rDNA-directed integration by an HIV-1 integrase—I-PpoI fusion protein

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ABSTRACT

Integrating viral vectors are efficient gene transfer tools, but their integration patterns have been associated with genotoxicity and oncogenicity. The recent development of highly specific designer nucleases has enabled target DNA modification and site-specific gene insertion at desired genomic loci. However, a lack of consensus exists regarding a perfect genomic safe harbour (GSH) that would allow transgenes to be stably and reliably expressed without adversely affecting endogenous gene structure and function. Ribosomal DNA (rDNA) has many advantages as a GSH, but efficient means to target integration to this locus are currently lacking. We tested whether lentivirus vector integration can be directed to rDNA by using fusion proteins consisting of the Human Immunodeficiency Virus 1 (HIV-1) integrase (IN) and the homing endonuclease I-PpoI, which has natural cleavage sites in the rDNA. A point mutation (N119A) was introduced into I-PpoI to abolish unwanted DNA cleavage by the endonuclease. The vector-incorporated IN-I-PpoI$_{N119A}$ fusion protein targeted integration into rDNA significantly more than unmodified lentivirus vectors, with an efficiency of 2.7%. Our findings show that IN-fusion proteins can be used to modify the integration pattern of lentivirus vectors, and to package site-specific DNA-recognizing proteins into vectors to obtain safer transgene integration.

INTRODUCTION

At present, the most efficient methods available for site-directed gene addition into human cells are based on DNA double-strand break (DSB)-enhanced homologous recombination (HR) (1). The site-specific cleavage of genomic DNA is catalysed using zinc finger nucleases (ZFNs), meganucleases or transcription activator-like effector nucleases (TALENs) (1–3). In the presence of a suitably designed homology-containing donor DNA molecule, insertion of exogenous sequences can occur at the cleaved site through homology-directed repair (HDR). Cellular expression of the nuclease protein is often achieved with DNA transfection methods, which can be difficult to translate into whole organisms [reviewed in (4)]. Integration-defective lentivirus vectors (IDLVs) have provided another means to enhance the delivery of both nuclease expression cassettes and the donor construct into cells (5). IDLVs can be used for both *in vitro* and *in vivo* transductions, but as a tool for delivering the recombination reaction components, they too suffer from limitations. First, the inability to control expression of the nuclease from the unintegrated vector is a drawback, and may lead to either over-expression-related cytotoxicity or inadequate enzyme levels in cells. Second, transduction of a target cell with the two to three required IDLVs simultaneously may be challenging. Moreover, any cDNA imported into the nucleus may become illegitimately integrated into the genome, possibly allowing constant expression of the imported genes. In the case of nucleases, this could predispose cells to genotoxicity and chromosomal instability.

A protein transduction method was recently applied for the cellular delivery of a meganuclease and its recombination substrate (6). The method relies on the expression of a HIV-1 Vpr fusion protein in vector-producing cells to obtain inclusion into virions (7). Although this *trans*-packaging method is efficient with regard to foreign protein incorporation, it predisposes transduced cells to undesired side effects of Vpr, which include induction of cell cycle arrest and apoptosis [for a review, see (8)]. Moreover, the Vpr *trans*-packaging approach requires induction of an extra plasmid into lentivirus vector production, as this dispensable gene has been deleted from the latest lentivirus vector (LVV) generations (9).

We have previously demonstrated that HIV-1 IN fusion proteins can be used as a *cis*-packaging method to deliver proteins of interest into transduced cells' nuclei while retaining some level of integration activity (10). IN-fusion proteins have been created before with the aim of directing transgene integration into predetermined sites (11–14). Despite their ability to target integration in *in vitro* reactions, IN-fusion proteins functioned only at a modest efficiency in cultured cells (15). The efforts to affect lentiviral integration patterns were redirected to modifying the DNA-specificity of the lens epithelium-derived growth factor(LEDGF/p75), after identifying its role in tethering IN to the chromatin (16–19).

We have generated new IN-fusion proteins with the aim of testing their applicability for further modifications of the protein content and the integration characteristics of third generation LVVs. I-PpoI is a dimeric 18–20 kDa homing endonuclease protein of the slime mold *Physarum polycephalum*, which has a natural 15-bp recognition site present in the highly conserved 28S ribosome RNA (rRNA) genes of eukaryotes (20,21). Each diploid human cell has about 600 copies of the rRNA genes in five clusters localized to the short arms of the acrocentric chromosomes 13, 14, 15, 21 and 22 (22). The tandemly repeated genes, collectively called the ribosomal DNA (rDNA), become transcribed in the nucleoli that form at the end of mitosis around rDNA (23). Owing to the wealth of rRNA genes and the presence of spacers between the gene repeats that likely confine natural insulator functions (24), the rDNA is an appealing safe harbour for transgene integration. We fused I-PpoI with HIV-1 IN to generate a fusion protein that would concomitantly answer two of our study questions: Can the *cis*-protein packaging strategy be used for the cellular delivery of a site-specific meganuclease, and can IN-fusion proteins promote targeted integration into a good GSH candidate, the rDNA.

MATERIALS AND METHODS

Plasmids

IN-fusion constructs were cloned as described (10). The cDNA for I-PpoI was PCR amplified from the plasmid pCNPpo6 (a kind gift from Dr. Raymond J. Monnat Jr), using the primers I-PpoI Forw (5′-ATTCACCACTAGTGC TCCAAAAAAAAAGCGC-3′) and I-PpoI Rev (5′-TATGG CCTCTCAGGCCATTATTATACCACAAAGTGACTGC C-3′). The N119A-mutated I-PpoI (25) was created with QuikChange® II XL Site-Directed Mutagenesis Kit

(Stratagene) using the primers N119A Forw (5′-GGGAGT CACTAGACGACGCCAAAGGCAGAAACTGGT GCC-3′) and N119A Rev (5′-GGCACCAGTTTCTGCCTT TGGCGTCGTCTAGTGACTCCC-3′). Expression cassette of the His-tagged IN-I-PpoI in the plasmid pBVboostFG were created for recombinant protein production in insect cells using the GATEWAY™ Cloning Technology (Gibco-BRL®, Life Technologies) (26). A double-stranded oligonucleotide containing the I-PpoI recognition sequence CT CTCTTAAGGTAGC was inserted into the EcoRV site of pBluescriptII (Stratagene) to prepare a plasmid containing a single cleavage site for I-PpoI. All oligonucleotides used in cloning were purchased from Oligomer Oy (Helsinki, Finland).

