Efficient Site-Specific Integration of Large Transgenes by an Enhanced Herpes Simplex Virus/Adeno-Associated Virus Hybrid Amplicon Vector

Qiang Liu, Claudio F. Perez, and Yaming Wang*

Department of Anesthesia, Brigham & Women’s Hospital, Harvard Medical School, Boston, Massachusetts 02115

Received 25 August 2005/Accepted 18 November 2005

We previously demonstrated that a herpes simplex virus type 1 (HSV-1)/adeno-associated virus (AAV) hybrid amplicon vector constructed by inserting the sequences of regulatory protein (rep) and inverted terminal repeats of AAV into an HSV amplicon vector resulted in the enhanced stability of transgene expression compared to the original HSV-1 amplicon vector. However, problems related to the expression of Rep compromised its therapeutic applications. We report here a new HSV/AAV hybrid amplicon vector system that not only solved problems associated with Rep expression but also markedly improved the stable transduction efficiency of this vector. This new HSV/AAV vector is designed in a way that little or no Rep would be expressed in packaging cells, but it can be expressed in transduced cells if Cre recombinase is provided. Furthermore, Rep expression will be automatically suppressed as a consequence of Rep-mediated integration. Our results showed that the new hybrid amplicon vector yielded titers comparable to those of standard amplicon vectors. When Cre-expressing 293 cells were transduced, a low level of Rep expression was detected, and stable transduction was achieved in ~22% of transduced cells; of those cells, ~70% transduction was achieved by Rep-mediated site-specific integration. In the majority of the stably transduced cells, Rep expression was no longer observed. Our results also proved that this vector system is capable of efficiently accommodating and site-specifically integrating large transgenes, such as the full-length dystrophin expression cassette. Thus, the new HSV/AAV vector demonstrated unique advantages in safe and effective delivery of long-lasting transgene expression into human cells.

Herpes simplex virus type 1 (HSV-1) amplicon vectors are simple plasmid-based vectors that contain a replication origin (OriS) and a packaging signal (pac) of HSV-1, in addition to the transgene(s) and plasmid backbone. In the presence of the HSV-1 genome, OriS initiates the vector to replicate into a linear concatemer, while pac allows the vector concatemer to be packaged into an HSV-1 virion (6, 19, 20). Up to 150 kb of the vector DNA can be packaged into one HSV-1 virion, which normally contains multiple copies of the given ampiclon plasmid. The packaged ampiclon vector possesses all of the infectious advantages of HSV-1 virions but not the toxicity of the virus, because there are virtually no HSV-1 genes introduced (5). However, gene transfers mediated by this vector system proved to be transient, thus significantly impeding their therapeutic applications, especially in cases where long-term transgene expression was demanded. The nonintegrative state of the vector genome in infected cells is most likely responsible for the instability of transgene expression. Strategies that altered the state of vector DNA in transduced cells have indeed prolonged transgene expression to some extent (3, 17, 18, 22). However, all of these strategies encountered certain problems that compromised their effectiveness (12). In order to meet the requirements for effective and long-lasting gene transfers, further modifications are necessary.

We, as well as others, created HSV/adeno-associated virus (AAV) hybrid vectors by incorporating the integrative elements of AAV, i.e., the rep gene and inverted terminal repeats (ITR), into HSV-1 amplicon vectors. We proved that the rep HSV/AAV hybrid vectors improved the stability of transgene expression primarily through an AAV-like site-specific genomic integration (1, 4, 7, 8, 23). However, expression of Rep resulted in a 10-fold titer reduction, and the frequency of stable transduction was modest (7, 23), 2 to 7%, depending on the cell type (23). Compelling evidence suggested that a high level of expression of Rep proteins during vector packaging was responsible for the titer reduction. We observed that the titers of HSV/AAV hybrid vectors were tightly correlated with the amount of Rep expressed. For example, when the rep gene was directly driven by the HSV-1 IE4/5 promoter, which was activated by VP16 that was expressed in high levels during packaging, barely any vector was produced. In contrast, when the rep gene was driven by its native p5 promoter and was placed far away from the IE4/5 promoter, up to 2 × 10^5 transduction units (TU)/ml vector particles were produced. Furthermore, when the rep gene was excluded from the vector, the rep hybrid vector yielded a titer equal to that of a standard amplicon vector (1 × 10^6 to 6 × 10^6 TU/ml) (23). Heister et al. previously reported a similar observation (7). Thus, presumably, elimination of the expression of Rep in packaging cells will restore normal vector production. In an attempt to improve stable transduction frequency (STF), in our previous study, we had added a pair of loxP sequences into the hybrid amplicon, with the intent to excise the ITR-transgene-containing fragment from the amplicon concatemer through Cre-loxP-mediated recombination. Theoretically, the excised circular-
ized ITR-transgene-containing structure, which resembles a circularized double-stranded recombinant AAV, would serve as the substrate for Rep and thus facilitate integration. However, when the loxP-containing hybrid vector was used to infect 293 cells that expressed Cre recombinase consecutively, we observed an STF similar to that seen in non-Cre-producing 293 cells (23). It is very possible that this was due to the fact that the p5 integration efficiency element (p5IEE), the cis element necessary for Rep-mediated integration, was not included in the ITR-transgene-containing structure (13, 14). However, due to the known toxicity and other potential problems of Rep, it is not possible to include a Rep expression unit inside the ITR-flanked fragment.

