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Site-Specific Integration of Functional Transgenes into the Human Genome by Adeno/AAV Hybrid Vectors

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Uncontrolled insertion of gene transfer vectors into the human genome is raising significant safety concerns for their clinical use. The wild-type adeno-associated virus (AAV) can insert its genome at a specific site in human chromosome 19 (AAVS1) through the activity of a specific replicase/integrase protein (Rep) binding both the AAVS1 and the viral inverted terminal repeats (ITRs). AAV-derived vectors, however, do not carry the *rep* gene and cannot maintain site-specific integration properties. We describe a novel hybrid vector carrying an integration cassette flanked by AAV ITRs and a tightly regulated, drug-inducible Rep expression cassette in the framework of a high-capacity, helper-dependent adenoviral (Ad) vector. Rep-dependent integration of ITR-flanked cassettes of intact size and function was obtained in human primary cells and cell lines in the absence of selection. The majority of integrations were site specific and occurred within a 1000-bp region of the AAVS1. Genome-wide sequencing of integration junctions indicates that nonspecific integrations occurred predominantly in intergenic regions. Site-specific integration was obtained also *in vivo*, in an AAVS1 transgenic mouse model: upon a single tail vein administration of a nontoxic dose of Ad/AAV vectors, AAVS1-specific integrations were detected and sequenced in DNA obtained from the liver of all animals in which Rep expression was induced by drug treatment. Nonrandom integration of double-stranded DNA can therefore be obtained *ex vivo* and *in vivo* by the use of hybrid Ad/AAV vectors, in the absence of toxicity and with efficiency compatible with gene therapy applications.

Key Words: gene transfer, viral vectors, adeno-associated virus, adenovirus, site-specific integration

INTRODUCTION

Gene therapy of many genetic and acquired disorders requires stable integration of appropriately regulated transgenes in human somatic cells. Gene transfer vectors derived from RNA oncoretroviruses or lentiviruses integrate at high efficiency in the human genome, but in an almost random fashion and with a preference for active genes [1,2]. Retroviral vectors have limited genome capacity, do not easily accommodate introns or complex regulatory elements, and are ill suited to *in vivo* use. Adenoviral (Ad) or adeno-associated viral (AAV) DNA vectors, on the other hand, do not normally integrate or do so sporadically and with unpredictable configurations [3]. Development of an integrating, large-capacity DNA viral vector is still an unmet goal of gene transfer technology.

Development of helper-dependent (HD), fully deleted Ad vectors has significantly reduced the limitations associated with first-generation vectors, such as acute toxicity, inflammatory and cytotoxic immune response against the viral proteins, and short-term persistence of gene expression [4–10]. AAV vectors have also been used to mediate long-term gene expression in nondividing cells. The wild-type AAV genome is a linear, single-stranded DNA filament of 4.7 kb, which contains at both ends a 145-base inverted terminal repeat (ITR), encoding the origin of replication and the packaging signal, and two AAV-specific genes, *rep* and *cap* [11]. *rep* encodes four overlapping proteins of 78, 68, 52, and 40 kDa, generated by alternative splicing of transcripts from two alternative promoters. The ITRs and either Rep78 or Rep68 are sufficient for replication of the AAV genome and mediating its integration into a specific site (AAVS1) on human chromosome 19q13.3–qter [12–14]. However, due to the very limited packaging size of AAV particles, AAV vectors are deleted of all viral genes and have

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therefore lost the Rep-mediated, site-specific integration property of the wild-type virus. AAV vectors maintain a residual capacity for ITR-mediated integration in some target cells (e.g., liver), although at low efficiency and with virtually no site specificity [15–18]. AAV ITRs and Rep functions have been previously incorporated into hybrid, large-capacity viral vectors, such as baculoviral and HSV-1 amplicon vectors [19–21], or into a split Ad/AAV vector system, in which an AAV ITR-flanked integration cassette and a Rep78/68 expression cassette were carried by two different HD-Ad vectors [22]. However, the inhibitory effect of Rep on viral replication [23–25] prevented or significantly affected the production of Ad and HSV-1 vectors incorporating all the elements of the AAV integration machinery [20–22].

In this report, we describe the development of single hybrid Ad/AAV vectors carrying a double reporter gene integration cassette flanked by AAV ITRs and tightly regulated, drug-inducible Rep expression cassettes. The hybrid vectors were successfully packaged and purified at a titer of $>10^9$ transducing units/ml. Upon infection at low multiplicity of susceptible human primary cells and cell lines, Rep-dependent, AAVS1-specific integration of ITR-flanked cassettes of intact size and function was obtained with an efficiency proportional the level of Rep expression and in the absence of cell selection. Site-specific integration was studied also *in vivo,* in transgenic mice carrying one copy of a 3.5-kb fragment of the AAVS1 on the X chromosome. Upon a single tail vein administration of a nontoxic dose (2×10^8 transducing units) of Ad/AAV vectors, AAVS1-specific integrations were mapped and sequenced in DNA obtained from the liver of animals in which activation of Rep expression was induced by drug treatment. Site-specific integration of a double-stranded DNA transgene into the human genome can therefore be obtained *ex vivo* and *in vivo* by a single administration of a HD-Ad/AAV vector.

RESULTS

Construction of HD-Ad/AAV Hybrid Vectors

To overcome the negative effects of Rep on packaging and genetic instability of AAV ITR-containing Ad genomes, we developed a series of alternative constructs in which the expression of Rep78 was controlled by a tetracycline-inducible activation/repression system [26]. The prototype vector (AR1) contained an AAV ITR-flanked integration cassette with two different reporter genes, the green fluorescent protein (GFP) and a truncated version of the p75 low-affinity nerve growth factor receptor (ΔLNGFR) under the control of the phosphoglycerokinase (PGK) and the SV40 early promoter, respectively, cloned into the STK-120 HD-Ad backbone (Fig. 1). Four different vectors were derived from AR1 in the STK-119 backbone by adding Rep78 expression cassettes with different types of regulation. The AR1-CMVRepTA/TS and AR1-CMVRepTS/TA vectors contained, in opposite transcriptional orientation, a Rep78 expression cassette under the control of the cytomegalovirus (CMV) immediate-early minimal promoter (−53) linked to the bacterial *tet* operator (*tetO*-CMV) and a bicistronic expression cassette in which the tetracycline-controlled reverse transcriptional activator (rtTA) and transcriptional silencer (tTS) genes were expressed under the control of the CMV promoter/enhancer (−675) and an internal ribosomal entry site (IRES) in either configuration (TA/TS or TS/TA) (Fig. 1). In the absence of the inducer, the tetracycline analogue doxycycline (Dox), the tTS protein binds to, and represses transcription from, the *tetO*-CMV element controlling Rep expression, while in the presence of Dox the rtTA protein allows activation of *tetO*-CMV transcription at high levels. The AR1-CMVRepTS vector contained only the tTS gene under the control of a CMV promoter, while in the AR1-TKRepTS vector the Rep78 gene was under the control of the minimal promoter (−81) of the HSV-1 thymidine kinase gene (TK) linked to the *tetO* element (*tetO*-TK) (Fig. 1). The control vector, CMVRep, contained only a Rep78 expression cassette driven by the CMV promoter/enhancer in the STK-120 backbone (Fig. 1).

Control of Rep78 Expression

We assessed expression and regulation of Rep78 for all vectors by transfecting 293*Cre*4 cells with the vector plasmids in the presence and absence of Dox. We harvested the cells 24 or 48 h after transfection and analyzed their nuclear extracts by Western blotting and using a rabbit anti-Rep polyclonal antiserum. Cells transfected with the vectors expressing both rtTA and tTS (AR1-CMVRepTA/TS and AR1-CMVRepTS/TA) accumulated high levels of Rep78, independent of the configuration of the rtTA–tTS expression cassette and the presence of the inducer (not shown). In contrast, in cells transfected with the vectors expressing only the tTS suppressor (AR1-CMVRepTS and AR1-TKRepTS), basal Rep78 expression was very well controlled and induced by Dox 48 h posttransfection at levels comparable with those obtained by an unmodified CMV promoter (Fig. 2A). These vectors were therefore chosen for further analysis.

