Integration of Adeno-Associated Virus Vectors in CD34+ Human Hematopoietic Progenitor Cells After Transduction


Gene transfer vectors based on adeno-associated virus (AAV) appear promising because of their high transduction frequencies regardless of cell cycle status and ability to integrate into chromosomal DNA. We tested AAV-mediated gene transfer into a panel of human bone marrow or umbilical cord-derived CD34+ hematopoietic progenitor cells, using vectors encoding several transgenes under the control of viral and cellular promoters. Gene transfer was evaluated by (1) chromosomal integration of vector sequences and (2) analysis of transgene expression. Southern hybridization and fluorescence in situ hybridization analysis of transduced CD34 genomic DNA showed the presence of integrated vector sequences in chromosomal DNA in a portion of transduced cells and showed that integrated vector sequences were replicated along with cellular DNA during mitosis. Transgene expression in transduced CD34 cells in suspension cultures and in myeloid colonies differentiating in vitro from transduced CD34 cells approximated that predicted by the multiplicity of transduction. This was true in CD34 cells from different donors, regardless of the transgene or selective pressure. Comparisons of CD34 cell transduction either before or after cytokine stimulation showed similar gene transfer frequencies. Our findings suggest that AAV transduction of CD34+ hematopoietic progenitor cells is efficient, can lead to stable integration in a population of transduced cells, and may therefore provide the basis for safe and efficient ex vivo gene therapy of the hematopoietic system.

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INTEGRATION OF AAV VECTORS IN CD34 CELLS

MATERIALS AND METHODS

Plasmids and vectors. All AAV vectors used in this study were derived from the previously described base vector, CWRSV, which contains bases 1-189 and 4045-4680 from wild-type AAV2, including the 5' and 3' ITRs and the endogenous AAV2 polyadenylation signal. CWRAP was constructed by inserting the thermostable human placental alkaline phosphatase (PLAP) gene from pDAP into the polylinker of pCWRSV as a 1900-bp SalI fragment, encoding the open reading frame from pDAP.27 Integration into any primary cells including CD34 cells.25 All analyses of vector sequences in transduced CD34 cells reported thus far have relied on polymerase chain reaction (PCR) amplification methods.26-28 Because PCR-based analyses of AAV transduction may be confounded by the survival of unintegrated episomal vector genomes, we reasoned that a direct assessment of vector integration into primary chromosomal DNA was imperative for the evaluation of AAV vectors for long-term human stem cell gene therapy.

In this study, we investigated the capacity of AAV vectors to transfer genes into human BM or umbilical cord blood-derived CD34+ hematopoietic progenitor cells both by transgene expression and vector integration into chromosomal DNA. Transduction of a panel of CD34 cells was tested both in suspension cultures and colony-forming assays, using a series of AAV vectors. Gene transfer and expression were observed in a significant fraction of CD34 cells and were not dependent on cytokine prestimulation of target cells. Both Southern and fluorescence in situ hybridization (FISH) analyses showed integration of vector sequences into chromosomal DNA in CD34 cells derived from different donors, suggesting that AAV-mediated gene transfer could result in the stable genetic modification of cells. These findings may provide the basis for the use of AAV vectors for the safe and efficient gene transfer into primitive hematopoietic progenitor cells.
AAV-mediated gene transfer into CD34 cells. We initially tested AAV transduction of primary BM-derived CD34 cells using vCWRAP, an AAV vector encoding the PLAP gene. Cells were transduced with vCWRAP (MOI, 0.5), a control vector, or vCWRHIVASVN or were left untransduced, before placement in suspension cultures in media containing IL-3 and IL-6. Figure 2A shows the frequency of PLAP-expressing cells from three donors on day 8 posttransduction. Specific transgene expression was observed in 16% to 58% of the CD34 cells, with 39% to 58% of the CD34 cells with little or no background observed in either the control-transduced (vCWRHIVASVN) or untransduced cells. Similar results were observed when cells were washed extensively 24 hours after transduction. PLAP expression above background was not detected when CD34 cells were exposed to lysates prepared from adenovirus or HSV-infected 293 cells transfected with vector plasmids in the absence of AAV rep and cap genes (data not shown). Additionally, no specific PLAP expression was observed when vector stocks were preclarified with anti-AAV antibodies and immunomagnetic beads (data not shown). These results suggest that gene transfer was mediated by AAV virions and that PLAP expression in CD34 cells was specific for vector transduction. The frequency of PLAP expression observed in BM-derived CD34 cells transduced with vCWRAP closely approximated the maximal level of transduction expected at an MOI of 0.5.