In an attempt to solve the above-mentioned problems, in the present study, we redesigned the HSV/AAV hybrid amplicon. This new generation of HSV/AAV hybrid amplicon contains all the elements in the previous rep− hybrid amplicon, including the loxP sequence, but the rep68/78 coding sequence together with a simian virus 40 late poly(A) were placed upstream of the 3′ ITR, the p5 promoter was separately placed downstream of the 5′ ITR, and both were placed in a backwards orientation. We expected that, in this construct, no Rep should be expressed unless Cre recombinase is provided and that Rep expression should be terminated automatically once Rep-mediated site-specific integration takes place. It is also anticipated that the Cre-loxP-mediated deconcatenation and the inclusion of p5IEE in the linear construct will result in a higher STF. Furthermore, formalization of the circularized ITR-transgene-rep-containing structure through Cre-loxP recombination could facilitate the integration of large transgenes.

In this study, we constructed and evaluated an enhanced green fluorescent protein (eGFP)-expressing hybrid amplicon, as well as a large hybrid amplicon with a 17.5-kb insert including the full length of dystrophin cDNA and muscle creatine kinase (MCK) promoter. The results of these studies confirmed our hypotheses.

**MATERIALS AND METHODS**

**Cell culture.** Human embryonic kidney 293 (HEK293) cells (wild-type [wt] 293) were obtained from the American Type Culture Collection (Manassas, VA). Vero 2-2 cells were provided by Rozanne Sandri-Goldin (University of California, Irvine, CA). A Cre recombinase-expressing HEK293 cell line (23) was generated as described previously (23). All the cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum (HyClone, Logan, UT). Vero 2-2 cell growth medium was further supplemented with 0.4 mg/ml G418 (Invitrogen). 293− cells were selected with 2 μg/ml puromycin periodically.

**Vector constructs.** (i) HLIGp5-R. To construct HLIGp5-R, the neomycin-resistant resistance cassette in the vector HLIGN, the rep− HSV/AAV hybrid amplicon vector described previously (23) was removed to generate the vector HLIG. The p5 promoter of AAV rep was inserted downstream of the 5′ ITR of HLIG in the 3′-to-5′ orientation. The open reading frame (ORF) of rep68/78 with a simian virus 40 late poly(A) was inserted upstream of the 3′ ITR of the HLIG-plus-p5 vector in the 3′-to-5′ orientation. The resulting vector, HLIGp5-R, has a 9.7-kb total size and a 5-kb ITR-flanked fragment.

(ii) HLIDGp5-R. To construct HLIDGp5-R, a 17.3-kb BssHII-flanked fragment containing a full-length mouse dystrophin cDNA driven by an MCK promoter derived from the pCVAA vector (a gift of J. S. Chamberlain) was inserted into HLIGN at the MluI site upstream of the human cytomegalovirus (CMV) promoter that drives the eGFP reporter gene. The resulting vector, HLIDGp5-R, has a 27-kb size, and the ITR-flanked fragment is 22.3 kb.

**Vector packaging.** The helper virus-free packaging system developed previously by Fraefel et al. (5) was used to package all the amplicons used in this study. In brief, Vero 2-2 cells were transfected with a mixture of a set of five cosmids, either plasmid HLIGp5-R or HLIDGp5-R, and Lipofectamine (Invitrogen). Amplicon vectors were harvested 60 h later. Amplicon vector stocks were titrated as transduction units per milliliter by infecting BHK cells and then counting the GFP+ cells.

**Western blot analysis.** The expression of Rep proteins was evaluated using Western blot analysis as described previously (23). Briefly, cells were lysed in buffer containing 1% NP-40, 50 mM Tris, pH 8.0, and 150 mM NaCl supplemented with protease inhibitors (Boehringer-Mannheim, Indianapolis, IN). Equivalent amounts of the extracted proteins (150 μg/lane) were then separated in a sodium dodecyl sulfate–10% polyacrylamide gel and transferred onto a nitrocellulose membrane (Bio-Rad). Rep protein was identified using a mouse monoclonal anti-AAV Rep antibody (Clone 303.9; ARP Scientific, Belmont, MA) that recognizes all four Rep proteins of AAV (40, 52, 68, and 78 kDa).