Packaging of the Ad/AAV Hybrid Vectors

We produced the HD-Ad vectors by transfection/infection of the 293*Cre*4 cell line, as previously described [22]. After five serial passages of viral amplification, the integrity of the episomal Ad vectors was controlled by Southern blot analysis of Hirt DNA extracted from crude lysates of the packaging cells. Both the AR1-CMVRepTS and AR1-TKRepTS viruses yielded the predicted restriction fragments when grown in the absence of Dox, while they rearranged when grown in the presence of Dox (results not shown). Both viruses correctly expressed the

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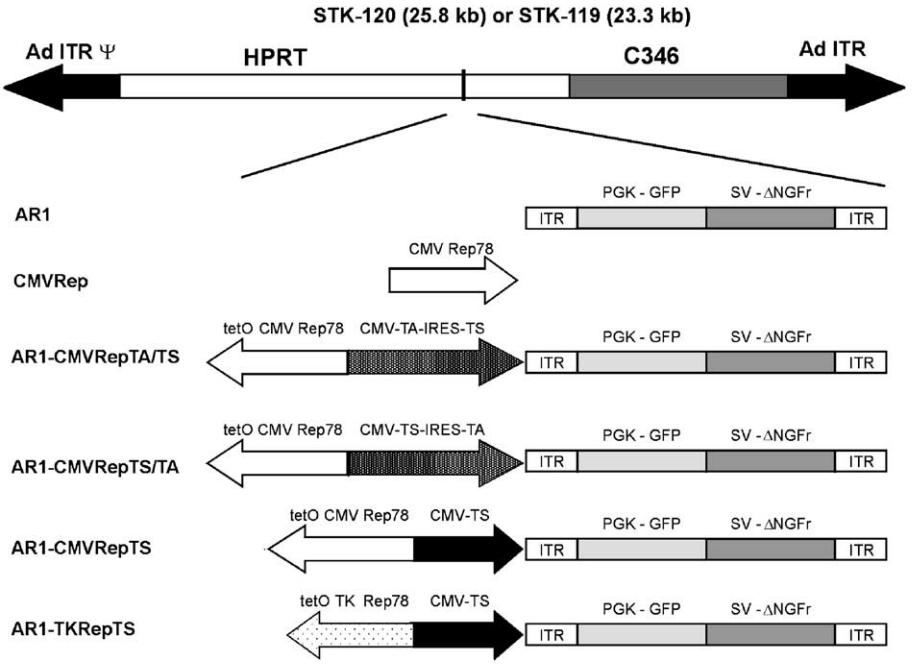

FIG. 1. Schematic representation of vector structures. Expression cassettes were inserted into the pSTK120 (25.8 kb) or pSTK119 (23.3 kb) HD-Ad plasmids. The prototype vector, AR1, contains an AAV ITR-flanked integration cassette in which expression of GFP and ΔLNGFR is driven by a PGK and a SV40 promoter, respectively. The CMVRep vector contains an AAV Rep78 ORF under the control of the CMV immediate-early enhancer/promoter (−675). In the other vectors, the AAV integration cassette is combined with an inducible Rep expression cassette, in which the Rep78 ORF is driven by a minimal CMV (−53) or TK (−81) promoter controlled by a bacterial *tet* operator (tetO) sequence, and a CMV-driven expression cassette for the tetracycline-controlled reverse transcriptional activator (TA) and transcriptional silencer (TS) genes in either configuration with respect to an internal ribosomal entry site (IRES) element (AR1-CMVRepTA/TS and AR1-CMVRepTS/TA) or the TS only (AR1-CMVRepTS and AR1-TKRepTS).

reporter genes (GFP and ΔLNGFR), as demonstrated by FACS analysis of infected HeLa cells, and Rep78, as indicated by a rescue and replication assay carried out on 293 cells superinfected with a first-generation Ad vector (not shown).

Medium-scale preparations of AR1-CMVRepTS and AR1-TKRepTS were comparable in terms of total viral particles (10^{11} vs 8.3×10^{10}), while the AR1-TKRepTS preparation was better in terms of transducing units (1.3×10^9 vs 2×10^8), virions per packaging cell (415 vs 280), and helper virus contamination (0.8% vs 5%). The different activity of the two *tetO*-controlled minimal promoters was demonstrated in HeLa cells infected at low (5 tu/cell) multiplicity of infection (m.o.i.) and maintained for 48 h in the presence or absence of Dox. Expression of Rep78 was assessed by Western blot analysis of crude nuclear extracts. Basal expression of Rep78 was low from the *tetO*-CMV promoter and virtually undetectable from the *tetO*-TK promoter (Fig. 2B). Expression of Rep78 was induced by Dox in both

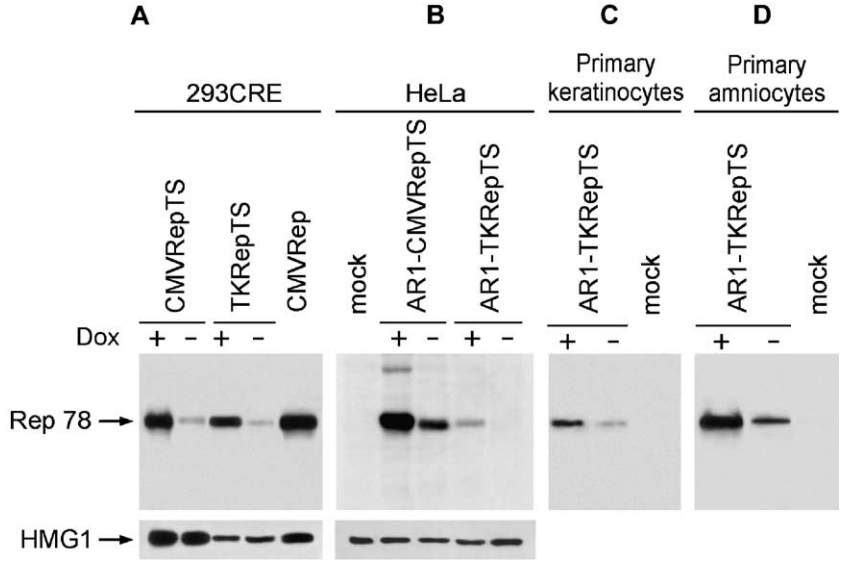

FIG. 2. Western blot analysis of Rep expression in crude nuclear extracts. (A) 293*Cre*4 packaging cells transfected with CMVRepTS and TKRepTS, in the presence (+) or absence (−) of Dox, and CMVRep. Signals were normalized with a monoclonal antibody against the chromatin-associated nuclear protein HMG1. (B) HeLa cells mock infected (mock) or infected with AR1-CMVRepTS or AR1-TKRepTS at an m.o.i. of 5 and analyzed after 48 h of culture ±Dox. (C, D) Human primary keratinocytes and amniocytes mock infected (mock) or infected with AR1-TKRepTS at an m.o.i. of 20 and analyzed after 5 days of culture ±Dox.

cases, although at much higher levels from the *tetO*-CMV promoter (Fig. 2B).