Transduction of human umbilical cord blood CD34 cells with vCWRAP (MOI, 0.25 to 0.30) was tested after transduction with vector in the form of lysates or after further purification through isopycnic cesium chloride gradients. Figure 2B shows transgene expression in 16% to 32% of cells at 1 week (donors RE and SE) posttransduction. Totals of 18% and 16% of cells from donor RE showed transgene expression on transduction with crude cell lysate or cesium-purified vector, respectively. Similarly, for donor SE, 17% of CD34 cells expressed PLAP on transduction with crude lysates as compared with 32% with cesium chloride-purified vector. AAV vector transduction of cord blood CD34 cells showed a greater donor-to-donor variability than that generally observed with BM.

Integration of AAV vector sequences in CD34 hematopoietic cells. We next analyzed the fate of the vector genomes in BM CD34 cells after AAV transduction in the absence of selective pressure. Southern hybridization analyses of PCR-amplified vector-specific sequences had previously shown the presence of vector sequences in high molecular weight (HMW) DNA from transduced cells (data not shown). How-
Fig 2. (A) PLAP transgene expression in CD34 cells from 3 BM donors in suspension cultures. Cells were transduced with either vCWRAP or vCWRHIVAPAP at an MOI of 0.5 or were left un-transduced before culture for 8 days when cells were assayed for PLAP expression. (B) PLAP transgene expression in CD34 cells from 2 separate human umbilical cord blood donors using either crude vector lysates or CsCl-purified vector stocks. Results shown represent the frequency of transgene-expressing cells in the cultures. The absolute numbers of PLAP-positive cells is shown as a fraction of the total enumerated above each histogram. Specific expression was calculated after background subtraction.

ever, the possibility of amplification from either integrated or unintegrated vector sequences precluded conclusions regarding the fate of the vector in the cell. Thus, it was important to evaluate integration by hybridization analysis of restriction enzyme-digested HMW cellular DNA. CD34 cells from four donors were transduced at an MOI of 2 with either vCWRSP-MDR (donor JM) or vCWRHIVAPAP (donors DS and KS) and were washed extensively after 24 hours. Cells were harvested 7 days posttransduction for integration analysis. HIIC21, a 293-based G418R clonal cell line derived after transduction with vCWRHIVASVN (a vector identical to CWRHIVAPAP, except for a substitution of the PLAP gene with a Neo gene), contains 1 copy of the vector genome per cell and served as a positive control. Genomic DNA from vCWRSP-MDR–transduced cells was digested with Sal I, which cuts once within the vector. The DNA from vCWRHIVAPAP-transduced cells was digested using either Sal I (single cutter) or BamHI (double cutter). After electrophoresis and capillary transfer to nitrocellulose, blots of restriction-digested DNA were hybridized to probes designed to specifically identify fragments containing vector-chromosome junctions (Fig 3A). A limited number (2 to 6) of major vector-specific bands were detected in each sample analyzed (Figs 3B-D). Of this limited set of bands, one or two usually hybridized more strongly to the probe than did the others. In each case, the HMW bands detected were
Fig 3.
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Fig 3. Integration of vector sequences in AAV-transduced CD34 cells. (A) Maps of vectors used for transduction of cells in integration analyses. The presumed integrated forms of the vectors are drawn to scale. Also shown are the locations of the restriction sites and probes used in the analyses. BglII (Bgl) denotes the 5' and 3' ends of the vector sequences. B, BamHI; S, SalI. (B) Southern hybridization analyses of DNA isolated from CD34 cells from donor JM, transduced with vCWRSP-MDR, and analyzed 7 days posttransduction. DNA was cut with SalI, which cleaves once within the vector, and probed with probe 1, specific for the 5' vector-chromosome integration junction. (C) The left-hand panel shows a Southern blot of DNA isolated from vCWRHIVAPAP-transduced CD34 cells from donor DS, digested with SalI, which cuts once within the vector, and probed for both 5' and 3' vector-chromosome integration junctions. The right-hand panel shows a Southern blot of DNA isolated from vCWRHIVAPAP-transduced CD34 cells from donor DS digested with BamHI, which cuts twice within the vector, and probed for the 3' vector-chromosome integration junction. (D) Southern blot from CD34 cells transduced with vCWRHlVAPAP from donor KS. DNA was digested with BamHI as in the right-hand panel of (C). The blot was probed for the 3' vector-chromosome junction sequences using probe 3. Size markers are indicated for each panel. H1L21, a clonal cell line containing vCWRHIVASVN at 1 copy per cell served as the positive control, and untransduced cells were provided as negative controls. The minor upper bands present in H1L21 DNA with probe 1 represents an incomplete digest.