### Recombinant IN-fusion protein production, purification and *in vitro* testing

Baculovirus and protein production in insect cells were carried out as described (26,27). The recombinant His-tagged IN-I-PpoI and wt IN proteins were purified from infected insect cells using the BD TALON™ Metal Affinity Resin (BD Biosciences). The elution fractions containing the largest amount of recombinant protein were identified with western blot and used for *in vitro* DNA cleavage testing. Digestion mixtures were set up using 300 ng of pBluescriptII containing the I-PpoI site and either control I-PpoI (Promega) or the purified recombinant proteins wt IN or IN-I-PpoI. Digestions were carried out at 37°C for 2 h, after which the ScaI buffer and enzyme (Fermentas) were added to compose a double digestion. Digestions were verified by agarose gel electrophoresis.

Vector and virus-like particle production

Vesicular stomatitis virus G-glycoprotein (VSV-G) pseudotyped third-generation HIV-1-based LVV stocks containing the IN-fusion proteins were prepared and titred as described (10). The core packaging plasmids used were pMDLg/pRRE, pMDLg/pRRE-IN$_{D64V}$, pMDLg/pRRE-IN-I-PpoI or pMDLg/pRRE-IN-I-PpoI$_{N119A}$ (Figure 1). Vectors containing mixed IN-molecule multimers were produced using two different packaging plasmids in equimolar amounts. Virus-like particles (VLPs) were produced with the same protocol but without a transfer construct.

Cells, transduction and cytotoxicity assay

Human embryonic kidney HEK 293 (ATCC: CRL-1573™), HeLa cells (ATCC: CCL-2™) and MRC-5 cells (ATCC: CCL-171™) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 1% Penicillin–Streptomycin (Sigma) and 10% Fetal Bovine Serum (FBS; Hyclone) at 37°C in a 5% CO_2-containing humidified atmosphere. The culture medium for MRC-5 additionally contained 1% Non-Essential Amino Acid Solution (Sigma) and 1% Sodium Pyruvate solution (Sigma). Cells were transduced with LVVs diluted into prewarmed culture media. The cytotoxicity tests were done on transduced HeLa and MRC-5 cells using the CellTiter-Glo Luminescent Cell Viability Assay (Promega).
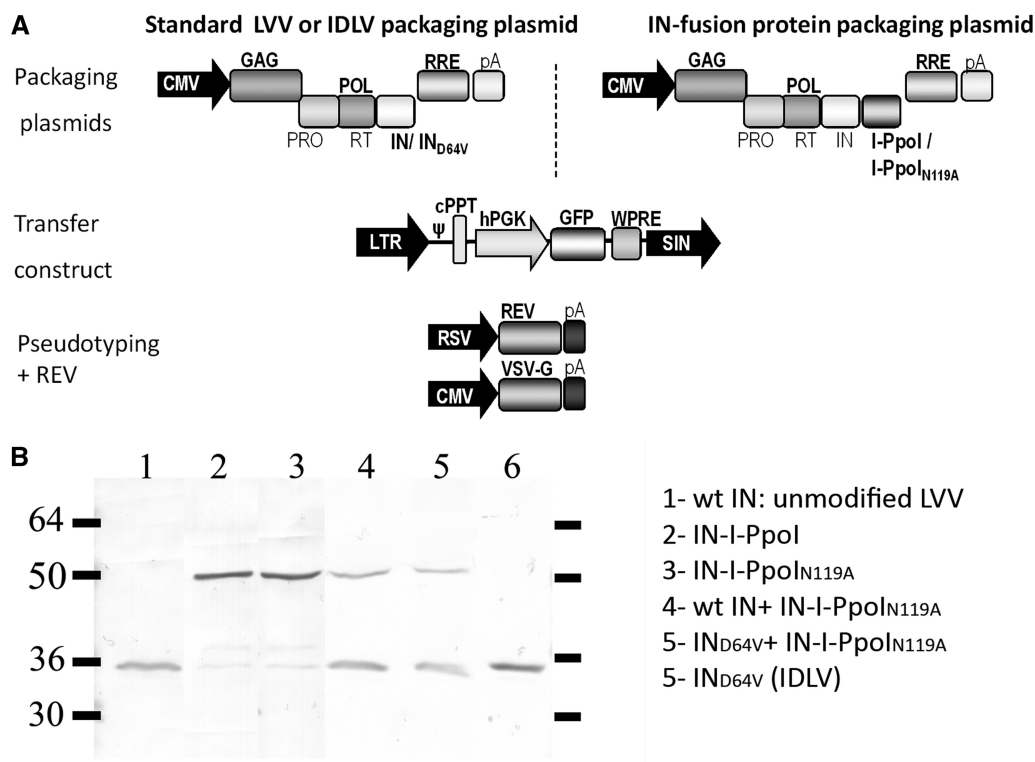
Figure 1. Production of lentivirus vectors and virus-like particles. (**A**) Lentivirus vector (LVV) and virus-like particle (VLP) production plasmids. The packaging plasmids were used either alone or mixed in equimolar amounts to generate LVVs and VLPs containing a single type of IN-molecule or mixed IN-multimers, respectively. (**B**) The IN-molecule content of LVVs was detected by an immunoblot using antiserum to HIV-1 IN. The vector-contained IN molecules on each lane are listed on the right. Expected molecular weights for IN/IN_{D64V}: 32 kDa; $IN-I-PpoI_{(N119A)}$ 51 kDa. PRO, protease; RT, reverse transcriptase; RRE, Rev-responsive element; pA, polyadenylation signal; CMV, human cytomegalovirus immediate-early enhancer/promoter; cPPT, central polypurine tract; hPGK, human phosphoglycerate kinase promoter; GFP, green fluorescent protein; WPRE, Woodchuck hepatitis virus post-transcriptional regulatory element; SIN, self-inactivated LTR; LTR, long terminal repeat; RSV, Rous Sarcoma Virus promoter; VSV-G, Vesicular stomatitis virus G glycoprotein; IDLV, integration deficient lentivirus vector; Wt, wild type.