Site-Specific Integration of the AAV ITR-Flanked Cassette in HeLa Cells

We infected HeLa cells with the AR1-TKRepTS vector at an m.o.i. of 20, cultured them for 4 weeks ±Dox, and scored them for the expression of GFP and ΔLNGFR. Of the cells cultured with Dox, 1.5–2.0% maintained reporter gene expression, while cells cultured without Dox, or transduced with the control Rep$^-$ AR1 vector, scored negative already 3 weeks after infection. To analyze vector integrations, we FACS-sorted GFP$^+$/ΔLNGFR$^+$ HeLa cells and cloned them by limiting dilution. We extracted genomic DNA from all clones and analyzed them by Southern blotting after restriction with *Acc*I, which releases a 3.1-kb fragment encompassing most of the chromosome 19 AAVS1 site. Hybridization with an AAVS1-specific probe showed a size shift of the 3.1-kb band in 9 of 17 (53%) independent HeLa clones, indicating rearrangement of the AAVS1 region in at least one chromosome 19 (Fig. 3). The rearranged bands hybridized also to a GFP-specific probe in 7 of the 9 clones, indicating the integration of the AAV ITR-flanked cassette in the AAVS1 region. Further restriction analysis indicated that the AAV ITR-flanked cassette had integrated as a monomer or a dimer in the AAVS1 site and conserved an intact structure in all cases (not shown). In 2 clones (45 and 57 in Fig. 3), the AAVS1 site showed a rearranged band that did not hybridize to the ITR probe, indicating Rep-mediated disruption of the AAVS1 region in the absence of integration. Conversely, 10 of 17 clones showed bands corresponding to intact AAV ITR-flanked cassettes integrated elsewhere in the genome. Two clones (18 and 31 in Fig. 3) showed both AAVS1-specific and nonspecific integrants.

To verify whether insertion of functional *rep* genes occurred together with integration of the AAV ITR-flanked cassette, we carried out a rescue and replication assay on GFP$^+$/ΔLNGFR$^+$ HeLa clones superinfected with wild-type Ad5 virus in the presence of Dox. Hirt DNA was extracted from infected cells and analyzed by Southern blotting with a GFP-specific probe. We detected no band corresponding to the replicated form of the AAV ITR-flanked integration cassette in any of the HeLa clones, indicating that no

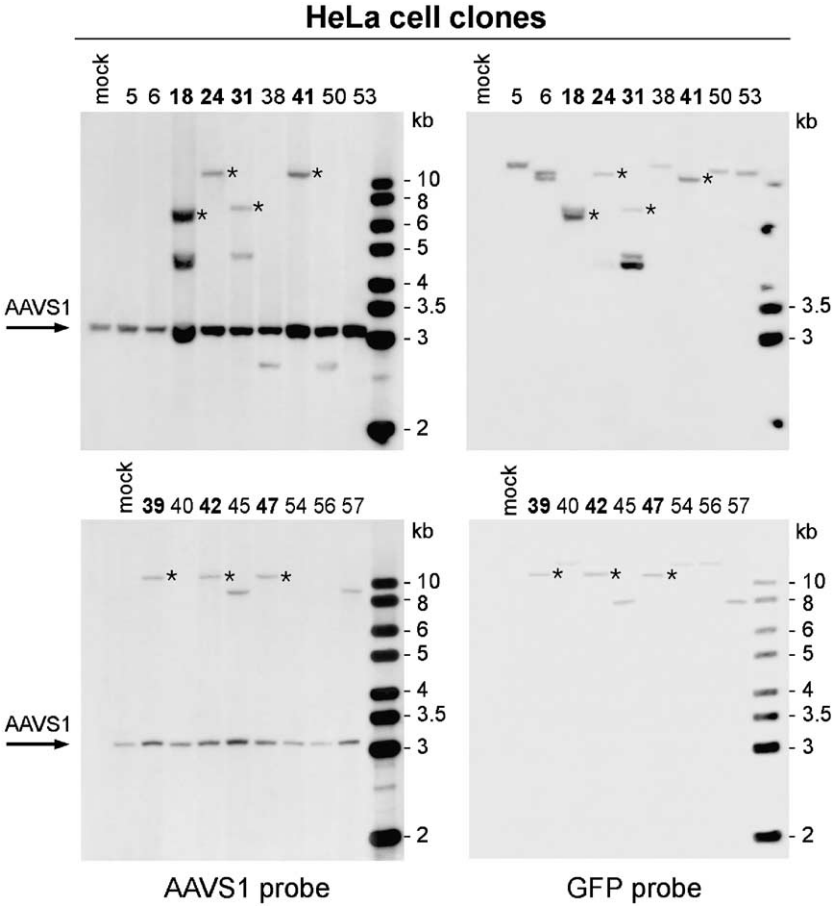

FIG. 3. Southern blot analysis of 17 independent HeLa cell clones (numbered) derived from a bulk culture of HeLa cells transduced with AR1-TKRepTS and grown in the presence of Dox. 20 μg of genomic DNA was digested with *Acc*I and hybridized to an AAVS1-specific probe (left). The 3.1-kb *Acc*I AAVS1 intact genomic fragment is indicated by an arrow. The same blots were rehybridized to a GFP probe to detect the ITR-flanked integration cassette (right). Rearranged bands hybridizing to both probes, and corresponding to AAVS1 site-specific integrations, are indicated by asterisks. HeLa clones containing site-specific integrants are indicated in bold. In all blots, the first lane contains DNA from mock-transduced cells and the last lane a size marker.

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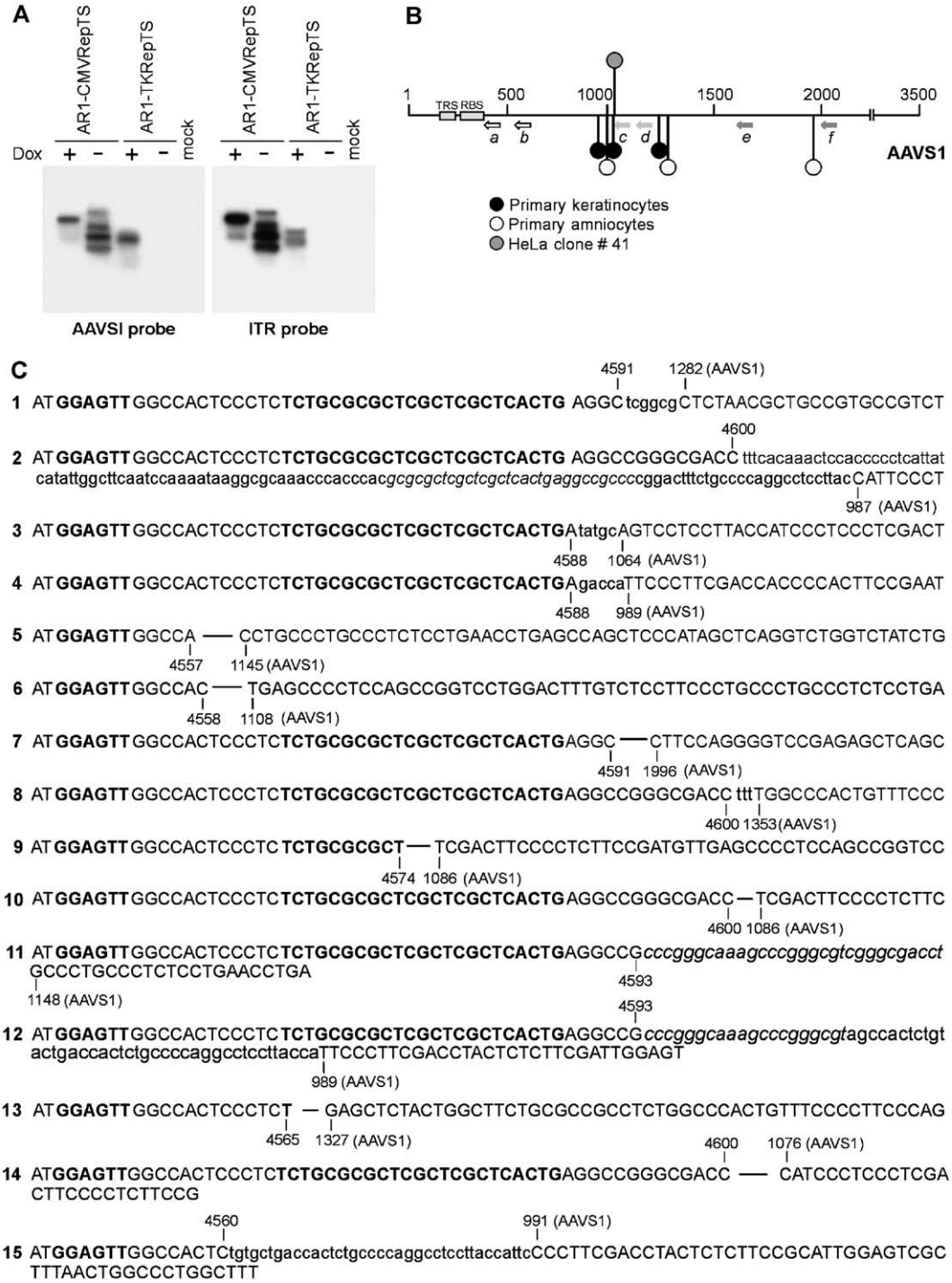