larger than those expected from free unintegrated vector or double-stranded intermediate forms. Table 1 shows the sizes of bands expected in each case from 1× and 2× episomal forms of the vector, 2× tandem integrants, and single-copy integrants, along with the sizes of the actual bands detected in the HMW portion of the radioautographs.

SalI-digested DNA from vCWRSP-MDR–transduced (4.9 kb) cells (donor JM) showed predominant vector-specific bands of approximately 8.6 kb and 15.8 kb when probed with probe 1, expected to identify the 5' vector-chromosome junction (Fig 3B). Because the hybridizing bands observed on the radioautograph do not match any of the expected episomal or free multimeric sizes, we conclude that they must represent junction fragments consisting of vector and chromosomal sequences.

Analysis of HMW SalI-digested DNA from vCWRHIVAPAP-transduced (4.4 kb) cells (donor DS) showed a principal band of approximately 7.4 kb and a minor band of approximately 2.9 kb when hybridized to probe 2 (Fig 3C). This probe straddles the cleavage site by 167 bp at the 5' end and by 251 bp at the 3' end (Fig 3A) and should detect at least two fragments. By PCR analysis, we confirmed that the SalI site within the integrated vector sequences was not methylated and that the restriction digest was complete (data not shown). Again, vector-specific bands detected were consistent with integrated vector sequences.

DNA from vCWRHIVAPAP-transduced cells (donor DS) was also digested with BamHI, which cleaves twice within the vector sequences, and was probed for the 3' vector-chromosome junction with probe 3 (Fig 3C). Two major hybridizing bands of approximately 7.6 kb and 4 kb representing the 3' integration junction region between vector and chromosomal sequences were identified. In addition, faint bands of approximately 3.4 kb and 3 kb were observed. Each single-copy integrant would be expected to yield vector-specific bands greater than 1851 bp. Because the major bands
detected in this blot were larger than this, we concluded that they were consistent with integrated copies of AAV vector sequences.

Finally, genomic DNA was extracted from vCWRHIVAPAP-transduced cells and digested with BamHI, from an additional donor (donor KS). Analysis of this radioautograph showed multiple bands of HMW. These bands ranged from approximately 2.3 to 7 kb in size, with predominant bands at 7 kb, 5.1 kb, and 3.6 kb. As for donor DS, our analysis showed bands greater than 1.85 kb, indicating integration of vector sequences. Although some BamHI bands less than 4.1 kb from either donor (DS or KS) may correspond to episomal 2× copies (Table 1), all bands greater than this 4.1-kb boundary must represent integrated junction fragments. It is also important to note that two donors transduced with the same vector, digested with the same restriction enzymes, and probed with the same probes yielded bands of different, albeit discreet, sizes. These differences are attributed to restriction site polymorphism in the chromosomal DNA.