The day before transduction, 6000 HeLa cells/well and 10 000 MRC-5 cells/well were seeded onto 96-well microplates (B&W Isoplate-96 TC, Perkin Elmer). Cells were transduced with LVVs using 2 and 10 ng of p24 per well. 24, 48 and 72 h after transduction, each plate was assayed by adding the CellTiter-Glo Reagent and reading the luminescence.

Western blot

Recombinant proteins and correct packaging of the different IN proteins into LVVs were verified by western blot using antisera to HIV-1 IN, amino acids 23–34 (Cat. No. #757) obtained though NIH AIDS Research & Reference Reagent Program, and the secondary antibody Goat Anti-Rabbit IgG (H+L)–AP Conjugate (Bio-Rad Laboratories). Lentiviral vector preparations were lysed in Laemmli buffer and denatured at 95°C for 5 min before separation on 10–12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gels. Proteins were transferred onto nitrocellulose membranes (0.2 µm, Trans-Blot Transfer Medium, Bio-Rad) and probed with antibodies.

Immunofluorescence staining and scanning confocal microscopy

MRC-5 cells grown on Poly-L-lysine (Sigma)-treated coverslips or Lab-Tek™ II Chambered Coverglasses were transduced with LVVs and VLPs using the same vector amounts as in the cytotoxicity assay, or treated with H_2O_2 (Sigma). After 4 to 6 h of transduction, cells were fixed and processed by immunocytochemistry using the directly labelled primary antibody Mouse IgG2b κ Alexa Fluor 647 Anti-H2A.X-Phosphorylated (Ser139) Antibody (BioLegend) and the primary antibody rabbit polyclonal to Fibrillarin (ab5821; Abcam) with the secondary antibody AF546 goat-anti-rabbit (Invitrogen). Nuclei were highlighted by mounting the samples with ProLong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA, USA). Confocal microscopy images were acquired at room temperature with a Zeiss LSM 700 confocal microscope operated with Zeiss Zen software (Carl Zeiss Microimaging, Jena, Germany) and combined using Adobe photoshop elements 5.0.

Flow cytometry

To measure GFP expression kinetics after LVV transduction, HeLa cells were transduced with varying amounts of different vectors to obtain similar initial fluorescence levels. The day before transduction, HeLa cells were plated at 1×10^5 cell/well onto six-well plates. Single-cell suspension samples were taken from the wells between days 1–28 post transduction, after which cells were replated. Samples were analysed with the BD FACS

Canto II and FACS Diva software (BD Biosciences). The relative integration efficiency was estimated as the percentage of GFP positive cells from the day 2 peak value, which can originate from transient expression.

Extracting genomic integration sites (IS)

MRC-5 cells were transduced with LVVs at varying multiplicities of infection (MOI). Cells were cultivated for 7–14 days, pelleted and stored at $-70°C$ until used. LM-PCR was carried out as described (28) with modifications. Briefly, 2–2.5 μg of genomic DNA from transduced MRC-5 cells was digested using AvrII, SpeI and NheI, purified and ligated to linkers. The ligation mixture was heat-inactivated, and diluted to a volume of 80–100 μl. Phire^{TM} or Phusion$^®$ hot start DNA polymerases (Finzymes Oy, Espoo, Finland) were used for LM-PCR with the following cycling: $1 \times 98°C$ 30 s; $7 \times 98°C$ 5 s, $72°C$ 1 min 20 s; $37 \times 98°C$ 5 s, $66°C$ 1 min 20 s; $1 \times 72°C$ 4 min; $4°C$. The primary LM-PCR products were diluted 1:50, and used for nested PCR with the two-step PCR conditions: $1 \times 98°C$ 30 s; $37 \times 98°C$ 5 s, $72°C$ 1 min 20 s; $1 \times 72°C$ 4 min. The secondary barcoded LM-PCR products were purified using the ChargeSwitch$^®$ PCR Clean-Up Kit (Invitrogen), pooled and subjected to next-generation sequencing (454 Life Sciences GS FLX Titanium pyrosequencing platform, Beckman Coulter Genomics MA, United States).

Bioinformatics methods

Paired-end pyrosequencing reads were first decoded using exact match to DNA barcodes included in the second round of PCR. The resulting collection of sequences was aligned against three different target sequences using BLAT [BLAST-like alignment tool, (29)] with >95% match score: (i) the LTR-specific ASB1 primer of the second PCR, (ii) the linker-specific ASB16 primer of the second PCR and (iii) 100 bp viral LTR sequence. For a read to be considered as a valid integration event, it was required to match all of the following filtering criteria: (i) must have a valid alignment to both primers starting within the first 5 bases, (ii) the alignment against viral LTR should contain the last 22 bps directly next to the genomic DNA junction and (iii) the summed span of alignment against primers and LTR sequence should be ≤95% of the total read size. Reads starting with ASB16 primers were reverse complemented to correct the genomic orientation. The curated reads were then processed as described (30). Additionally, to control for contamination or false positive decoding resulting from sequencing errors, each IS was checked for presence in more than one sample. The sample hosting the IS with higher sequence abundance was given priority over other samples sharing the same IS. In cases of ties, the IS was removed altogether. The short arms of the acrocentric chromosomes that contain the rDNA are not included in the human genome assembly NCBI36/Hg18. Integration sites in the rDNA were therefore analysed by BLAT-aligning reads to the human genome assembly GRCh37/Hg19 and counting unique ISs in the unplaced supercontig ChrUn_gl000220 (GenBank: GL000220.1).