FIG. 4. Analysis of the AAV ITR-AAVS1 junctions in human primary cells infected with AR1-CMVRepTS and AR1-TKRepTS. (A) PCR amplification of ITR-AAVS1 junctions from amniocyte DNA by AAV ITR- and AAVS1-specific nested primers (c and d in B) hybridized to AAVS1 (left) and ITR (right) probes. (B) Schematic representation of the 3.5-kb AAVS1 locus. Locations of Rep-binding (RBS) and terminal resolution (TRS) sites are indicated. a–f indicate AAVS1-specific primer pairs. Integration hot spots into keratinocyte, amniocyte, and HeLa cell DNA are indicated by circles. (C) Sequences of integration junctions in DNA from amniocytes transduced by AR1-CMVRepTS (sequences 2–4) or AR1-TKRepTS (5–10) and from keratinocytes transduced with AR1-TKRepTS (11–13) or AR1-CMVRepTS (14, 15). Sequence 1 is the junction in HeLa clone 41 used as a standard in the quantitative PCR assay (Fig. 5). Breakpoints on both ITR and AAVS1 are indicated. The ITR RBS and TRS are indicated in bold. Sequence insertions at the junctions between ITR and AAVS1 are in lowercase. Partial, inverted sequences of ITR origin are in italic.

functional *rep* sequence had integrated in their genomes (not shown).

Site-Specific Integration in Human Primary Cells

We infected early passage amniocytes and keratinocytes with AR1, AR1-CMVRepTS, and/or AR1-TKRepTS at an m.o.i. of 20 and grew them for 5 days in the presence or absence of Dox. We tested for induction of Rep78 expression by Western blotting of crude nuclear extracts 5 days after infection (Figs. 2C and 2D). We extracted high-molecular-weight DNA from these cells and analyzed it by nested-set PCR with AAVS1- and AAV ITR-specific primers (c and d in Fig. 4B). As shown in Fig. 4A, DNA bands recognized by both the AAVS1 and the AAV ITR probes were amplified from amniocytes transduced by the AR1-CMVRepTS vector in the presence and absence of Dox and by the AR1-TKRepTS vector only in the presence of Dox (Fig. 4A). We detected no signal in cells transduced by the control Rep$^-$ AR1 vector (not shown).

We analyzed the distribution of integrants throughout the AAVS1 region by PCR using three couples of nested primers annealing to different positions within AAVS1 (a and b at nt 526–615, c and d at nt 1175–1349, and e and f at nt 1612–2089, see Fig. 4B). Specific DNA bands (200 to 700 bp long) were amplified from all cells only by the c + d and e + f sets of primers (not shown).

To characterize further the integrations, we cloned and sequenced PCR-amplified bands. Analysis of the integration junctions showed that the insertions clustered within a 1-kb region of the AAVS1 (nucleotides 987 to 1996), downstream of the Rep-binding site (RBS; nucleotides 398–413) (Fig. 4C). We detected two major breakpoints in the AAV ITR, around nucleotides 4600 (10 insertions) and 4560 (5 insertions). Ten breakpoints occurred downstream of the ITR RBS (nucleotides 4565–4587), 2 within the RBS, and 3 between the RBS and the terminal resolution site (TRS; nucleotides 4547–4552) (Fig. 4C). We observed DNA insertions of variable length at seven ITR–AAVS1 junctions (1, 2, 3, 4, 11, 12, and 15 in Fig. 4C). Interestingly, 3 of these insertions correspond to partial AAV ITR sequences in reverse orientation (in italic in 2, 11, and 12 in Fig. 4C).

To provide an estimate of the frequency of Rep-mediated, site-specific integration we carried out a quantitative, real-time PCR assay of DNA extracted from amniocytes and keratinocytes transduced by both vectors at an m.o.i. of 20 and grown for 5 days in the presence or absence of Dox. The analysis was carried out with a primer annealing to nt 4522–4548 in the right-side ITR of the integration cassette (17s) and a primer and an internal fluorescent probe annealing to nt 1612–1593 (e in Fig. 4) and 1562–1583 of the AAVS1, respectively. We used DNA from a HeLa clone (No. 41) with an integration cassette inserted as a dimer at position 1282 of the AAVS1 as a standard of 2 integrated copies per genome in 100% of the cells. The estimated proportion of cells harboring an AAVS1-specific integration ranged from 0.1% in keratinocytes transduced with AR1-TKRepTS to 16.0% in amniocytes transduced with AR1-CMVRepTS. No site-specific integration was detectable in cells transduced with the AR1-TKRepTS vector in the absence of Dox. Since the couple of primers used to run the real-time PCR assay detects only a subset of the integration sites observed by sequence analysis (Fig. 4) and only one orientation of the integrated cassette, these should be considered only as relative estimates, indicating a direct correlation between integration efficiency and Rep expression levels in the transduced cells (see Fig. 2).

Analysis of Non-Site-Specific Integrations in Human Primary Cells

To analyze the Rep-mediated, non-AAVS1-specific integrations of the AAV ITR-flanked cassette in human primary cells, we subjected genomic DNA extracted from keratinocytes transduced by the AR1-CMVRepTS vector to linker-mediated (LM) nested PCR using a combination

TABLE 1: Distribution of Rep-mediated, non-site-specific integration sites in the genome of human primary keratinocytes transduced by AR1-CMVRepTS in presence of Dox

ID	ITR breaks	Chromosome location	Target
1	nt 4597	1q25.1; nt 171222634–171222654	Intergenic
2	nt 4624	2p21; nt 45289588–45289691	Intergenic
3	nt 4601	3p21.1; nt 53429734–53429817	Intergenic
4	nt 4597	3p12.3; nt 77984746–77984836	Intergenic
5	nt 4596	3p12.3; nt 77984738–77984837	Intergenic
6	nt 4598	6q16.3; nt 103924210–103924365	Intergenic
7	nt 4593	10q26.2; nt 129830641–129830664	Intergenic
8	nt 4599	12p11.2; nt 27683849–27683908	Intergenic
9	nt 4603	12q21.3; nt 86363480–86363554	NM003622, introns 4–5
10	nt 4593	Xq28; nt 148325730–148325840	Intergenic
11	nt 4593	Xq28; nt 148326006–148326150	Intergenic
12	nt 4623	Xq21.1; nt 76115269–76115177	Intergenic

The nucleotide position of the breakpoint on the AAV ITR and the chromosomal location, nucleotide position, and length of amplified sequence at the breakpoints on genomic DNA are indicated.