No vector-specific bands were ever detected in untransduced CD34 cells. Although FISH analysis is not strictly quantitative, our results suggest that AAV vector transduction resulted in integration of vector sequences into chromosomal DNA in CD34 cells.

FISH analyses of transduced cells. We next confirmed AAV vector integration and quantitated integration frequencies in transduced umbilical cord blood CD34 cells by FISH analysis of cytogenetically prepared chromosomal spreads. The vCWRHIVASVN clonal cell line, HIIC21, served as a positive control (Fig 3A). The parent cell line (293) did not hybridize to vector-specific probes (Fig 4A). Each metaphase spread of HIIC21 showed the presence of vector-specific signal (hybridized to an Hpa I-SnaBI fragment of vCWRHIVASVN) on each sister chromatid of a single chromosome per cell (Fig 4B).

CD34 cells from two separate cord blood samples (LC and FG) were transduced with vCWRHIVASVN (MOI, 0.2) and were maintained in culture in the absence of G418 selection for either 1 (LC) or 2 (FG) weeks. Before harvest for FISH, the cells were stimulated to divide by culture in media containing higher concentrations of cytokines (see the Materials and Methods). During this stimulation period, the cells were blocked in metaphase by treatment with colcemid. Representative metaphase spreads are shown in Fig 4C through F. To avoid false-positive results, metaphases were scored as positive only if hybridization signals were observed visually on both sister chromatids of a given chromosome.

One week after transduction at an MOI of 0.2, 3 of 110 (2.7%) nuclear spreads examined from donor LC had positive signals similar to those shown in Fig 4D. The corresponding untransduced cells had no such signals (Fig 4C). Approximately 3.5% positive nuclei (7 of 198) were observed in donor FG 2 weeks after transduction (Fig 4F). As before, the untransduced CD34 cells were negative (Fig 4E), indicating that the detected signals were specific for the vector.

Although FISH analysis is not strictly quantitative, our results suggest that a minimum of 3% of cord blood CD34 cells in metaphase had integrated vector-specific signals at the same location on both sister chromatids when transduced at an MOI of 0.2, 1 to 2 weeks posttransduction in the absence of selective pressure. Additionally, the presence of integrated transgene sequences on both sister chromatids in metaphase spreads, indicated successful replication of the integrated vector during cellular DNA synthesis. Thus, the results of the FISH data corroborate the results of the Southern analyses and show that the AAV vectors are capable of integrating into the chromosomes in a population of transduced CD34 cells.

Neotransduction of CD34 progenitors of colony-forming cells (CFCs). We next tested whether AAV transduction resulted in gene transfer into BM-derived CD34 hematopoietic progenitor cells capable of myeloid colony formation.

### Table 1. Expected Sizes of Vector-Specific Bands in Transduced CD34 Cell Populations

<table>
<thead>
<tr>
<th>Vector Enzyme</th>
<th>Probe</th>
<th>Bands detected</th>
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<tbody>
<tr>
<td>CWRSP-MDR SalI</td>
<td>Probe 1</td>
<td>JM</td>
</tr>
<tr>
<td>CWRHIVAPAP BamHI</td>
<td>Probe 2</td>
<td>DG</td>
</tr>
<tr>
<td>CWRHIVAPAP BamHI</td>
<td>Probe 3</td>
<td>DS KS</td>
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Sizes of the predicted vector-specific bands are shown in base pairs for various configurations of AAV vector genomes. Also shown are the actual sizes of bands observed in Southern analyses of transduced CD34 cells (Fig 3).
Fig 4. FISH of vector sequences in CD34+ cells. (A) The parent untransduced 293 cell line (human embryonic kidney cells) and (B) a transduced cell line, HICZ1, bearing one copy of integrated vector, vCWRHIVASVN, serve as negative and positive controls for FISH. (C and D) Representative FISH analysis of CD34 suspension cultures from donor LC at 1 week after culture initiation and transduction: (C), untransduced cell; (D), metaphase from cells transduced with vCWRHIVASVN at an MOI of 0.2. (E and F) FISH analysis of CD34 suspension cultures from donor FG 2 weeks after culture initiation and transduction: (E), untransduced CD34 cell; (F), metaphase from cell transduced with vCWRHIVASVN at an MOI of 0.2. All untransduced and their corresponding transduced cells were photographed using the same exposure time to guarantee similar levels of background. A 3.6-kb HpaI-SnaBI fragment was isolated from pCWRHIVASVN, was labeled with digoxigenin-deoxyuridine triphosphate by nick-translation (Boehringer Mannheim), and was used as the probe.