**Southern blot analysis.** The genomic DNA from each of the cellular colonies was digested with either HindIII or NdeI and sequentially cloned into the plasmid pCR2.1 TOPO (Invitrogen) for sequencing.

**PCR amplification of integration junction.** Nested PCR was performed to amplify the integration junctions. Primers AAVS1R1 (5′-AAGTGCTCCGGAAAGAGCATCCCTGTG-3′) and CMV (5′-AATCTAAGGACCCCTTAAGT-3′) or AAVS1R2 and R1 (5′-AAGCTGCTCCAAAACACCAGGAT-3′) were used in the first round to amplify integrations in the flip or flop orientation, respectively. Primers AAVS1R2 (5′-CAGAAAGCAGCTTAGACCTCAAGTG-3′) and P5 (5′-TTAGGTCACCTGAGTAAGTGTT-3′) or AAVS1R2 and R2 (5′-AAGCTGATAGGGATAGT-3′) were used in the second round to amplify integrations of both orientations. The conditions for both rounds were as follows: 94°C for 4 min followed by 40 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 10 min. The product of the second PCR amplification was analyzed by 1% agarose gel. The DNA from positive bands was extracted and subsequently cloned into the plasmid pCR2.1 TOPO (Invitrogen) for sequencing.

**RESULTS**

**Design and construction of the new hybrid amplicon vector.** In this study, we developed a constructional strategy in an attempt to address the issues related to Rep expression encountered in the previous rep− HSV/AAV hybrid amplicon (23). In the previous construct, the transgene cassettes were flanked by ITRs and then further flanked by loxP. A rep68/78 cassette driven by the p5 promoter was placed downstream of the 3′ loxP. In such a construction, Rep68/78 was expressed in packaging cells. In order to eliminate Rep expression, in this modified HSV/AAV hybrid amplicon, the p5 promoter and rep ORF were separately inserted into the ITR-flanked fragment. The p5 promoter was placed downstream of the 5′ ITR in the backwards orientation. The rep ORF was placed upstream of the 3′ ITR, also in a backwards orientation (Fig. 1A). This configuration leaves rep68/78 promoterless in the linear construct and precludes its expression during packaging. However, if the Cre recombinase is provided, the combination of the two loxP sites, which flank the AAV ITRs, will form the circularized ITR-rep-transgene-containing structure, defined as the preintegration unit, and the circularized vector backbone. In the preintegration unit, the p5 promoter is located directly in
The Rep expression seen in HILGp5-R-producing Vero 2-2 cells could be the result of the recombination of either two ITRs, both placing the p5 promoter in the front of the Rep ORF, thus allowing restoration of Rep expression (Fig. 1B). Next, because the usual consequence of the Rep-mediated integration is that it frequently occurs at the ITR or p5 promoter region, Rep expression will be automatically terminated after integration as a consequence of the reseparation of the p5 promoter and Rep ORF (Fig. 1C).

A new hybrid amplicon that expresses GFP, HLIGp5-R, was constructed and packaged with a five-cosmid helper virus-free packaging system (5). As anticipated, the vector yielded titers of ~1 × 10^8 TU/ml in crude stock, which was about a 10-fold increase from the titers of the previous rep⁺ hybrid vector, HLIRGN (0.4 × 10^5 to 2 × 10^5 TU/ml), and was only slightly lower than the titers of the standard amplicon (1 × 10^6 to 6 × 10^6 TU/ml) (15).

**Dynamics of Rep expression.** To assay the expression of Rep during the packaging process, Vero 2-2 cells (packaging cells) were transfected with five cosmids containing the whole HSV-1 genome, except the packaging signal (pac) (5), together with either the previous rep⁺ hybrid amplicon (HLIRGN) (23) or the new hybrid amplicon (HLIGp5-R). Using Western blot analysis, the expression of Rep was evaluated 48 h after transfection. The result showed that there was a sharp decrease of Rep68/78 expression in the Vero 2-2 cells that were producing the HLIGp5-R vector (Fig. 2A, lane 4, and B, lane 3) compared to the Vero 2-2 cells that were producing HLIRGN vector (Fig. 2A, lane 5, and B, lane 2), thus confirming that the construct of HLIGp5-R did largely limit the expression of Rep. The Rep expression seen in HLIGp5-R-producing Vero 2-2 cells could be the result of the recombination of either two loxPs or two ITRs, both placing the p5 promoter in the front of the rep sequence, thus resulting in the formation of Rep expression units.