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of ITR- and linker-specific primers, followed by cloning and sequencing of the PCR products. Twelve of 20 sequences met our validity criteria (presence of a primer-containing ITR sequence and a genomic sequence featuring a unique best hit with ≥95% identity to the human genome), and all were unambiguously mapped by Ensembl BLAST analysis on chromosomes 1, 2, 3, 6, 10, 12, and X (Table 1). Only one of these sequences corresponded to an integration into a known gene, introns 4–5 of the PPFIBP1 gene on chromosome 12p11.23 (NM003622), indicating that Rep-mediated integration has a general preference for intergenic regions. We observed inverted ITR sequences at the vector–cellular DNA junction in the majority of these insertions. We obtained no valid sequence by LM-PCR analysis of DNA extracted from keratinocytes transduced by the Rep$^-$ AR1 vector.

Site-Specific Integration in AAVS1 Transgenic Mice *in Vivo*

To analyze the ability of the hybrid vectors to mediate site-specific integration *in vivo*, we injected 2×10^8 transducing units of either AR1-TkRepTS or AR1-CMVRepTS into the tail vein of two groups of transgenic mice (four mice per group) carrying 1 copy of a 3.5-kb fragment of the AAVS1 in the X chromosome [27]. Half of the mice were given Dox in the drinking water from day −4 to +12. We injected control mice with 2×10^8 to

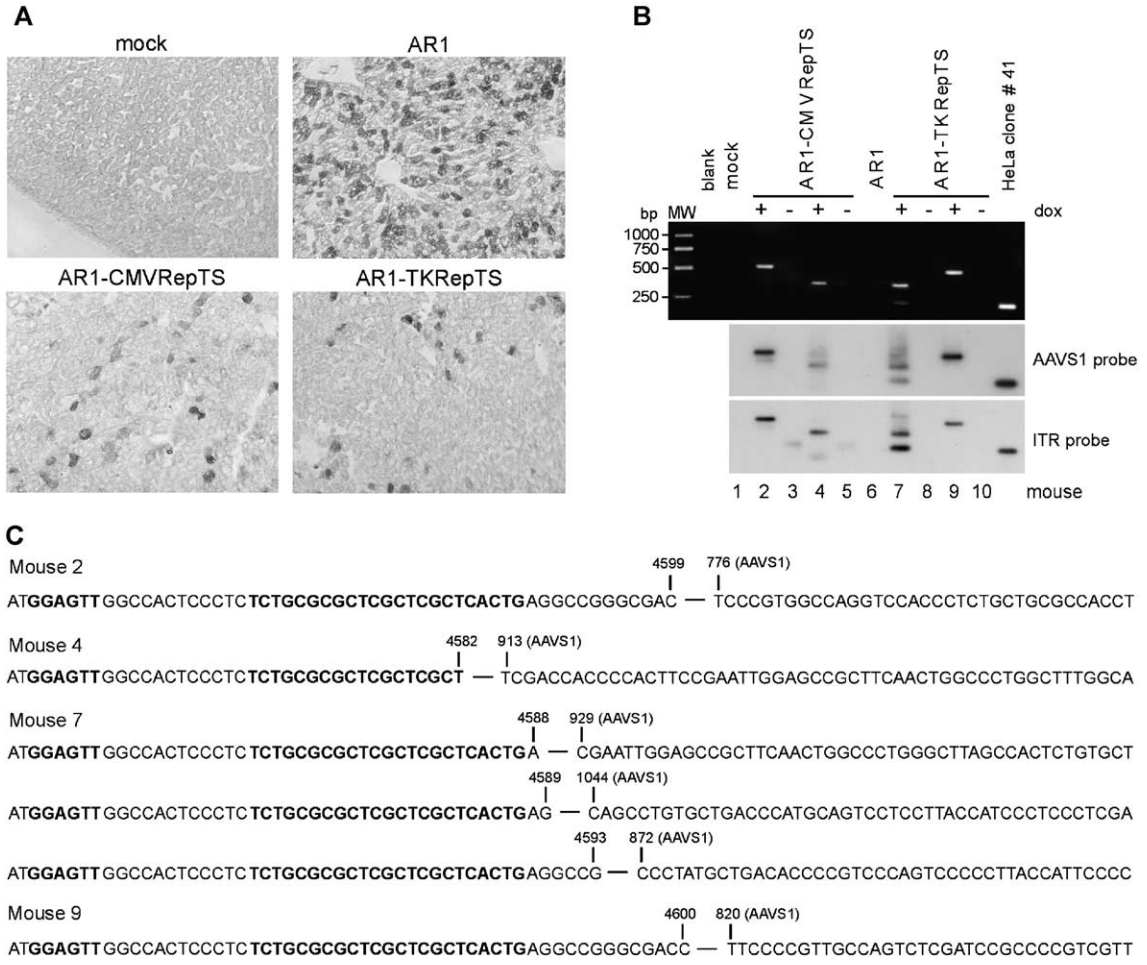

FIG. 5. Site-specific integration in the liver of AAVS1 transgenic mice upon systemic administration of AR1-CMVRepTS, AR1-TKRepTS, and AR1 vectors. (A) Immunohistochemical analysis of GFP expression in the liver of mice injected with AR1 (10^9 tu), AR1-CMVRepTS (2×10^8 tu), and AR1-TKRepTS (2×10^8 tu). A section from a mock-infected mouse is shown for comparison. (B) AAVS1-specific PCR assay on genomic DNA extracted from the liver of a mock-infected mouse (mouse 1) or mice injected with the Ad/AAV vectors ±Dox treatment (mice 2–5 and mice 7–10) or with the AR1 control vector (mouse 6). One-fifth of the amplification products were fractionated on a 1.5% agarose gel (top) and hybridized to AAVS1-specific (middle) and ITR-specific (bottom) probes. PCR of DNA from HeLa clone 41 was used as positive control. (C) Sequences of integration junctions obtained from the liver of mice systemically injected with AR1-CMVRepTS (mice 2 and 4) or AR1-TKRepTS (mice 7 and 9) and treated with Dox. Breakpoints on both ITR and AAVS1 are indicated. The ITR RBS and TRS are indicated in bold.

10^9 tu of the Rep$^-$ AR1 vector or saline. We sacrificed all the mice 12 days after injection and analyzed their livers by immunohistochemistry for GFP expression (Fig. 5A). Transduction efficiency in liver hepatocytes averaged 3 (lower dose) and 30% (higher dose) in mice infected with AR1 and 3% in mice infected with AR1-TkRepTS or AR1-CMVRepTS. We detected AAVS1-specific integration by PCR with a standard set of nested primers (ITR primers and c + d genomic primers, see Fig. 4) in genomic DNA extracted from the liver of all mice infected with AR1-TkRepTS and AR1-CMVRepTS and treated with Dox (Fig. 5B, mice 2, 4, 7, and 9). The PCR was always negative in the DNA extracted from animals not exposed to Dox (Fig. 5B, mice 3, 5, 8, and 10), mock infected (Fig. 5B, mouse 1), or infected with the Rep$^-$ AR1 vector (Fig. 5B, mouse 6).

To confirm the existence of AAVS1-specific integrants, we cloned and sequenced the PCR products. As shown in Fig. 5C, AAVS1-specific sequences were obtained from all mice, with breakpoints within or downstream of the RBS of the AAV ITR and between nucleotides 776 and 1044 in the AAVS1 sequence. No extra nucleotide was detected at the breakpoints (Fig. 5C). To provide a relative estimate of the frequency site-specific integration, we carried out a quantitative, real-time PCR assay on the DNA extracted from the livers of mice 2, 4, 6, 7, and 9, using nested primers (ITR primers and c + d genomic primers) and an internal fluorescent probe annealing to nt 1125–1141 of the AAVS1 (Fig. 6). We used plasmid DNA carrying an ITR–AAVS1 junction cloned from the liver of mouse 2 (Fig. 5) as standard. Estimated integration frequencies ranged from 0.2 to 2.0% of haploid liver genome (Fig. 6).