(colony-forming unit–granulocyte-macrophage) in vitro. Cells isolated from three individuals were transduced with vCWRHIVASVN, containing the NeoR gene under control of the SV40 early promoter, at an MOI of 3. After 4 days of suspension culture without G418, cells were placed in methylcellulose either with or without G418. The concentration of G418 used, 1,000 μg/mL (active drug), had previously been determined to be 100% lethal to CD34 progenitor cells. Figure 5A shows colony formation by transduced and untransduced cells grown in the presence of IL-3 alone, GM-CSF alone, or both. The absence of colony formation by untransduced CD34 cells in the presence of G418 confirmed that an appropriate concentration of G418 was used (Fig 5A, group 4). In contrast to untransduced cells, significant colony formation by transduced CD34 cells was observed in the presence of G418 (Fig 5A, group 1), indicating the expression of the NeoR transgene in these cells. With either IL-3 alone or GM-CSF alone, colony formation by
transduced cells in G418 was equal to or slightly lower than that in the absence of G418. However, in the presence of both IL-3 and GM-CSF, many more colonies were obtained, a result attributed to experimental variability. The transduction efficiency of vCWRHIVASVN was calculated as the fraction of colonies derived from transduced cells in the presence of G418 relative to those in the absence of G418 (Fig 5A, groups 1 and 2). A mean of 175 colonies in the presence of G418 after culture in the presence of IL-3, IL-6, and GM-CSF, vCWRHIVASVN-transduced and untransduced cells served as controls. Figure 6 shows transduction frequencies of 67% when cells were transduced before cytokine exposure, as compared with those of 68% when cells were transduced 48 hours after culture with cytokines. Similar frequencies of transgene-expressing cells in both treatment groups at 7 days posttransduction suggested that prestimulation of CD34 cells with cytokines was not required for optimal AAV-mediated gene transfer. Adherent cells in the cultures, including macrophages, also showed a similar frequency of PLAP-expression in both groups. Analogous results were obtained when G418-resistant myeloid colony formation was analyzed from CD34 cells transduced with vCWRHIVASVN either before or 48 hours after cytokine stimulation (data not shown). These results suggest that cytokine prestimulation was not required for optimal AAV transduction.

**DISCUSSION**

This study shows that a series of AAV vectors encoding different transgenes efficiently transduced CD34+ cells derived from either normal human BM or human umbilical cord hematopoietic progenitor cells obtained from 20 different donors. Transduction of CD34 cells resulted in transgene expression in a significant population of cells when analyzed 1 to 3 weeks after transduction. Additionally, evaluation by two independent methods suggests that AAV transduction resulted in integration of vector sequences into CD34 chromosomal DNA within 7 days after transduction. Finally, these studies also show that, unlike retroviral vector transduction, transduction of CD34 cells using AAV vectors does not require cytokine prestimulation.