We reported previously that when 293⁺ cells, which constitutively express nuclear localized Cre, were infected with loxP-containing HSV-1 amplicon vectors, the amplicon concatemers were effectively deconcatenated (23). In this study, 293⁺ cells and wt 293 cells were infected with HLIGp5-R at an MOI of 1, and 24 h later, the cells were lysed to determine Rep expression. The expression of Rep68/78 in 293⁺ cells was confirmed by Western blot analysis (Fig. 2A, lane 3), which indicated the formation of preintegration units and proved the feasibility of the p5 promoter’s ability to transcribe the rep gene even though the ITR-loxP-ITR was sandwiched in between (Fig. 1B) the promoter and rep68/78 cDNA. In the lysate of infected wt 293 cells, a very faint band of Rep68/78 was observed (Fig. 2A, lane 2), which likely corresponded to the residues of Rep68/78 from the vector solution.

The protein extracts from 11 293⁺ colonies stably transduced by HLIGp5-R were also assessed for the expression of Rep by Western blot analysis. Rep68/78 expression was undetectable in 8 out of the 11 colonies analyzed, and in the remainder of the colonies, only a low level of Rep expression was observed (Fig. 2A, lanes a to d, and B, lanes e to I). This result indicated that Rep expression could be terminated in the majority of stably transduced cells as a result of Rep-mediated integration.

**Site-specific integration.** Having confirmed that Rep expression could be induced by Cre, we then examined whether or not the amount of Rep expressed was adequate to mediate site-specific integration. Southern blot analysis was performed to determine the frequency of AAVS1 site-specific integration. The genomic DNA of 32 colonies of 293⁺ cells that were stably transduced with HLIGp5-R was analyzed. The DNA was first digested with HindIII, which generates a 6-kb AAVS1 band in the wt human genome, 0.6- and 9.1-kb fragments in HLIGp5-R plasmid, and 0.5- and 4.2-kb fragments in the preintegration unit. The cuts are at the 3’ site of the GFP gene (Fig. 1A). The blots were probed with the transgene probe derived from a 0.7-kb PmeI-flanked GFP cDNA (Fig. 1A). GFP-positive

---

**FIG. 1.** Schematic of the new HSV/AAV hybrid vector design. (A) The eGFP expression hybrid vector. The p5 promoter and rep ORF in the backwards orientation are placed separately at the ends of the loxP-ITR-flanked transgene fragment. (B) As a result of Cre-loxP recombination, the packaged hybrid amplicon vector will generate multiple copies of two types of circularized structures: (i) the preintegration unit, containing the transgene and Rep expression cassette with ITR-loxP-ITR in between the promoter and ORF, and (ii) the backbone of the vector. (C) As a result of Rep-mediated site-specific integration, the preintegration unit will be linearized at the ITR or p5 promoter region, and then rep expression will be interrupted.

**FIG. 2.** Rep expression profile. (A and B) Lane 1, control; (A) lane 2, wt 293 cells infected with HLIGp5-R; (A) lane 3, 293⁺ cells infected with HLIGp5-R showing a low level of Rep68/78 expression; (A) lane 4 and (B) lane 3, 2-2 cells that are producing HLIGp5-R; (A) lane 5 and (B) lane 2, 2-2 cells that are producing HLIRGN, our first Rep⁺ hybrid vector, showing abundant Rep68/78 expressed compared to the 2-2 cells that are producing HLIGp5-R, shown in (A) lane 4 and (B) lane 3. SM, the size marker, showing the position of 60 kDa. (A) lanes a to d and (B) lanes e to I, 293⁺ clones that were stably transduced by HLIGp5-R. In 3 of the 11 clones, there was a low level of Rep expression observed.
bands in various sizes were seen in different colonies (see Fig. 3B for examples). None of the 32 colonies showed an intense 9.1-kb GFP-positive band, the sign of concatemer integration that occurred during standard ampiclon integration. After the radioactive signal had been stripped off, the blots were rehybridized with the AAVS1 probe derived from a 1.1-kb BamHI/PvuII AAVS1 fragment, which was located in the 5′ end of the AAVS1 locus. Shifted AAVS1 bands were detected in 26 of the 32 colonies (see Fig. 3A for examples). In 18 of those 26 colonies, the shifted AAVS1 bands had the same size as their GFP counterbands, indicating that in 56% of the 32 colonies, the transgene was integrated into the AAVS1 locus. Since the Rep-mediated integration could occur in either the flip or flop orientation with equal frequency, by digesting the genomic DNA with HindIII that cuts at the 3′ site of GFP gene and probing the blot with the AAVS1 probe that only presented a small proportion of 5′ AAVS1 sequence, one could demonstrate the occurrences of site-specific integration in the flip orientation only. Therefore, we next used NdeI, which cuts the vector at the 5′ site of the GFP gene (Fig. 1A), to digest the genomic DNA of the colonies that did not show matched GFP+ and shifted AAVS1 bands in the Southern blot result. Using the same procedure and probes, the Southern blot result showed that three more clones now had matched GFP+ and AAVS1 bands (Fig. 3C and D, clones 2, 5, and 7). In addition, another two clones with GFP+ bands that were very close to the size of wt AAVS1 most likely also had the transgene integrated in the AAVS1 site because shifted AAVS1 bands were shown in previous Southern blot results. Altogether, the results demonstrated that in more than 70% (23/32) of the stably transduced 293+ cellular clones, the preintegration unit was integrated at the AAVS1 site, an efficacy which has previously been achieved only by wt AAV infection. These data strongly suggested that the amount of Rep expressed through Cre-loxP recombination in the configuration of the new hybrid vector was sufficient to carry out the function of site-specific integration. To assess whether the backbone of the vector integrated into the cell genome, all the blots were also stripped and hybridized with a probe derived from the vector backbone. With this probe, there was no positive signal detected in any of the 32 colonies analyzed (data not shown).

