

Cell type differences in activity of the *Streptomyces* bacteriophage ϕ C31 integrase

Christof Maucksch^{1,2}, Manish Kumar Aneja¹, Elisabeth Hennen¹, Alexander Bohla¹, Florian Hoffmann¹, Markus Elfinger^{1,2}, Joseph Rosenecker¹ and Carsten Rudolph^{1,2,*}

¹Department of Pediatrics, Ludwig-Maximilians-University, 80337 Munich and ²Free University of Berlin, Department of Pharmacy, 14166 Berlin, Germany

Received June 30, 2008; Revised July 31, 2008; Accepted August 1, 2008

ABSTRACT

Genomic integration by the *Streptomyces* bacteriophage ϕ C31 integrase is a promising tool for non-viral gene therapy of various genetic disorders. We investigated the ϕ C31 integrase recombination activity in T cell derived cell lines, primary T lymphocytes and CD34⁺ haematopoietic stem cells in comparison to mesenchymal stem cells and cell lines derived from lung-, liver- and cervix-tissue. In T cell lines, enhanced long-term expression above control was observed only with high amounts of integrase mRNA. Transfections of ϕ C31 integrase plasmids were not capable of mediating enhanced long-term transgene expression in T cell lines. In contrast, moderate to high efficiency could be detected in human mesenchymal stem cells, human lung, liver and cervix carcinoma cell lines. Up to 100-fold higher levels of recombination product was found in ϕ C31 integrase transfected A549 lung than Jurkat T cells. When the ϕ C31 integrase activity was normalized to the intracellular integrase mRNA levels, a 16-fold difference was found. As one possible inhibitor of the ϕ C31 integrase, we found 3- to 5-fold higher DAXX levels in Jurkat than in A549 cells, which could in addition to other yet unknown factors explain the observed discrepancy of ϕ C31 integrase activity.

INTRODUCTION

Promising clinical gene therapy trials have been undertaken recently to treat X-linked severe combined immunodeficiency (X-SCID) using replication-deficient retroviruses for gene delivery of the common cytokine receptor γ -chain (1,2). Autologous CD34⁺ haematopoietic stem cells were transduced *ex vivo* and retransfused into patients, which led to a reconstitution of the bone marrow and normal immune responses in 17 out of 20 treated patients (3). However, out of 17 X-SCID patients, which

responded to the treatment, five patients developed T cell leukaemia within 3 years after treatment due to insertional mutagenesis (4–6). As an alternative to viral gene delivery systems, non-viral gene therapy holds great promise for the treatment of monogenetic diseases like X-SCID without causing such assimilable severe side effects. Though transfection efficiency and transgene stability with non-viral vectors still lags behind viral systems, new strategies for genomic integration of the transgene have been developed, which reduce the risk of insertional mutagenesis events (7). One such tool to achieve safe and stable non-viral gene delivery is the *Streptomyces* bacteriophage derived ϕ C31 integrase, which mediates site-specific integration of plasmid DNA (pDNA) into mammalian host genomes (8,9). We have recently shown that the ϕ C31 integrase system could have potential for the treatment of lung diseases, which was demonstrated by long-term transgene expression in lung cells *in vitro* and *in vivo* without selection pressure (10). Others have previously shown recombination activity of the ϕ C31 integrase and resulting long-term transgene expression in tissues like murine liver (9), rat retina (11) or human skin (12). So far there has been only one study that addressed ϕ C31 integrase activity in haematopoietic cells. Ishikawa *et al.* (13) characterized several known and new integration sites and observed enhanced gene expression mediated by the ϕ C31 integrase in T cell derived Jurkat cell line under selection with G418. The goal of this study was to compare the activity of the ϕ C31 integrase in the haematopoietic system with cells derived from other tissues. One focus of our study was to avoid application of cell-selection procedures, which may not be applicable to clinical protocols. Though we could confirm that the ϕ C31 integrase system is active in haematopoietic cells, we could not observe any long-term luciferase reporter gene expression mediated by co-transfection of ϕ C31 integrase expression plasmids. Only co-delivery of high amounts of integrase mRNA resulted in enhanced long-term transgene expression in one T cell line. Moreover, the activity of ϕ C31 integrase for episomal recombination was low in any haematopoietic cell type investigated. To determine if the ϕ C31 integrase