In all cases described here, gene transfer was evident, independent of the transgene or selective pressure. Use of one vector encoding the NeoR selectable marker under the SV40 early promoter control allowed for positive selection of transduced cells. Another vector, encoding the thermostable human PLAP gene, did not confer a growth advantage.
Integration of AAV Vectors in CD34 Cells

Transgene expression was observed in myeloid colonies differentiating in vitro from transduced CD34 cells, with the numbers of transduced CFCs closely approximating the maximal number expected at the multiplicity of transduction used in each experiment. Importantly, both the recombinant vector titer as well as assessment of transduction frequencies were determined by actual transgene expression. This method of titering allowed for selective quantitation of only intact vector genomes that were competent for both encapsidation and transduction. Using functional titers to calculate the multiplicity of transduction, the incidence of transgene expression was found to be directly proportional with the MOI on most cells. Equivalent levels of colony formation by both transduced and untransduced cells grown in the absence of selective pressure suggested that AAV transduction did not inhibit myeloid differentiation in vitro. Importantly, no deleterious or toxic effects of AAV transduction were observed as judged by colony number, size, and morphology. To date, we have tested AAV-mediated gene transfer into the BM and human umbilical cord-derived CD34 cells from more than 50 individuals of varying ethnic backgrounds, with significant levels of transduction being observed in most samples. These results suggest that AAV may use a relatively conserved mode of entry into human CD34 cells. However, cells from a few donors (notably, cord blood donors) showed lower transduction frequencies than expected, indicating a possible genetic or physiological basis of AAV transduction.

Equivalent levels of AAV-mediated gene transfer ob-
erved when cells were transduced either before cytokine treatment or 48 hours poststimulation indicated that cytokine-mediated induction of proliferation and/or differentiation was not obligatory for AAV transduction. Similar results were recently reported with cord blood CD34 cells. These findings are in concordance with recent reports from our laboratory showing that AAV-mediated transduction of cells arrested in DNA synthesis was equivalent to that of those in logarithmic growth. These results have several implications for the use of AAV vectors for the genetic modification of the hematopoietic system. Pluripotent hematopoietic stem cells are postulated to be quiescent, with only a limited number being in active cell cycle at any given time. To date, the only other integrating viral vector systems available for gene therapy are retrovirus-based and require active cell division for stable integration. Thus, retrovirus-mediated gene transfer into hematopoietic progenitor cells requires cytokine prestimulation. Because specific cytokines and culture conditions that maintain the totipotential characteristics of stem cells are yet to be defined, prestimulation with cytokines may pose a significant impediment to stem cell gene therapy. Cytokine-mediated activation of hematopoietic stem cells renders them accessible to retroviral transduction; however, such treatment also results in lineage commitment, thus altering the self-renewal capacity and the ultimate fate of transduced cells.

Unlike the findings of Alexander et al., in which the induction of DNA damage was required for efficient AAV transduction of nondividing human diploid fibroblasts, our results and those of others indicate that transduction efficiencies ranging from 50% to 100% are observed in a reproducible fashion in unmanipulated, normal CD34 cells that have not undergone prior treatment with DNA-damaging agents. Also contrary to the findings of Halbert et al., our results suggest that AAV vectors transduce primary human cells at high efficiencies. Recently, efficient AAV transduction of cord blood hematopoietic progenitor cells was shown by Zhou et al., who assayed for Neo transgene expression and by DNA for PCR analyses. Wild-type AAV infection of nonhuman primate CD34 cells was shown by PCR analysis. AAV transduction of the Fanconi anemia complementation group C into peripheral blood CD34 cells was shown by clonogenic assays and RNA PCR analysis. Miller et al. have also described AAV transduction of the γ-globin gene into human erythroid CFCs. However, direct analyses of AAV vector integration into chromosomal DNA of primary CD34 cells from multiple donors have not yet been reported.