In an attempt to understand the integration mechanism, nested PCR was performed to amplify the integration junctions, followed by sequencing analysis. The genomic DNA of 293+ cells that were infected by HLIGp5-R at an MOI of 1 was extracted 48 h after infection. In order to amplify the junctions of both flip and flop integrations, two sets of primers for the transgene, one specific for p5 and another specific for the rep open reading frame, were used against a set of primers for the 5′ site of the AAVS1 site. The PCR products were cloned, and seven clones with different sizes, most likely representing seven individual junctions, were sequenced. The results showed that these clones contained either AAVS1, ITR, and p5 sequences in tandem or AAVS1 and rep sequences in tandem. In some of the clones, the ITR sequence was not recognizable, and for others, the remaining ITRs were always only partial. There was always a short unidentifiable sequence in between the ITR and AAVS1. It could be the mutated loxP sequence. If the rep ORF was included, the sequence of rep was intact. However, if p5 was included, deletions in the 5′ region of p5 were always observed. There were small ranges of deletions, insertions, and mismatches seen in the adjacent AAVS1 sequence. Figure 4 shows the junction arrangement. They represent the typical Rep-mediated integration junctions, indicating that the conventional AAV-like integration mechanism took place in the process. Although it has been previously reported (13, 14) that p5 but not the ITR is the necessary cis element for Rep-mediated site-specific integration, we found that in four out of five clones in which the p5 sequence was detected, there was an ITR sequence in between AAVS1 and p5, indicating that the ITR was involved in the integration.

Stability of gene transduction. We next evaluated the STF of the vector HLIGp5-R in 293+ cells in comparison with the STF in wt 293 cells. As additional controls, the STFs of two vectors,
that the STF of HILGp5-R in 293 STF, we compared the STFs yielded in 293/H11001 when these vectors were used. Though only a small amount of Rep was expressed transiently in the deconcatenated HSV-1 amplicon vectors, even integration mechanism of wild-type AAV worked very efficiently. These results strongly demonstrated that the Rep-mediated integration mechanism of wild-type AAV worked very efficiently in the deconcatenated HSV-1 amplicon vectors, even though only a small amount of Rep was expressed transiently when these vectors were used.

To assess whether increasing the MOI could further improve STF, we compared the STFs yielded in 293+ cells by HLIGp5-R at MOIs of 0.1, 1, and 5, respectively. The results showed that there was no significant difference in the STF at different MOIs (Table 1). In fact, at an MOI of 5, significantly fewer total colonies were counted when the same number of cells was plated, suggesting that more cells were initially lost at higher MOIs (data not shown). This could be related to the excessive expression of GFP and/or Rep, both of which were toxic to cells (10, 16), and/or that a significant amount of host shutoff proteins were brought into the cells by multiple virions (9).