The unplaced contig was analysed to contain one full and one slightly shortened rRNA gene repeat by BLAT-aligning different rRNA gene features from the Human ribosomal DNA complete repeating unit (GenBank: U13369.1) to GRCh37/Hg19. Integration sites for sequences that matched equally well to either of the rRNA genes on the contig were placed on the first (full) rRNA gene repeat. ChrUn_gl000220 was given priority in cases where an IS could match equally well to the unplaced contig in Hg19 and to rRNA gene fragments scattered in the non-acrocentric chromosomes in Hg18. IS in the rDNA were visualized by adding IS information-containing custom tracks (Supplementary Methods) to the UCSC Genome Browser on Human Feb. 2009 Assembly (GRCh37/hg19) (31). The total number of IS for different data sets in NCBI36/Hg18 was corrected with the ChrUn_gl000220 localized hits. Genomic I-PpoI sites were searched for by using NCBI BLASTN 2.2.26+ (32) on the reference assembly GRCh37.p5. Variable-sized windows around each IS from IN-I-PpoI$_{N119A}$-containing vectors were tested to detect the abundance of I-PpoI sites in the region. Owing to sparse location of the sites, the abundance was saturated around a megabase window. New sequences were stored in the NCBI GenBank sequence database (accession numbers JS886887–JS920506).

Statistics

The cytotoxicity data were analysed using one-way ANOVA with Dunnett's multiple comparison post test. For the rDNA-targeted IS study, all vector sets were compared with each other. Statistical analysis was performed using the Fisher's exact test when comparing data sets with $n < 500$ with each other, and with the Chi-square test when the larger data sets were part of the comparison. Analyses were performed using GraphPad Prism version 5.01 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com. Statistics used to analyse the genomic heatmap data (Figure 6) are described in Brady *et al.* (33).

RESULTS

Generation and characterization of LVVs containing the IN-I-PpoI fusion protein

Before IN-I-PpoI was packaged into LVVs, the ability of the fusion protein to specifically cleave the I-PpoI recognition sequence was verified in a plasmid cleavage assay. The recombinant IN-I-PpoI fusion protein proved to be as efficient a restriction enzyme as the commercial I-PpoI enzyme used as a positive control (Supplementary Figure S1). The third-generation LVV packaging plasmids were then modified to contain the I-PpoI reading frame 3' to IN and used in vector production (Figure 1A). In addition to wild type I-PpoI, an inactivated version of the meganuclease was fused to IN. This IN-I-PpoI$_{N119A}$ protein was designed to allow the study of integration site selection in the absence of target DNA cleavage, which is unnecessary for IN-catalysed integration reactions. The remaining three plasmids used in LVV

production were unmodified: the transfer construct, which forms the vector RNA genome and encodes for a GFP marker protein under a PGK promoter; the VSV-G plasmid, which encodes a heterologous envelope protein to pseudotype the vector; and a plasmid encoding for REV, which is needed both for the expression of the *gag* and *pol* genes and the accumulation of packageable vector transcripts (34). Fusions to IN's C terminus may be detrimental for integration catalysis, but IN molecules mutated at different domains are capable of complementing each other's functions to restore integration (35). Mixed multimer vectors containing the unmodified wild type IN (wt IN) or the integration-defective IN_{D64V} in addition to $IN-I-PpoI_{N119A}$ were therefore also generated. The sizes of the IN-fusion protein bands were as expected, with minimal unspecific protein degradation (Figure 1B). Congruent with previous studies (13,36), we found mixed multimers of the IN molecules obligatory for vector integration (Figure 2). The vectors containing wt IN in addition to $IN-I-PpoI_{N119A}$ promoted long-term transgene expression better than vectors complemented with IN_{D64V}.

Nucleolar rDNA is cleaved after IN-I-PpoI protein transduction

To test the ability of the newly created lentivirus vectors to gain access to and carry out specific DNA cleavage on the nucleolar rRNA genes, we transduced MRC-5 lung fibroblasts with vectors and virus-like particles (VLPs) containing the IN-fusion proteins. Sites of DSB formation were visualized through γH2A.X immunocytochemistry (37). Confocal microscopy analyses revealed parallel and overlapping localization of the DSB marker with the nucleolus marker fibrillarin, indicating rRNA gene cleavage by the IN-I-PpoI-containing vectors and VLPs (Figure 3 and Supplementary Table S1). In addition, distinctive