DISCUSSION

Random integration of gene transfer vectors is becoming a major concern for clinical application of gene therapy. Both retroviral and lentiviral vectors were shown to integrate preferentially into active genes [1,2], and the occurrence of T cell leukemia in patients treated by gene therapy for severe combined immunodeficiency has been correlated with the integration of a retroviral vector into a T cell proto-oncogene [28]. For therapy of non-life-threatening disorders, an increased risk of cancer could result in an ethical barrier to the use of randomly integrating vectors. In this report, we describe the development of a new generation of hybrid DNA vectors that combine the large capacity and infectivity of adenoviral vectors with the ability of the AAV Rep protein to direct the integration of AAV ITR-flanked sequences at specific sites into the human genome.

A major difficulty in building viral vectors containing Rep expression cassettes is the negative effect of Rep on viral replication [24,25], which prevents packaging or reduces titers to values unacceptably low for practical use. To overcome these problems, we have developed a tight, drug-inducible transcriptional regulation system based on a minimal promoter under the control of the

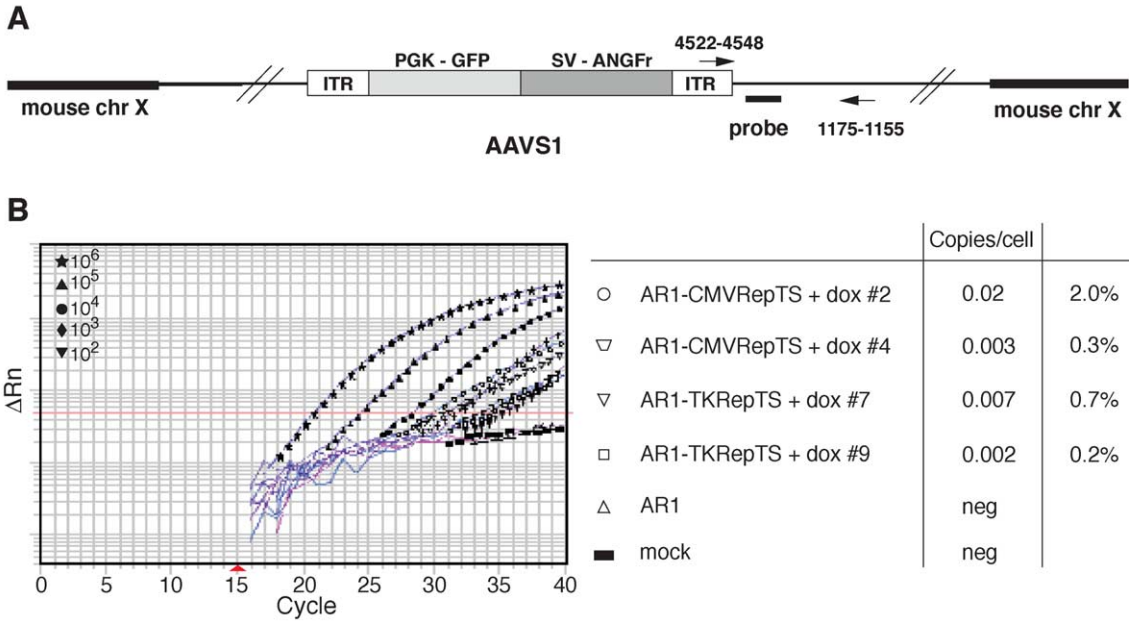

FIG. 6. Real-time PCR assay for quantification of AAVS1-specific integration in the transgenic mouse livers. (A) Schematic map of an integrated ITR cassette. Arrows indicate ITR- and AAVS1-specific primers. The probe (1125–1141 in the AAVS1) is indicated by a bar. (B) Standard curves were generated using serial dilutions (10^6–10^2 copies) of plasmid DNA carrying an ITR–AAVS1 junction cloned from the liver of mouse 2 (Fig. 5). The table shows the efficiency of site-specific integration of the AR1-CMVRepTS, AR1-TKRepTS, and control AR1 vectors as copies per cell and % of haploid liver genome.

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tetO element and a constitutively expressed tTS suppressor. This combination allowed efficient packaging of a single HD-Ad vector containing both a Rep expression and an AAV ITR-based integration cassette. Drug-induced activation of Rep allowed us to integrate intact copies of the ITR-flanked cassette into the AAVS1 site in human primary cells, at very low (1 or 2) copy number, in the absence of selective pressure and with efficiency higher than previously reported for other Rep-based integration systems. In an animal model of targeted gene delivery, an AAVS1 transgenic mouse, we could demonstrate AAVS1-specific integration in liver DNA 12 days after administration of a single dose of Ad/AAV vector transducing less than 5% of the hepatocytes. Drug-induced, site-specific integration of medium-size transgenes can therefore be obtained *ex vivo* and *in vivo* by administration of nontoxic doses of hybrid Ad/AAV vectors containing a drug-inducible Rep expression cassette.

Sequencing of the ITR–AAVS1 junctions showed that the Rep-mediated integration is remarkably precise both *in vitro* and *in vivo,* without significant loss or rearrangement of either ITR or genomic sequences at the breakpoints. The ITR sequences were interrupted downstream or within the Rep-binding sequence, as observed for wild-type AAV integration. In some of the breakpoints cloned from primary cells transduced *in vitro,* we observed the insertion of small, inverted sequences of ITR origin at the vector–cellular DNA junction. Scrambled alignments of vector and genomic sequences were previously observed at the junction of AAV vectors into mouse liver DNA and interpreted as the consequence of Rep-independent replication of the single-stranded AAV vector genome during integration [29], most likely mediated by a transcription-coupled, double-strand break DNA repair mechanism leading to preferential integration into transcribed chromatin regions [30,31]. Our data would indicate that DNA replication is strictly associated with integration also in the case of Rep-mediated integration starting from a double-stranded template.

A LM-PCR-based cloning and sequencing approach confirmed the existence of non-AAVS1-specific integration sites in primary cells and indicated their almost random distribution with a preference (11 of 12) for intergenic, nontranscribed regions. Interestingly, 2 of these integrations occurred at a distance of 8 bp from each other on chromosome 3 and 2 at a distance of 276 bp on chromosome X. Furthermore, inverted ITR sequences such as those observed at the ITR–AAVS1 junctions were detected also in the majority of the insertions outside the AAVS1. These findings indicate that Rep played a role in mediating both AAVS1-specific and nonspecific integrations and suggest the existence of additional, less frequent preferential sites in the human genome. Although the small size of the sample does not allow us to draw statistically significant conclusions, the observed preference for nontranscribed regions appears opposite to that observed for recombinant AAV vectors in a sample of genomic integrations of comparable size [30]. A possible explanation for this difference is that Rep-mediated integration starting from a double-stranded template would no longer require coupling to the transcriptional machinery, as postulated in the case of single-stranded AAV templates [31].

The efficiency of AAVS1-specific integration was proportional to the level of Rep78 expressed by the vector, both *in vitro* and *in vivo*. Higher Rep levels, obtained for instance by the use of more potent promoters, would probably further increase integration efficiency, although there is a trade-off caused by the Rep-induced toxicity for the target cells. Constitutive expression of Rep was reported to induce caspase-3-mediated, p53-independent apoptosis and cell cycle arrest in mammalian cells [32]. Our study indicates that in ~10% of stably transduced HeLa clones the AAVS1 locus is rearranged even in the absence of an integrated cassette. This is probably due to the ability of Rep to bind to the RBS in AAVS1, induce nicking at the nearby TRS, and start unscheduled DNA replication before binding to, replicating, and eventually integrating an ITR-containing genome [33]. In the presence of exceedingly high or prolonged synthesis of Rep, the AAVS1 site could become a hot spot of chromosome fragility or genetic recombination in transduced cells, an obviously undesirable side effect of a Rep-based integration system. Drug-controlled Rep expression is expected to reduce these potential genotoxic effects, an additional safety characteristic of our hybrid vectors.