**Integration of large transgenes.** In the HSV/AAV hybrid vector context, the ITR-flanked transgene cassette is embedded into the vector genome that is analogous to an integrated AAV genome. In order to move the ITR-flanked transgene cassette to the cellular genome, the first step would be to release the cassette from the vector genome, a process known as rescue. Although the underlying mechanism of the rescue remains elusive, a recent report has suggested that the so-called rescue was achieved by Rep-mediated replication that was initiated at the ITR (24). Since the size of AAV is relatively small, 4.7 kb, it is questionable whether or not Rep is capable of replicating genes that are larger than the size of AAV itself. In this new version of hybrid vector, through Cre-loxP recombination, the fragments containing ITRs and the transgene(s) will be excised from vector concatemers, and therefore, the need for the rescue step will be eliminated. To test this hypothesis, we added a 17.3-kb fragment of a full-length mouse dystrophin cDNA driven by a 3.3-kb full-length muscle MCK promoter into the ITR-flanked locus of HLIGp5-R. The resulting vector, HLIDGp5-R (Fig. 5A), yielded titers of up to 8 x 10^5 TU/ml in unconcentrated vector solution that were only slightly lower than the titers of HLIGp5-R. Since GFP was also expressed, this vector was evaluated for its STF in 293+ cells, and this was compared to the STF of the same vector in wt 293 cells using the same approach as described above. The results showed that at an MOI of 1, HLIDGp5-R yielded 12.4% (±2.5%) STF in 293+ cells and 2.6% (±1.3%) STF in 293+ cells. We also assessed the site-specific integration frequency of HLIDGp5-R using Southern blot analysis. The genomic DNA of 17 clones of 293+ cells stably transduced by HLIDGp5-R was digested by NdeI and then hybridized by the AAVS1 site probe followed by the GFP probe, as described above (Fig. 5A). The results showed that shifted AAVS1 bands were observed in 10 out of the 17 clones and that 5 of those were also positive for the GFP probe (Fig. 5B and C), indicating that in 29% of the tested colonies, the transgene was integrated into the AAVS1 site in the 3'-to-5' orientation. Presumably, there would be about the same number of colonies integrated into AAVS1 in the reverse orientation, which could not be shown by this set of probes. To demonstrate whether or not the 22-kb insert was integrated as a whole, the genomic DNA of the same 17 clones was digested with KpnI, and the blot was hybridized with a dystrophin probe derived from an 8-kb NdeI fragment from HLIDGp5-R (Fig. 5A). The expected 12-kb positive band was observed in 16 out of the 17 colonies, confirming the genomic integration of the entire length of mouse dystrophin cDNA (Fig. 5D). There was no radioactive signal observed in the control 293+ genomic DNA, thus excluding the possibility that the human dystrophin gene is hybridized by the probe. These data demonstrated that the strategy described here not only allows reasonable vector production but also circumvents the rescue step and thus permits the possibility of stably transducing large transgenes into the human genome in a site-specific manner using the wt AAV integration mechanism.
DISCUSSION

Although Rep-mediated site-specific integration is desirable, the toxicity and potential harmfulness of Rep have undermined its potential. In rep-containing adenoviral and HSV-1 hybrid vectors, the expression of Rep in packaging cells has been shown to severely reduce vector production (7, 15, 23). In addition, high-level constitutive expression of Rep in transduced cells might retard cellular proliferation, disturb cellular function, and disrupt the sequence of the AAVS1 locus (16). Even for a latently integrated Rep expression unit, the potential risk of losing transgene expression due to the reactivation of Rep expression remains a concern. In a situation such as helper virus infection or other drastic stressors, the rep gene could be activated and the integrated transgene could be excised from the cell genome. In this study, we have demonstrated that all of the above-described potential problems could be alleviated, at least in the context of the HSV-1/AAV hybrid amplicon.

The strategy described enables not only the precise regulation of Rep expression but also the deconcatenation of amplicon genomes in a single step, thus circumventing the dilemma associated with Rep expression as well as facilitating Rep-mediated stable transduction. The new hybrid vector yielded a markedly improved STF in 293<sup>+</sup> cells compared to the STF produced by our first rep<sup>+</sup> hybrid amplicon (22% versus 7%) (23). However, in wt 293 cells, the new hybrid vector had the same STF as a standard amplicon (2%) (23). Clearly, the Cre-loxP-mediated deconcatenation played a central role in facilitating this increase. In addition to inducing Rep expression, which was certainly the most important factor, other factors resulting from deconcatenation could have contributed to the marked improvement of STF as well. First, deconcatenation generates more substrates for the Rep-mediated integration. Although multiple copies of the transgene(s) are delivered with a single HSV-1 virion, the concatemer of the packaged HSV-1 amplicon vector genome acts no differently than a single copy of the plasmid. Deconcatenation disconnects the transgene cassettes from each other, and as a result, multiple copies of the freed transgene could behave independently. It was reported that in wt AAV, the integration frequency depended on the number of viral copies (11). This should be the same for the hybrid vector, and thus, the increased number of transgene copies through deconcatenation should increase the STF. Second, deconcatenation should eliminate the necessity of rescuing the ITR-transgene fragment from the amplicon concatemer, which was likely to have been a limiting step of the integration mediated by our first rep<sup>+</sup> hybrid amplicon. In wt AAV, Rep-mediated amplification takes place only in the lytic stage of the life cycle, where Rep expression surges due to the existence of helper virus. Otherwise, low-level Rep expression normally results in a latent infection where the viral genome goes to integration without amplification (2). It is questionable whether the amount of Rep expressed from this hybrid vector is adequate to amplify enough copies of ITR-flanked transgene cassettes for integration and whether Rep is capable of amplifying transgenes that
are larger than wt AAV itself. In the new hybrid vector, through Cre-loxP recombination, the ITR-rep-transgene-containing preintegration unit, which resembles the circularized double-stranded wt AAV, will be preformed and ready for integration. Thus, the amplification step is no longer needed. Third, and perhaps most importantly, the sequence of the p5 promoter was included in the preintegration unit. In our previous study, we observed that the deconcatenation of the first rep + hybrid ampiclon resulted in no further increase in the STF compared to the nondeconcatenated ampiclon vector. This was probably because in the first rep + hybrid ampiclon, the rep cassette was placed outside of the loxP sites, and therefore, the p5 promoter was not included in the preintegration unit.