ring-like nucleoli were detected in these cells, suggestive of fibrillarin re-organization in response to nucleolus-directed DNA damage (Supplementary Table S1, Supplementary Figure S2). As expected, $IN-I-PpoI_{N119A}$ caused fewer and less visible DSBs. In addition to the well-known I-PpoI cleavage sites in the 28S rRNA gene, eight perfect full-length (Supplementary Table S2) and several degenerate (38) I-PpoI sites can be found in the human genome. Three of the perfect I-PpoI sites mapped by us in the reference assembly GRCh37.p5 (Supplementary Table S2) have been previously described (39,38), and six of them are found in LSU (large subunit) rRNA repeat elements. The non-nucleolar sites can be cleaved in up to 25% of cells at 6 hours after I-PpoI enzyme induction (38). In line with this result, we also detected γH2A.X signals
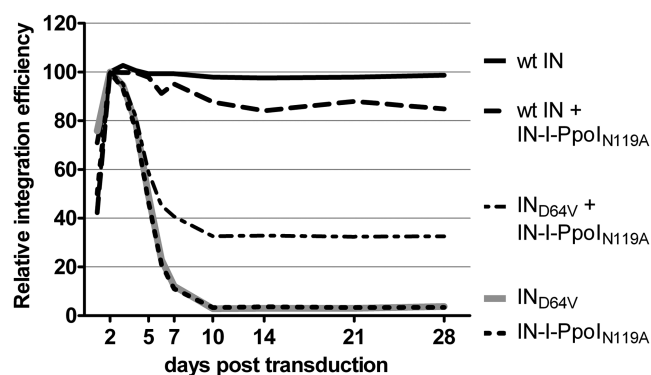
Figure 2. The relative integration efficiency of different IN-modified vectors. HeLa cells transduced with LVVs containing different IN molecules were assayed by flow cytometry. For each vector the amount of GFP-positive cells in different time points was normalized to the value of day two, when GFP expression generally reaches its highest value (set to 100%). The integration efficiency of different vectors can be evaluated by looking at values after day 10, by which expression from unintegrated vector genomes has dropped close to zero.
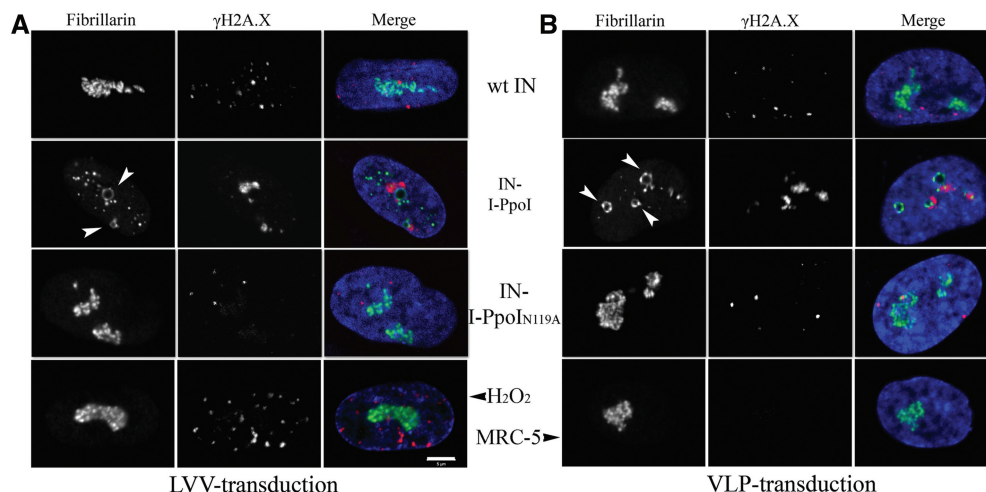
Figure 3. DSBs caused by LVV- or VLP- contained IN proteins. MRC-5 cells were transduced with LVVs (**A**) or VLPs (**B**) containing different IN molecules (middle). The DSB sites were detected by confocal microscopy using the antibody to γH2A.X (red). Nucleoli were labelled using the antibody to fibrillarin (green). Nuclei were visualized using DAPI (blue). The white arrowheads point to distinct shell-like nucleoli with adjacent DSB markers seen in cells transduced with the IN-I-PpoI-containing LVVs and VLPs. Hydrogen peroxide (H_2O_2; A) treatment was used as a positive control for DSBs. Scale bar: 5 μm. MRC-5, untreated MRC-5 cells (B). LVV, lentivirus vector; VLP, virus-like particle; DSB, DNA double-strand break.

outside the nucleoli in cells transduced with IN-I-PpoI-containing LVVs or VLPs after 4–6 hours of treatment (Figure 3, Supplementary Figure S2 and Supplementary Table S1).

The emergence of concurrent DSBs at many genomic loci can be detrimental to a cell's survival. Constitutive I-PpoI expression results in the cleavage of about 10% of the rDNA I-PpoI sites, and is known to be cytotoxic in human cells (21). Although lentivirus particles have been estimated to contain only 15–250 molecules of IN (40,41), we noticed a distinct morphology in LVV or VLP IN-I-PpoI transduced cells, indicative of I-PpoI's cytotoxicity. Indeed, the viability of cells transduced with these vectors was found to decrease after transduction (Figure 4). In conclusion, the IN-fusion protein strategy is an efficient means to package site-specific nucleases into LVV or VLP particles, which can deliver their protein cargo into transduced cell nuclei to obtain genome cleavage. In the case of I-PpoI, which has several cleavage sites both in the nucleolar rRNA genes and in other genomic locations, the extent of DNA cleavage was cytotoxic.