Expression of Rep, a likely immunogenic protein, could potentially elicit a cytotoxic immune response against cells transduced *in vivo* by a Rep-expressing vector. The drug-controlled expression system is expected to reduce the immunogenicity of Rep to a minimum, although it introduces an additional immunogenic factor, the constitutively expressed Tet repressor protein. The immunogenic potential of the Tet repressor is a controversial issue, although it is conceivable that in cells or tissues in which episomal Ad vectors persist for long time (liver, for example) this might eventually turn out to be a problem. Anti-Rep and anti-Tet immune responses therefore need to be addressed in specific animal models for each vector delivery route.

Adenovirus-based integrating vectors could find practical application in both *ex vivo* and *in vivo* gene transfer protocols. The large capacity of the Ad vector backbone allows the incorporation of large genes and complex regulatory systems into the vectors, a current limitation of retroviral vector technology. A high proportion of site-specific integration and a general preference for nontranscribed regions would reduce both the risk of insertional oncogenesis and the problem of position-

dependent variability or silencing of transgene expression. The relatively low vector titers obtained with the currently available HD-Ad packaging system still appear as a limitation for the *in vivo* use of Ad/AAV hybrid vectors. However, progress is being rapidly made in the Ad vector field and should allow these potential limitations to be overcome in the near future.

MATERIALS AND METHODS

Cell culture. HeLa, HepG2, 293 (ATCC), and 293*Cre*4 (Merck, West Point, PA, USA) cell lines were propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Hyclone). Human primary amniocytes were obtained from prenatal amniocentesis and maintained in Chang's medium B (Irvine Scientific, Santa Ana, CA, USA). Human primary keratinocytes were isolated from skin biopsies and maintained in Defined Keratinocyte-SFM (Gibco Invitrogen Co., UK).

Construction and packaging of the HD-Ad/AAV hybrid vectors. The pSTK120 HD-Ad vector backbone was used to generate the pCMVRep vector, carrying a Rep78 cDNA under the control of the CMV immediate early promoter/enhancer sequence (−675 to +41), and the pAR1 vector, containing a GFP expression cassette (pEGFP-C3; Clontech, Palo Alto, CA, USA) driven by the human PGK promoter and a cDNA coding for a carboxy-terminal truncation of the human p75 low-affinity nerve growth factor receptor (ΔLNGFR) under the SV40 early promoter, all flanked by AAV ITRs (3.8 kb). The pSTK119 backbone was used to generate vectors containing both the AAV ITR-flanked integration cassette and the Rep78 regulated expression systems. In the pAR1-CMVRepTA/TS and pAR1-CMVRepTS/TA vectors, the Rep78 gene was driven by a CMV immediate early minimal promoter (−53 to +41) fused to the bacterial *tet* operator (*tetO*-CMV), while the two Dox-regulated transcription factors rtTA and tTS (from the pTet-On and pTet tTS plasmids; Clontech) were inserted in either configuration in a CMV promoter-driven bicistronic transcriptional unit containing the IRES of the encephalomyocarditis virus, derived from the pIRESneo expression vector (Clontech). The TA/TS and TS/TA cassettes were cloned in opposite transcriptional orientations with respect to the *tetO*-CMVRep78 cassette. In the pAR1-CMVRepTS and pAR1-TKRepTS plasmids, the Rep78 gene was under the control of the *tetO*-CMV promoter or a minimal HSV-TK promoter (−81 to +52) linked to the *tetO* element (*tetO*-TK), respectively. Both plasmids contained a CMV-driven tTS expression cassette in opposite transcriptional orientation with respect to the Rep78 cassettes. Rescue and amplification of the HD-Ad viruses were carried out by transfection of 293*Cre*4 and infection with AdH14 first-generation helper virus (Merck, West Point, PA, USA), as previously described [22]. Infectious particle titer was established on HeLa cells.

Analysis of Rep78 expression. Nuclear extracts were prepared by lysing cells in 10 mM Hepes, pH 7.9, 600 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, and a standard cocktail of protease inhibitors, run in 5-μg aliquots on 8% polyacrylamide/SDS gels, electrotransferred to nitrocellulose membranes, and incubated with an anti-Rep polyclonal rabbit antiserum (a gift from Carlo Toniatti) in PBS + 0.1% Tween 20 for 1 h at room temperature. Rep78-specific bands were visualized by a chemiluminescence kit (ECL Amersham Pharmacia Biotech, UK).

Southern blot analysis. High-molecular-weight DNA was extracted from 5×10^6 HeLa cells, digested in 20-μg aliquots with *Acc*I, run on a 0.8% agarose gel, transferred to a nylon membrane (Duralon; Stratagene, TX, USA), and hybridized to AAVS1- and GFP-specific probes according to standard methods.

Amplification and sequencing of ITR–AAVS1 junctions. Human primary amniocytes and keratinocytes were infected at an m.o.i. of 20 with AR1-CMVRepTS and/or AR1-TKRepTS virus and harvested 5 days after infection. Site-specific integration was determined by PCR of total genomic DNA by using nested primer pairs specific for the right AAV ITR (16s, 5′-GTAGCATGGCGGGTTAATCA-3′, and 17s, 5′-TTAACTACAAGGAACCCCTAGTGATGG-3′) and the AAVS1 region (a, 5′-GCCCCACTGCCGCAGCTGCTCCC-3′; b, 5′-CCGCACAGGCCGCCAGGAACTCG-3′; c, 5′-GCGCG-CAGAAGCCAGTAGAGC-3′; d, 5′-CTGGCTCAGGTTCAGGAGAGG-3′; e, 5′-CGCTCAGAGGACATCACGTG-3′; f, 5′-GGGACACAGGATCCCTGGA-GG-3′). Genomic DNAs (500 ng) were preamplified in 50 μl of polymerase buffer with 2.5 U of AmpliTaq Gold polymerase (Applied Biosystems, Roche, NJ, USA), 200 μM dNTPs, 1.5 mM $MgCl_2$, and 300 nM each primer, and 1/100 of the amplified DNA was used as template for a second round of PCR as previously described [22]. Finally, one-tenth of the PCR product was run on 1.5% agarose gel, transferred to Hybond N^+ membrane, and hybridized with AAVS1- or ITR-specific probes. The amplified junctions were cloned into pCR 2.1-TOPO vector (Invitrogen, UK) and sequenced.

Quantitative real-time PCR for determination of AAVS1-specific integration. Quantitative real-time PCR of ITR-AAVS1 junctions in primary keratinocytes and amniocytes was carried out by a two-step PCR assay [34] with the ITR primer 17s, the AAVS1 primer e, and an AAVS1 5′ fluorescein-labeled probe (5′-TTTCCGGAGCACTTCCTTCTCG-3′) annealing to nucleotides 1612–1593 of the AAVS1. Quantitative real-time PCR of ITR–AAVS1 junctions in AAVS1 transgenic mouse liver was carried out by nested PCR with the ITR primers 16s and 17s, AAVS1 primers c and d, and a fluorescent probe (5′-TCCTGGACTT-TGTCTCC-3′) annealing to nt 1125–1141 of the AAVS1.

Mapping of non-AAVS1-specific integration sites. Integration junctions were amplified, cloned, and sequenced by LM nested PCR [2] using two nested AAV ITR-specific primers (18s, 5′-CCTAGTGATGGAGTTGGCCACTCC-3′, and 19s, 5′-GAGTTGGCCACTCCCTCTCTGC-3′). Human DNA sequences were BLAST searched against the public database by the Ensembl browser. Targeted gene search based on the chromosomal localization of each integration site was done using the Human Contig View of Ensembl.