In other words, that preintegration unit was equivalent to a recombinant AAV. Philpott et al. demonstrated that a 138-bp cis element inside the p5 promoter sequence, namely, the p5EE, is a necessary element for Rep-mediated site-specific integration (13, 14). Most likely, the increased STF seen in the present study was the result of the inclusion of the p5 promoter in this preintegration unit.

We examined whether the new hybrid vector can stably transduce the full-length dystrophin cDNA to human cells efficiently through site-specific genomic integration. We first constructed a vector containing a GFP-fused full-length human dystrophin cDNA under the transcriptional control of a CMV promoter and infected 293 cells. Despite the fact that we did observe the formation of GFP+ colonies and even detected dystrophin expression in these cells, we observed a high rate of cell death, which made the quantitative analysis difficult (data not shown). We reasoned that the forced expression of dystrophin in 293 cells, which normally do not express this protein, could be the cause of cell death. Alternatively, we constructed another hybrid vector in which a GFP reporter gene was expressed from a CMV promoter and a full-length mouse dystrophin was expressed from a full-length MCK promoter so that no dystrophin would be expressed in 293 cells. This vector stably transduced 12% of infected 293+ cells. Of those, ~50% were integrated into the AAVS1 site. Although the STF yield in the large vector was less than that in the small vector (~12% versus ~22%), it is the first demonstration of efficient integration of a large transgene into the human genome in a site-specific manner that is feasible. The lower STFs seen in the large-gene transduction could be the result of fewer copies of preintegration units generated through Cre-loxP recombination. A large ampiclon contains fewer copies of preintegration units than a small ampiclon. Furthermore, Cre might function less efficiently in large constructs than in smaller ones, since loxP sites are farther apart in large constructs. Another possibility is that Rep might integrate large preintegration units less efficiently. The 27-kb vector size should not be the upper limit, because an HSV-1 ampiclon vector can hold up to a 150-kb DNA fragment (21). When Cre-loxP is used to form preintegration units, there is a high probability that the whole genome of the packaged ampiclon vector could be integrated as a single piece at the specific locus of the human genome, which would provide us enormous freedom in achieving regulated gene transduction.

While the strategies of efficiently delivering Cre recombination and selectively excluding cells in which the transgene is not integrated in AAVS1 site need to be developed, we have demonstrated in this study that the new HSV/AAV hybrid ampiclon vector system is an advanced gene delivery tool capable of mediating long-lasting and safe transgene expression for probably any given gene described so far. Regulated Rep expression made it possible to utilize only the function of Rep without its deleterious effects. The facts that up to ~22% of infected cells were stably transduced by this vector and that more than 70% of those cells were stably transduced with the transgene being targeted to the AAVS1 site suggest that the system allowed the integration mechanism of wt AAV to reach its full potential, as long as adequate Cre was supplied. With the combination of the AAV-like transduction stability and the multiple distinct advantages of HSV-1 ampiclon vectors, the hybrid HSV-1 ampiclon vector system now has a much broader spectrum of applications than any other viral vector system. In particular, its ability to stably express large transgenes, such as the full-length dystrophin, in dividing and nondividing cells with minimal risk caused by genomic insertion will have an important impact in the realm of gene therapy.

ACKNOWLEDGMENTS

We thank P. D. Allen for scientific advice and critical reading of the manuscript; W. Liu, R. Hirsch, S. Mukherjee, and A. F. Flint for technical assistance; and M. Niemann for English editing. This work was supported by grants from MDA to Y.W., NIAMS K01 AR02148 and K02 AR051181 to Y.W.