Directed integration into rDNA by IN-I-PpoI$_{N119A}$

To study whether the IN-fusion protein disabled for DNA cleavage would have an impact on the vector integration site selection, MRC-5 cells were transduced with LVVs wt IN/IN$_{D64V}$+IN-I-PpoI$_{N119A}$ and cellular ISs were extracted using LM-PCR and 454 sequencing. ISs were mapped to rDNA by counting BLAT hits in the unplaced supercontig ChrUn_gl000220 that contains one full and one slightly shortened rRNA gene repeat. To determine the level of background rDNA integration by an unmodified HIV-1 lentivirus vector, the abundance of ISs in rDNA was studied using two published vector data sets (Figure 5). No integrants were found in rDNA for the smaller data set that was generated with the same restriction enzymes as the data described here. For the larger data set, 0.1% of the vector's 40604 cellular ISs were localized to the rDNA. In contrast, an rDNA-targeting efficiency of 2.7% was found for the vectors containing IN$_{D64V}$+IN-I-PpoI$_{N119A}$, the difference to the control vectors being significant. The second IN-modified vector tested, wt IN+ IN-I-PpoI$_{N119A}$, yielded an rDNA-targeting efficiency of 0.2%. The reason why it failed to target integration into rDNA above the background levels may be due to an uneven distribution of the fusion protein in newly formed vector particles; if only wt IN containing LVVs are generated along with wt IN+ IN-I-PpoI$_{N119A}$ particles, these would integrate more efficiently and randomly, affecting the result. The intact catalytic core domain of the wt IN molecule may also compete in DNA binding with I-PpoI$_{N119A}$. Integration targeting towards the I-PpoI recognition sites residing in the non-nucleolar chromosomes (Supplementary Table S2) was not observed for either of the IN-I-PpoI$_{N119A}$-containing vectors. The majority of the IN-modified vectors ISs in rDNA localized to 18S and 28S rRNA genes (Supplementary Figure S3). For the control vector,
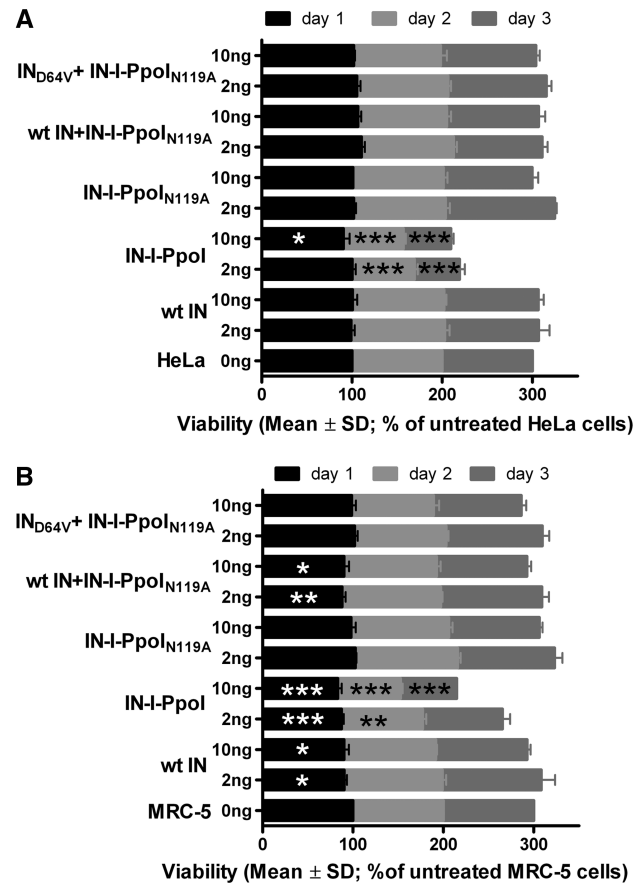
Figure 4. Cytotoxicity of IN-I-PpoI containing LVVs. HeLa (**A**) and MRC-5 (**B**) cells were transduced with two vector concentrations (2 and 10 ng of p24 per well) of LVVs containing different IN molecules (left). Cellular viability was measured 24, 48 and 72h after transduction (day 1, 2 and 3). Viability of the untreated cells at each time point is set to 100%. Viability of the vector-treated cells in a given time point is shown as the percentage of the untreated cells' values. Differences between vector-treated groups and the untreated cell values were analysed at each time point using one-way ANOVA and the Dunnett's Multiple Comparison Test. ***$P < 0.001$, **$0.001 < P < 0.01$, *$0.01 < P < 0.05$.
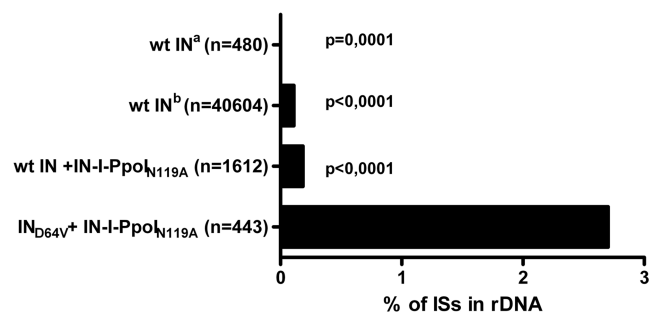
Figure 5. Integration frequency in the rDNA. Integration frequency in the rDNA is shown. Previously published data sets of HIV-1 integration sites are shown as a reference. All vector sets were compared with each other. Differences between vectors were statistically significant only between IN$_{D64V}$+IN-I-PpoI$_{N119A}$ and the rest of the vectors; the *P*-values for these comparisons are shown. IS, integration site. [a]HIV-1 vector integration sites (53) generated with the same restriction enzymes as the IN-fusion protein data sets. [b]HIV-1 vector integration sites generated with the restriction enzymes AvrII and MseI (43).

many ISs also localized to parts of the contig in which no rRNA gene-related annotations were found.

Taken together, IN-I-PpoI$_{N119A}$ increased transgene integration into rDNA when complemented with IN$_{D64V}$. Because significantly less IS were found in rDNA for the control vectors, this shift is addressable to the DNA recognition properties of I-PpoI$_{N119A}$.

