human parvovirus. In the absence of helper virus, the wild-type AAV genome integrates in a site-specific manner via its inverted terminal repeats (ITRs) into the q13.3-qter of human chromosome 19 (19, 20, 34). Although recombinant AAV vectors appear to lack this property (8, 17, 28), there is as yet no evidence that AAV integration leads to insertional mutagenesis (8, 25). Furthermore, the relatively higher stability, viral titers, and transduction efficiency of AAV add to the desirable features of this type of vector (25–27). We have developed a recombinant AAV vector to determine whether AAV-mediated delivery and expression of mouse *ecoR* cDNA in human cells could enhance retroviral transduction efficiency. Here, we present evidence that infection of established human cell lines,

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FIG. 1. Construction of the recombinant AAV-*ecoR* vector plasmid and virions. The overall strategy for construction of the recombinant plasmid pKY-1 containing the RSV-LTR promoter-driven murine *ecoR* cDNA (2.85 kb) in between AAV ITRs is depicted here and described in the text. The recombinant AAV genome was rescued from the plasmid and packaged as recombinant vRSVp-*ecoR* virions as previously described (22, 28, 29, 38, 41).

as well as of primary human bone marrow-derived CD34⁺ cells, with a recombinant AAV vector containing the *ecoR* gene does indeed lead to subsequent efficient transduction with a retroviral vector. This may represent a model system for efficient retrovirus infection of normally resistant organs and primary cells.

AAV-mediated transfer of *ecoR* cDNA renders human HeLa and KB cells susceptible to infection by a recombinant retroviral vector. The cDNA sequences of the mouse *ecoR* gene, driven by the Rous sarcoma virus long terminal repeat (RSV-LTR) promoter from plasmid ψ N61, were subcloned in between the AAV ITRs derived from plasmid *psi*201 (33), to construct a recombinant AAV plasmid, designated pKY-1, by the strategy depicted in Fig. 1. Recombinant AAV virions (vRSVp-*ecoR*) were generated from plasmid pKY-1 and purified on CsCl equilibrium density gradients as previously described (22, 28, 29, 38, 41). The recombinant AAV viral titers ($\sim 2 \times 10^{10}$ /ml), as well as the extent of contamination with the wild-type AAV ($\sim 0.1\%$), were determined on quantitative DNA slot blots as described previously (27–29, 36, 41). The physical particle-to-infectious virus ratio was calculated to be ~ 500 (data not shown). The ecotropic retrovirus-producing cell line, F5B, has been described previously (13). It consists of a retroviral vector, N2, containing a gene for resistance to neomycin (RV-Neo^r), in the psi-2 packaging cell line (2, 13). F5B cells were maintained in Iscove's modified Dulbecco's medium with 10% fetal bovine serum. Recombinant retrovirus stocks were harvested from confluent packaging cells, filtered through 0.22- μ m-pore-size filters, and used immediately for infection. The titers of the recombinant retrovirus stocks were determined to be approximately 10^6 CFU/ml (13).

Human cell lines, HeLa (a cervical carcinoma cell line) and KB (a nasopharyngeal carcinoma cell line), were infected with

TABLE 1. Effect of AAV-mediated *ecoR* gene transfer in HeLa and KB cells on retrovirus transduction^a

vRSVp- <i>ecoR</i> (MOI)	No. (%) of G418-resistant colonies in cells	
	HeLa	KB
0	0 (0)	0 (0)
1	11 $\pm$ 1 (9)	25 $\pm$ 3 (11)
10	21 $\pm$ 1 (16)	53 $\pm$ 8 (24)
100	47 $\pm$ 3 (37)	103 $\pm$ 2 (47)

^a HeLa or KB cells were seeded in 12-well dishes and were either mock infected, or infected with vRSVp-*ecoR* at various MOIs for 2 h at 37°C. Forty-eight hours later, the cells were incubated with the murine ecotropic retroviral vector RV-Neo^r at 100 CFU/cell for 2 h in the presence of 8 μ g of polybrene/ml. The medium was replaced, and 2 days following retrovirus infection, G418 was added to cultures. Individual clones were enumerated 14 days postinfection, as described in the text. The data represent the means $\pm$ standard errors of the means from three independent experiments.

vRSVp-*ecoR* at a multiplicity of infection (MOI) of 0, 1, 10, or 100 for 2 h at 37°C. Forty-eight hours after infection with recombinant AAV, cells were either mock treated or infected with the ecotropic retroviral vector RV-Neo^r at 100 CFU/cell for 2 h at 37°C in the presence of 8 μ g of polybrene/ml. Forty-eight hours after retrovirus infection, cells were exposed to the drug G418, a structural analog of neomycin, at a final active concentration of 400 μ g/ml for HeLa cells and 600 μ g/ml for KB cells. Cultures were maintained for 10 to 14 days, and G418-resistant colonies were enumerated. The results are summarized in Table 1. It is evident that whereas the murine ecotropic retrovirus did not infect human HeLa or KB cells, prior infection of these cells with vRSVp-*ecoR* rendered them permissive for retrovirus infection. Infectibility by the ecotropic retrovirus reached maxima of approximately 37% in HeLa cells and 47% in KB cells. It is perhaps not surprising that a 10-fold higher MOI of the recombinant AAV titer resulted in an increase of only approximately twofold in retroviral transduction efficiency, presumably because of efficient transduction and expression of the *ecoR* gene mediated by the recombinant AAV. Since the efficiency of retroviral transduction is generally less than 50% among permissive cells (21), even under optimal conditions, these data, nonetheless, suggest that AAV-mediated *ecoR* gene transfer efficiently allows for ecotropic retroviral transduction of nonpermissive human cells.