REFERENCES

1. Bakowska, J. C., M. V. Di Maria, S. M. Camp, Y. Wang, P. D. Allen, and X. O. Breakefield. 2003. Targeted transgene integration into transgenic mouse fibroblasts carrying the full-length human AAVS1 locus mediated by HSV/AAV rep (+) hybrid ampiclon. Gene Ther. 10:1691–1702.
2. Berns, K. L., and C. Giraud. 1996. Biology of adeno-associated virus. Curr. Top. Microbiol. Immunol. 218:1–23.
3. deFelice, P., M. Izquierdo, F. Wandosell, and F. Lim. 2001. Integrating retroviral cassette extends gene delivery of HSV-1 expression vectors to dividing cells. BioTechniques 31:394–402, 404–405.
4. Fraelich, C., D. R. Jacoby, C. Ljung, H. Hiderbrand, J. Y. Chow, F. W. Alt, X. O. Breakefield, and J. A. Majozub. 1997. Gene transfer into hepatocytes mediated by helper virus-free HSV/AAV hybrid vectors. Mol. Med. 3:813–825.
5. Fraelich, C., S. Song, F. Lim, P. Lang, L. Yu, Y. Wang, P. Wild, and A. I. Geller. 1996. Helper virus-free transfer of herpes simplex virus type 1 plasmid vectors into neural cells. J. Virol. 70:1790–1797.
6. Geller, A. I., and X. O. Breakefield. 1988. A defective HSV-1 vector expresses Escherichia coli beta-galactosidase in cultured peripheral neurons. Science 241:1667–1669.
7. Heister, T., I. Heid, M. Ackermann, and C. Fraelich. 2002. Herpes simplex virus type 1/adeno-associated virus hybrid vectors mediate site-specific integration at the adeno-associated virus preintegration site, AAVS1, on human chromosome 19. J. Virol. 76:7163–7173.
8. Johnston, K. M., D. Jacoby, P. A. Pechan, C. Fraelich, P. Borghesani, D. Schuback, R. J. Dunn, F. I. Smith, and X. O. Breakefield. 1997. HSV/AAV hybrid ampiclon vectors extend transgene expression in human glioma cells. Hum. Gene Ther. 8:359–370.
9. Kwong, A. D., J. A. Kruper, and N. Frenkel. 1988. Herpes simplex virus virion host shutoff function. J. Virol. 62:912–921.
10. Liu, H. S., M. S. Jan, C. K. Chou, P. H. Chen, and N. J. Ke. 1999. Is green fluorescent protein toxic to the living cells? Biochem. Biophys. Res. Commun. 260:712–717.
11. McCarty, D. M., S. M. Young, Jr., and R. J. Samulski. 2004. Integration of adeno-associated virus (AAV) and recombinant AAV vectors. Annu. Rev. Genet. 38:819–845.
12. Oehmig, A., C. Fraelich, X. O. Breakefield, and M. Ackermann. 2004. Herpes simplex virus type 1 ampiclons and their hybrid virus partners, EBV, AAV, and retrovirus. Curr. Gene Ther. 4:385–408.
13. Philpott, N. J., C. Giraud-Wali, C. Dupuis, J. Gomos, H. Hamilton, K. I. Berns, and E. Falck-Pedersen. 2002. Efficient integration of recombinant adeno-associated virus DNA vectors requires a p5-rep sequence in cis. J. Virol. 76:5411–5421.
14. Philpott, N. J., J. Gomos, K. I. Berns, and E. Falck-Pedersen. 2002. A p5 integration efficiency element mediates Rep-dependent integration into AAVS1 at chromosome 19. Proc. Natl. Acad. Sci. USA 99:12381–12385.
15. Recchia, A., R. J. Parks, S. Lamartina, C. Toniatti, L. Pieroni, F. Palombo,
16. Schmidt, M., S. Afione, and R. M. Kotin. 2000. Adeno-associated virus type 2 Rep78 induces apoptosis through caspase activation independently of p53. J. Virol. 74:9441–9450.

17. Sena-Esteves, M., J. A. Hampl, S. M. Camp, and X. O. Breakefield. 2002. Generation of stable retrovirus packaging cell lines after transduction with herpes simplex virus hybrid amplicon vectors. J. Gene Med. 4:229–239.

18. Sena-Esteves, M., Y. Saeki, S. M. Camp, E. A. Chiocca, and X. O. Breakefield. 1999. Single-step conversion of cells to retrovirus vector producers with herpes simplex virus–Epstein-Barr virus hybrid amplicons. J. Virol. 73:10426–10439.

19. Spaete, R. R., and N. Frenkel. 1982. The herpes simplex virus amplicon: a new eucaryotic defective-virus cloning-amplifying vector. Cell 30:295–304.

20. Spaete, R. R., and N. Frenkel. 1985. The herpes simplex virus amplicon: analyses of cis-acting replication functions. Proc. Natl. Acad. Sci. USA 82:694–698.

21. Wade-Martins, R., E. R. Smith, E. Tyminski, E. A. Chiocca, and Y. Saeki. 2001. An infectious transfer and expression system for genomic DNA loci in human and mouse cells. Nat. Biotechnol. 19:1067–1070.

22. Wang, S., and J. M. Vos. 1996. A hybrid herpesvirus infectious vector based on Epstein-Barr virus and herpes simplex virus type 1 for gene transfer into human cells in vitro and in vivo. J. Virol. 70:8422–8430.

23. Wang, Y., S. M. Camp, M. Niwano, X. Shen, J. C. Bakowska, X. O. Breakefield, and P. D. Allen. 2002. Herpes simplex virus type 1/adeno-associated virus rep+ hybrid amplicon vector improves the stability of transgene expression in human cells by site-specific integration. J. Virol. 76:7150–7162.

24. Ward, P., P. Elias, and R. M. Linden. 2003. Rescue of the adeno-associated virus genome from a plasmid vector: evidence for rescue by replication. J. Virol. 77:11480–11490.