To carefully determine whether transgenes can be stably inherited by the progeny of genetically modified hematopoietic stem cells, evaluation of any gene transfer system must include analyses of the fate of the vector genome after cellular transduction. Because AAV is a single stranded DNA virus, the second strand must be synthesized before transgene expression. Such double-stranded episomal copies of the AAV vector genome may be adequate for gene expression or PCR analysis but do not predict the integration status of vector genomes. Thus, transgene expression quantified by either protein or RNA analysis is an insufficient measure of vector integration. Similarly, previous work from our laboratory and those from others suggests that PCR-based integration analyses performed shortly after transduction with a stable DNA vector such as AAV must be interpreted with caution. To circumvent these difficulties, we chose to perform direct integration analysis of transduced cells using Southern and FISH analyses. Southern hybridization of vector-Chromosome junction fragments, after restriction digests and using vector-specific probes, showed the presence of integrated sequences in CD34 chromosomal DNA. In each case, the detection of vector-specific sequences in bands larger than either unit length vector or the expected size fragments from double-stranded unintegrated vector DNA was suggestive of chromosomal integration. Integrated vector sequences were detectable within 7 days after transduction. Interestingly, a limited number of predominant vector-specific bands was observed in transduced CD34 cells. Whether the presence of this restricted number of vector-Chromosome junction fragments was caused by a preferential proliferation of a few transduced clones or a nonrandom vector integration into a finite number of sites, possibly hot spots for recombination, is currently unclear. However, given the presence of cytokines in the medium, it is unlikely that a limited number of clones outgrew the cultures in 7 days. Thus, although wild-type free, rep-negative AAV vectors do not appear to integrate into AAVS1, the possibility of vector integration into a finite number of other sites can not yet be excluded. Additionally, the presence of extrachromosomal vector sequences in unpredicted rearranged forms cannot be ruled out. However, for such structures to be detected as predominant bands by Southern analysis, it is expected that they would have to represent a significant portion of the vector genomes, thus ruling out random rearrangements. Interestingly, our results suggest that some degree of restriction site polymorphism may occur in the chromosomal flanking sequences. The mechanism of vector integration and proteins possibly involved in this event are as yet unknown. Whether the AAV-encoded rep 78 protein, which has been implicated in site-specific AAV integration, also plays a role in vector integration is unclear. There are recent reports of the association of rep 78 with certain classes of AAV virions. Whether such structures contribute to vector integration requires further investigation. Additionally, the analysis of vector-Chromosome junction sequences may provide further insight into the mechanism of vector integration.

To directly assess AAV vector integration and differentiate between the possibilities discussed above, we performed metaphase FISH analyses of transduced cord blood cells. The results of these studies definitively confirmed integration of vector sequences into CD34 cells. In accordance with the results of the Southern analyses, integrated vector sequences were detected within 7 days after transduction in the absence of selective pressure. Because only metaphases could definitively be scored as having integrated sequences, nondividing and slowly dividing interphase cells were, by nature, excluded from analysis. Additionally, although the criterion of scoring only positive sister chromatids may represent a conservative estimate of integration frequency, it was needed to exclude episomal vector genomes. The larger population
of transgene-expressing CD34 cells as compared with the fraction showing integrated vector sequences suggests that a portion of transduced cells must carry double-stranded episomal vector genomes. Whether vector sequences in some of these cells would eventually integrate or be lost before integration is unclear. We are currently determining the proportion of vector genomes that survive episomally relative to those that integrate in CD34 cells.

Most importantly, FISH analyses showed the presence of integrated vector sequences on both sister chromatids in metaphase chromosomes, indicating that vector sequences, along with chromosomal DNA, could be replicated during cellular DNA synthesis. These results suggest that the progeny of the transduced cells in which integration has occurred could also carry AAV-encoded transgenes, suggesting the use of AAV vectors for stem cell gene therapy.

AAV vectors have recently been approved for use in clinical gene therapy for cystic fibrosis based on recent observations of long-term in vivo expression of an AAV-vector-encoded cystic fibrosis transmembrane conductance regulatory gene in rabbit airway epithelial cells.44 The ability of AAV vectors to transduce and integrate in CD34+ hematopoietic progenitor cells in the absence of selective pressure suggests that AAV vectors may serve as attractive vehicles for safe and stable long-term gene therapy of the hematopoietic system. The proficiency of AAV vectors in gene transfer into primitive hematopoietic progenitor cells in long-term BM cultures44 as well as in murine BM transplantation models is currently under investigation.46

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