AAV-mediated transduction leads to stable integration and long-term expression of the murine *ecoR* gene in human cells.

It was of interest to examine whether the murine *ecoR* was stably integrated into the chromosomal DNA of transduced human cells. Several independently isolated G418-resistant clones from different plates of HeLa cells infected at various MOIs of vRSVp-*ecoR* were expanded and grown separately for 4 weeks. Total genomic DNA was isolated from each of the clones, and equivalent amounts were digested with *Kpn*I (three sites in the AAV proviral genome) or *Nhe*I (no sites in the AAV proviral genome), electrophoresed on agarose gels, and analyzed on Southern blots with ³²P-labeled murine *ecoR* cDNA-specific probe as previously described (35). These data for four clones each are presented in Fig. 2. As can be seen in Fig. 2a, the probe detected DNA fragments of the expected sizes (~ 4.8 , 0.94, and 0.38 kb) in each of the clones infected at various MOIs. Mock-infected cells lacked these fragments, as expected. When DNA samples from two representative clones each were digested with *Nhe*I, the probe detected a high-*M*_r DNA band in transduced cells (Fig. 2b), indicating that the

FIG. 2. Southern blot analysis for recombinant AAV-mediated transduction and integration of murine *ecoR* cDNA in HeLa cells. Total genomic DNA samples isolated from HeLa cells following mock infection or vRSVP-*ecoR* infection at various indicated MOIs followed by transduction with RV-Neo^r vector were isolated 4 weeks posttransduction, digested with *KpnI* (a) or *NheI* (b), and analyzed on Southern blots with the murine *ecoR* cDNA probe, as described in the text.

recombinant proviral genome was stably integrated into the host chromosomal DNA.

It was next of interest to examine the level of expression of the transduced murine *ecoR* gene in each of the clonal isolates. Equivalent amounts of total cellular RNA isolated 30 days posttransduction from these clones as well as from mock-infected cells were analyzed on Northern blots with ³²P-labeled murine *ecoR* and human β -actin cDNA probes, as previously described (32). These blots are shown in Fig. 3. It is evident that the *ecoR*-specific probe detected two RNA transcripts in each of the clones (Fig. 3a), an observation which is consistent with previously published reports (23, 42–44). However, interclonal variations in the level of expression of the *ecoR* gene were readily apparent, and the level of expression did not appear to correlate with the viral MOI. That the RNA loads were roughly equivalent was substantiated by probing the same blot with the human β -actin cDNA-specific probe (Fig. 3b). These results, nonetheless, indicate that the recombinant

FIG. 3. Northern blot analysis for expression of the transduced murine *ecoR* gene in HeLa cells. Total cellular RNA samples from HeLa cells were isolated following mock infection or vRSVP-*ecoR* infection at various indicated MOIs followed by transduction with RV-Neo^r vector 1 month posttransduction and were analyzed on Northern blots with the murine *ecoR* cDNA probe (a). The blot was stripped of the *ecoR* probe and reprobed with the human β -actin cDNA probe (b) as described in the text.

FIG. 4. Retroviral vector-mediated Neo^r gene transfer in primary human CD34⁺ cells following transduction with recombinant vRSVP-*ecoR*. Approximately 10⁵ human CD34⁺ cells were plated in Iscove's modified Dulbecco's medium with 10% fetal bovine serum and 2 mM glutamine and were infected with vRSVP-*ecoR* at an MOI of 10 for 2 h without prestimulation with cytokines (–Cytokines). Forty-eight hours later, cells were exposed for 2 h to the murine ecotropic RV-Neo^r at 100 CFU/ml in the presence of 4 mg of polybrene/ml. Two days after retrovirus infection, the cells were cultured, as previously described (45), in the presence of the following cytokines: recombinant human erythropoietin, stem cell factor, interleukin-3, and freshly prepared human plasma. Hematopoietic progenitor cell assays were performed in the absence and presence of the drug G418 at 1.5 mg/ml for 14 days. In some experiments, CD34⁺ cells were prestimulated with cytokines for 48 h prior to infection with vRSVP-*ecoR* (+Cytokines). The rest of the steps were the same as described above. These data are from two separate experiments.

AAV-mediated transduced *ecoR* gene was transcriptionally active for at least 30 days.