IN-I-PpoI$_{N119A}$ changes the typical integration pattern of LVVs

Dimerization and folding of the IN-attached I-PpoI may sterically inhibit the interaction of IN with its important cellular cofactor LEDGF/p75. Differences seen in the vectors' integration frequency with regard to specific genomic features argues in favour of this theory (Table 1 and Figure 6). HIV-1 normally prefers AT-rich sequences close to integration sites, likely resulting from the

DNA-binding specificity of LEDGF/p75 through its AT-hook (42), and disfavours integration in CpG islands (43). In contrast to the control vector, both of the IN-modified vectors showed favoured integration into CpG islands and GC-rich DNA close to the integration site (Figure 6). This shift is also seen in LEDGF/p75-depleted cells, where the lentiviral integration pattern starts to resemble that of simple retroviruses (44). The similarity of the change in integration pattern implies that I-PpoI blocks the interaction of IN with LEDGF/p75 or alternatively competes in DNA binding with LEDGF/p75, tethering IN to more GC-rich DNA. Parts of the rRNA genes have a high GC content and CpG island frequency (Supplementary Figure S3), but this is unlikely to explain the difference because the majority of vector IS reside in non-nucleolar DNA. With respect to integration within oncogenes, no differences were found
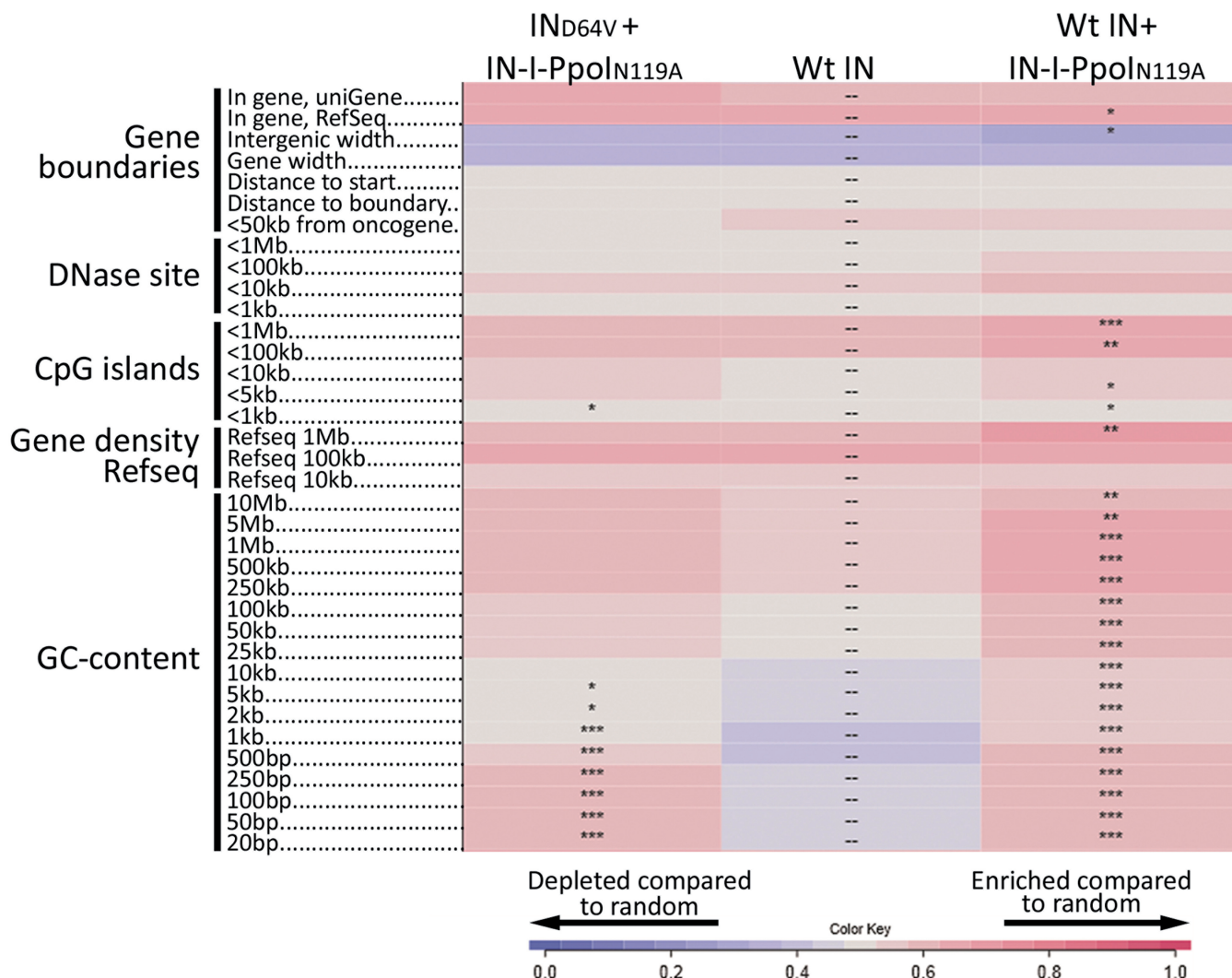
Figure 6. Integration frequency in different genomic features. A heat map summarizes the relationships of vector integration site data sets (indicated above the columns) to selected genomic features (left of the corresponding row of the heat map). Tile colour indicates whether integration by different vectors is favored (increasing shades of red) or disfavored (increasing shades of blue) in a given feature relative to their matched random controls, as detailed in the colored receiver operating characteristic area scale at the bottom of the panel. The p-values shown as asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) emerge from significant departures from the wt IN data set (53). The base pair values in the row labels indicate the size of the genomic interval used for analysis. Statistical methods and detailed naming of the genomic features: Berry *et al.* (30) and Brady *et al.* (33,54).

Table 1. Summary of integration frequency in genomic features

Genomic feature	Vector			Matched random controls		
	$IN_{D64V}+$ IN-I-PpoI$_{N119A}$	wt IN+ IN-I-PpoI$_{N119A}$	wt IN (Ref. 53)	$IN_{D64V}+$ IN-I-PpoI$_{N119A}$	wt IN+ IN-I-PpoI$_{N119A}$	wt IN (Ref. 43)
Total sites	443	1612	480	1308	4827	6237
In RefSeq genes (%)	69.7***	73.5***	69.8***	41.4	40.6	39.1
In CpG islands (<2 kb)[a]	2.8***	1.5*	0.6	1	1	1
GC% at IS (<2 kb)	41.6	42.6***	39.6***	41.2	41.2	41
GC% at IS (<20 bp)	44.8***	46.5***	39.8	41.1	41	41
In allOnco (%)[b]	9.0**	9.4***	8.3***	4.9	4.7	4.1

Integration sets and their genomic distributions are shown. Significant deviation from matched random controls is shown. Statistical analysis between vectors and their matched random sets: Fisher's exact test for in/out or <XX-bp based annotations and the Wilcoxon rank sum test used for Counts/GC-based annotations.
[a]Frequency: ISs/matched random controls.
[b]The allOnco list: http://microb230.med.upenn.edu/protocols/cancergenes.html.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

between the modified vectors and the control vector (Table 1).