Animal studies. Four- to 5-week-old female/male AAVS1 transgenic mice [27] were injected iv with 2×10^8 tu of AR1CMVRepTS (group 1; $n = 4$;) or AR1TKRepTS (group 2; $n = 4$) virus. Four mice (two from each group) were given drinking water containing doxycycline at a concentration of 200 μg/ml. Two mice were injected iv with 2×10^8 or 10^9 tu of AR1, and one was mock infected. Site-specific integration was determined by PCR on liver genomic DNA 12 days postinfection by nested primer pairs specific for the right AAV ITR and the AAVS1 region (c and d). Livers were fixed in 4% paraformaldehyde, embedded in OCT, frozen in liquid nitrogen-cooled isopentane, and cut into 10-μm serial sections. Immunohistochemical staining for GFP was performed using an avidin–biotin–peroxidase technique. The primary antibody (α-GFP rabbit IgG fraction A-11122; Molecular Probes, Eugene, OR, USA) and the biotinylated goat anti-rabbit secondary antibody (Vectastain, Vector Laboratories) were diluted 1:1500 in PBS containing 0.1% Triton X-100 and 1% goat serum. Positive signals were detect by DAB staining.

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REFERENCES

1. Schroder, A. R., Shinn, P., Chen, H., Berry, C., Ecker, J. R., and Bushman, F. (2002). HIV-1 integration in the human genome favors active genes and local hotspots. *Cell* **110:** 521–529.
2. Wu, X., Li, Y., Crise, B., and Burgess, S. M. (2003). Transcription start regions in the human genome are favored targets for MLV integration. *Science* **300:** 1749–1751.
3. Thomas, C. E., Ehrhardt, A., and Kay, M. A. (2003). Progress and problems with the use of viral vectors for gene therapy. *Nat. Rev. Genet.* **4:** 346–358.
4. Chen, H. H., Mack, L. M., Kelly, R., Ontell, M., Kochanek, S., and Clemens, P. R. (1997). Persistence in muscle of an adenoviral vector that lacks all viral genes. *Proc. Natl. Acad. Sci. USA* **94:** 1645–1650.
5. Schiedner, G., *et al.* (1998). Genomic DNA transfer with a high-capacity adenovirus vector results in improved in vivo gene expression and decreased toxicity. *Nat. Genet.* **18:** 180–183.
6. Morsy, M. A., *et al.* (1998). An adenoviral vector deleted for all viral coding sequences results in enhanced safety and extended expression of a leptin transgene. *Proc. Natl. Acad. Sci. USA* **95:** 7866–7871.
7. Morral, N., *et al.* (1998). High doses of a helper-dependent adenoviral vector yield supraphysiological levels of alpha1-antitrypsin with negligible toxicity. *Hum. Gene Ther.* **9:** 2709–2716.
8. Morral, N., *et al.* (1999). Administration of helper-dependent adenoviral vectors and sequential delivery of different vector serotype for long-term liver-directed gene transfer in baboons. *Proc. Natl. Acad. Sci. USA* **96:** 12816–12821.
9. Thomas, C. E., Schiedner, G., Kochanek, S., Castro, M. G., and Lowenstein, P. R. (2000). Peripheral infection with adenovirus causes unexpected long-term brain inflammation in animals injected intracranially with first-generation, but not with high-capacity, adenovirus vectors: toward realistic long-term neurological gene therapy for chronic diseases. *Proc. Natl. Acad. Sci. USA* **97:** 7482–7487.
10. Kim, I. H., Jozkowicz, A., Piedra, P. A., Oka, K., and Chan, L. (2001). Lifetime correction of genetic deficiency in mice with a single injection of helper-dependent adenoviral vector. *Proc. Natl. Acad. Sci. USA* **98:** 13282–13287.
11. Berns, K. I., and Linden, R. M. (1995). The cryptic life style of adeno-associated virus. *Bioessays* **17:** 237–245.
12. Kotin, R. M., *et al.* (1990). Site-specific integration by adeno-associated virus. *Proc. Natl. Acad. Sci. USA* **87:** 2211–2215.
13. Samulski, R. J., *et al.* (1991). Targeted integration of adeno-associated virus (AAV) into human chromosome 19. *EMBO J.* **10:** 3941–3950.
14. Linden, R. M., Ward, P., Giraud, C., Winocour, E., and Berns, K. I. (1996). Site-specific integration by adeno-associated virus. *Proc. Natl. Acad. Sci. USA* **93:** 11288–11294.
15. Miao, C. H., *et al.* (1998). The kinetics of rAAV integration in the liver. *Nat. Genet.* **19:** 13–15.
16. Ponnazhagan, S., *et al.* (1997). Lack of site-specific integration of the recombinant adeno-associated virus 2 genomes in human cells. *Hum. Gene Ther.* **8:** 275–284.
17. Yang, C. C., *et al.* (1997). Cellular recombination pathways and viral terminal repeat hairpin structures are sufficient for adeno-associated virus integration in vivo and in vitro. *J. Virol.* **71:** 9231–9247.
18. Miller, D. G., Rutledge, E. A., and Russell, D. W. (2002). Chromosomal effects of adeno-associated virus vector integration. *Nat. Genet.* **30:** 147–148.
19. Palombo, F., Monciotti, A., Recchia, A., Cortese, R., Ciliberto, G., and La Monica, N. (1998). Site–specific integration in mammalian cells mediated by a new hybrid baculovirus-adeno-associated virus vector. *J. Virol.* **72:** 5025–5034.
20. Wang, Y., *et al.* (2002). Herpes simplex virus type1/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.
21. Heister, T., Heid, I., Ackermann, M., and Fraefel, C. (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.
22. Recchia, A., *et al.* (1999). Site-specific integration mediated by a hybrid adenovirus/adeno-associated virus vector. *Proc. Natl. Acad. Sci. USA* **96:** 2615–2620.
23. Antoni, B. A., Rabson, A. B., Miller, I. L., Trempe, J. P., Chejanovsky, N., and Carter, B. J. (1991). Adeno-associated virus Rep protein inhibits human immunodeficiency virus type 1 production in human cells. *J. Virol.* **65:** 396–404.
24. Hermonat, P. L. (1992). Inhibition of bovine papillomavirus plasmid DNA replication by adeno-associated virus. *Virology* **189:** 329–333.
25. Weitzman, M. D., Fisher, K. J., and Wilson, J. M. (1996). Recruitment of wild-type and recombinant adeno-associated virus into adenovirus replication centers. *J. Virol.* **70:** 1845–1854.
26. Freundlieb, S., Schirra-Muller, C., and Bujard, H. (1999). A tetracycline controlled activation/repression system with increased potential for gene transfer into mammalian cells. *J. Gene Med.* **1:** 4–12.
27. Rizzuto, G., *et al.* (1999). Development of animal models for adeno-associated virus site-specific integration. *J. Virol.* **73:** 2517–2526.
28. Hacein-Bey-Abina, S., *et al.* (2003). LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* **302:** 415–419.
29. Nakai, H., Iwaki, Y., Kay, M. A., and Couto, L. B. (1999). Isolation of recombinant adeno-associated virus vector–cellular DNA junctions from mouse liver. *J. Virol.* **73:** 5438–5447.
30. Nakai, H., Montini, E., Fuess, S., Storm, T. A., Grompe, M., and Kay, M. A. (2003). AAV serotype 2 vectors preferentially integrate into active genes in mice. *Nat. Genet.* **34:** 297–302.
31. Russell, D. W. (2003). AAV loves an active genome. *Nat. Genet.* **34:** 241–242.
32. Schmidt, M., Afione, S., and Kotin, R. M. (2000). Adeno-associated virus type 2 Rep78 induces apoptosis through caspase activation independently of p53. *J. Virol.* **74:** 9441–9450.
33. Young, S. M., Jr., and Samulski, R. J. (2001). Adeno-associated virus (AAV) site-specific recombination does not require a Rep-dependent origin of replication within the AAV terminal repeat. *Proc. Natl. Acad. Sci. USA* **98:** 13525–13530.
34. Huser, D., Weger, S., and Heilbronn, R. (2002). Kinetics and frequency of adeno-associated virus site-specific integration into human chromosome 19 monitored by quantitative real-time PCR. *J. Virol.* **76:** 7554–7559.