AAV-mediated transfer of murine *ecoR* cDNA allows subsequent retrovirus transduction of primary human CD34⁺ hematopoietic progenitor cells. Although these studies indicated that transduction by a recombinant AAV vector expressing the *ecoR* gene could obviously increase retroviral transduction efficiency in human cell lines, we next wished to examine this possibility with primary human hematopoietic cells. Human bone marrow-derived CD34⁺ hematopoietic cells were chosen because they are considered desirable target cells for somatic cell gene therapy (8, 10, 36, 39, 45). However, retroviral vector-mediated gene transfer into these cells has been reported to be at a disappointingly low frequency of less than 1% when the infection is performed with retroviral supernatants only (8). We and others have demonstrated that AAV-mediated gene transfer into primary human hematopoietic progenitor cells is highly efficient (8, 10, 36, 39, 45). Human CD34⁺ cells were isolated from bone marrow of normal donors upon informed consent and cultured as previously described (45). The infection protocol was similar to that for HeLa and KB cells, except that CD34⁺ cells were infected with vRSVP-*ecoR* at an MOI of 10 followed by infection with the RV-Neo^r vector. Mock-infected and infected cells were used in hematopoietic progenitor cell assays in the absence and presence of G418 as previously described (45). In some experiments, CD34⁺ cells were preincubated with various cytokines for 48 h prior to infection with vRSVP-*ecoR*. The results of these experiments are shown

FIG. 5. Southern blot analysis of PCR products from hematopoietic colonies derived from vRSVP-*ecoR* plus RV-Neo^r-infected primary human CD34⁺ cells. PCR amplification was carried out with DNA samples obtained from individual hematopoietic colonies derived from mock-infected (lane 2) or vRSVP-*ecoR*-plus RV-Neo^r-infected (lanes 3 to 12) primary human CD34⁺ cells by using the AAV-Rt.Jn. fragment primer pair as previously described (28). DNA from a cell line latently infected with AAV (D5; lane 1) was used as a positive control (34). The arrow indicates the PCR product of the expected size that hybridized with the AAV-Rt.Jn. fragment-specific DNA probe (28, 34).

in Fig. 4. It is evident that human CD34⁺ cells were resistant to transduction with the murine ecotropic retrovirus. Prior infection with vRSVP-*ecoR* increased retroviral gene transfer from less than 1% to approximately 7% in the absence of prestimulation with cytokines, and up to 14% with prestimulation with cytokines. In comparison, previous studies with amphotropic retroviral vectors have consistently documented the transduction efficiency of primary human hematopoietic cells to be <1% (8).

We also wished to document the physical presence of the recombinant AAV-mediated transduced *ecoR* gene, presumably stably integrated into the genomic DNA, in G418-resistant myeloid and erythroid cell colonies. Ten individual colonies were picked from vRSVP-*ecoR*-plus RV-Neo^r-infected CD34⁺ cell cultures and were subjected to 35-cycle PCR amplification with an AAV- and human chromosome 19-specific synthetic oligonucleotide primer pair as previously described (28, 34). These primers, with the sequences 5'-ATAAGTAG CATGGCGGGTTA-3', specific for AAV nucleotides 4498 to 4517 (P1), and 5'-CGCATAAGCCAGTAGAGC-3', specific for the AAV-chromosome 19 right junction (Rt.Jn.) fragment (P2), have been used successfully in previous studies (28, 34). Amplified DNA products were electrophoresed on 1.2% agarose gels and analyzed on Southern blots with a ³²P-labeled Rt.Jn. fragment-specific DNA probe as described above. The results shown in Fig. 5 indicate that of the 10 colonies tested, a fragment of the predicted size that hybridized to the Rt.Jn. fragment-specific DNA probe was detected in only 1 colony. These results are consistent with recent studies by us and others that found that recombinant AAV genomes do not generally appear to integrate preferentially into chromosome 19 (17, 28).

The increased rate of retroviral transduction of CD34⁺ cells was relatively low compared with rates for HeLa and KB cells. One possible explanation is that retroviral vectors require active cell division for efficient transduction (24). Indeed, when cells were prestimulated with cytokines prior to retroviral infection, the transduction efficiency was increased significantly, more than twofold higher than that without prestimulation. In this context, it is noteworthy that increased retroviral transduction in cultured cells following adenovirus-mediated transfer of the amphotropic receptor cDNA has recently been reported

(21). Although a four- to eightfold higher transduction efficiency was noted, adenovirus-mediated gene transfer is transient in nature, since the viral genome does not undergo stable integration into host cell chromosomal DNA.

More recently, recombinant AAV-mediated transfer and high-efficiency expression of *ecoR* in HeLa cells have also been reported (5). However, expression of the transduced gene was transient, and the AAV proviral genome was integrated in less than 10% of the clones. In contrast, we were able to document stable integration as well as long-term expression of the transduced *ecoR* gene in our studies. Whether the use of different reporter or selectable marker genes to isolate clonal populations of cells in the two studies accounted for the observed differences remains to be established. These differences notwithstanding, these studies suggest that the combined use of AAV- and retrovirus-based vectors may have important clinical implications for ex vivo and in vivo human gene therapy.

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