DISCUSSION

Targeted integration of transgenes to predetermined genomic sites presents one of the most important goals in current vector development. The ability of DNA repair proteins to incorporate exogenous DNA with homology arms to nuclease-catalysed DSBs has been harnessed in the majority of recent methods. Although good results can be obtained by transfecting the nuclease-encoding and donor DNA-carrying plasmids into cells, a broader applicability of the DSB–HDR mechanism requires better vectorization of its components. To this end, these sequences have been transferred into IDLVs (6,45–49), which promote transient expression in dividing cells. Such a setting can lead to site-specific transgene integration at high-efficiency *in vitro*, although in many cell types it has remained below 5% (45–49). Cellular expression of the nuclease from an IDLV also holds potential for unwanted genotoxicity through off-target activity, or inadvertent integration of the expression construct. In addition, IDLV delivery of ZFNs and the donor molecule generally rely on generating and using three different vectors, which is impractical in terms of maximized transduction efficiency and is difficult to apply for *in vivo* use.

The IN-fusion protein, or *cis*-packaging strategy, described here is a method by which both a desired protein and a transgene construct can be simultaneously delivered into transduced cells within one vector particle. Consequently the targeting protein does not need to be expressed in transduced cells, but is delivered at fixed amounts. In contrast to the HIV-1 Vpr-based *trans*-packaging method (7), the IN-fusion protein approach does not require increasing the number of plasmids transfected into vector-producing cells to obtain foreign protein incorporation. This may enhance the levels of vector production and avoids optimization of new transfection schemes. We generated vectors that contained both an

IN-fusion protein with DNA-cleaving activity and a fusion protein where only the DNA-binding activity of I-PpoI was retained. With such vectors, we were able to demonstrate that the *cis*-packaging method is applicable for both the nuclear delivery of a meganuclease and altering the integration pattern of LVVs with increased transgene integration in the rDNA.

As a target for transgene integration, rDNA seems like an interesting GSH candidate owing to the many unique features it bears in comparison with non-nucleolar DNA. First, rRNA genes are isolated on five short chromosome arms where they reside far away from protein-coding genes with oncogenic potential. Second, the numerous copies of rRNA genes can compensate for the loss of one gene due to transgene integration. Third, the spacer regions between rRNA gene repeats may limit the transcriptional status of the transgene from spreading to the surrounding chromatin, and vice versa. rDNA clusters are subject to meiotic rearrangements at a high frequency, which leads to considerable variation in rDNA cluster size between healthy individuals (22). Under mitosis, however, the gene cluster architecture is ordinarily well preserved (50). It is therefore not likely that transgenes inserted in the rRNA genes of somatic cells will become eliminated, translocated or multiplied due to rDNA cluster recombination. Assessing the long-term stability of rDNA-inserted transgenes is nonetheless important to fully characterize the locus' suitability as a GSH.

With vectors containing the IN-I-PpoI$_{N119A}$ fusion protein, we found 2.7% of the cellular integration sites to localize to rDNA, which is a significant increase to the 0.1% analysed for unmodified lentivirus vectors. Previously the rDNA of human cells has been targeted for transgene integration using electroporated homologous donor molecules (51). The actual frequency of HR in this system (1×10^{-5}) is, however, typically considered too low for practical applicability. When compared to a similar strategy that used an IN-fusion protein to target the cellular E2C site, our targeting efficiencies are 2-3 fold higher (15). However, differences in analyzed integration site numbers impede full comparability of the results.

Nucleases are efficient tools to acquire targeted genome modifications, but integration of a single vector copy in the correct locus may represent only a fraction of all possible outcomes after DSB induction. Even donor molecules lacking homology can be incorporated into or close to the cleaved site with surprising efficiency through non-homologous end joining (NHEJ) (47). With homology-containing donor molecules, 10% of the targeted integration reactions were found to result from the combined action of HDR with NHEJ and 7% of the analysed donors had integrated randomly. Concatameric donor molecule insertions are also frequently seen in experiments targeting transgene integration into a cellular DSB (45,46,6). This highlights the need for in-depth analysis of all potential recombination events in the nuclease-treated cells to avoid problems that could arise from random integration, incorporation of unintended vector sequences through NHEJ or disruption of an already corrected sequence. IN-catalysed lentivirus vector integration is not known to associate with concatameric insertions, enzyme-dependent cytotoxicity or genomic rearrangements. However, because lentiviruses tend to integrate within expressed genes, their applicability for therapeutic gene integration would be improved by efficiently targeting integration to safer genomic areas.

In conclusion, the data presented here show that IN-fusion proteins can be used as an alternative to Vpr to package DNA-cleaving proteins into lentivirus vectors, but also to increase IN-catalysed transgene integration at a pre-determined genomic locus. An IN-fusion protein with fewer genomic cleavage sites than found for I-PpoI could shed light on the question of whether this approach could also be used to enhance site-specific gene insertion through HDR and NHEJ. Also, the efficiency of IN-catalysed integration targeting may be increased with alternative DNA-binding proteins targeting the rDNA, or other genomic sites proposed as GSHs (52).

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SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–2, Supplementary Figures 1–3 and Supplementary Methods.

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