*To whom correspondence should be addressed. Tel: +49 89 5160 7711; Fax: +49 89 5160 7846; Email: carsten.rudolph@med.uni-muenchen.de

system is less efficient in the haematopoietic system in general, we examined primary haematopoietic cells and cell lines in comparison with primary cells and cell lines of various other tissues. We found that the activity of the ϕ C31 integrase is strongly cell type specific. Particularly in haematopoietic cells only low activity was observed, whereas in cells of all other tissues investigated moderate to high activity of the ϕ C31 integrase was detected. It has previously been shown that DAXX protein interacts with the ϕ C31 integrase and inhibits recombination (14). We further detected higher amounts of DAXX protein in Jurkat T cells than in A549 lung cells, which could be one reason for the reduced ϕ C31 integrase activity in haematopoietic cells.

MATERIALS AND METHODS

Plasmids

pCMVInt has been previously described (8,9,12) and was kindly provided by M. P. Calos (Stanford University, USA). pCAG-Int(NLS) and pPGK-Int(NLS) coding for an integrase fused with a C-terminal nuclear localization signal (NLS) of SV40 T antigen, driven by CAG and PGK promoters, respectively, and pSVpaxattP50-attB53, comprising an 'expression blocking' sequence flanked by *attB* and *attP* sites between a P1 promoter and a lacZ gene, were kindly provided by R. Kuehn (Helmholtz Zentrum Munich, German Research Centre for Environmental Health, Germany). pC31-Intopt was obtained from Addgene Inc. (Cambridge, MA, USA). pVAX1-Int was constructed by excising the integrase cDNA from pCMV-Int by PstI-XhoI (Fermentas, St. Leon-Rot, Germany) digestion and cloning into respective sites of pVAX1 (Invitrogen, Paisley, UK). pdelCpG-GFP was obtained from Genart (GENEART AG, Regensburg, Germany) and contains the same expression cassette as pVAX1 but has reduced number of CpG motifs. The GFP expression cassette was removed from this plasmid by HindIII-BamHI digestion and self-circularized to obtain pdelCpG. Fragment containing the integrase was excised from pVAX1-Int by PmeI digestion and cloned into PmeI site of pdelCpG to generate pdelCpG-Int. mRNA coding for the ϕ C31 integrase was produced by CureVac (CUREVAC GmbH, Tuebingen, Germany). pVAX1-Luc was constructed as a shuttle vector by excising firefly luciferase cDNA from pGL3-Basic vector (Promega, Madison, WI, USA) by HindIII-XbaI digestion and cloning into the corresponding sites of pVAX1. A HindIII-PmeI fragment from pVAX1-Luc was then cloned into the same sites of pdelCpG-GFP, thus replacing GFP with luciferase and generating pdelCpG-Luc. ϕ C31 integrase recognition sequence *attB* was excised from pTA-*attB* (provided by M. P. Calos) by EcoRI digestion and was cloned blunt ended into the BglII site of pdelCpG-Luc to obtain pdelCpG-Luc-*attB*. pEGFPLuc*attB* was kindly provided by T. W. Chalberg (Stanford University, USA). Briefly, the plasmid codes for the fusion protein of EGFP and luciferase driven by the CMV promoter and contains the *attB* recognition site for the integrase in the plasmid backbone adjacent to the ampicillin selection marker. All constructs used in this study are schematically represented in Figure 1.

Cell culture

A549 (alveolar type II cells), HepG2 (hepatocytes), HeLa (epithelial cervix carcinoma cells) and Jurkat (T cells) cell lines were obtained from German Collection of Microorganisms and Cell Cultures (DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). BEAS-2B (bronchial epithelia cells) cell line was purchased from the ATCC (American Type Culture Collection, Rockville, MD, USA). Human primary CD3⁺ T cells were kindly provided by T. Magg (Ludwig-Maximilians-University, Germany). Human primary mesenchymal stem cells (Cambrex Corporation, East Rutherford, NJ, USA) were kindly provided by C. Karow (Ludwig-Maximilians-University, Germany) and grown in Dulbecco's Modified Eagle-Medium (PPA Laboratories, Linz, Austria) containing 10% fetal bovine serum (PPA Laboratories, Austria). ED-7R cell line (15) was kindly provided by S. Kumaki (Tohoku University, Japan). Umbilical cord blood samples were kindly provided by Bavarian Stem Cell Bank (Bayerische Stammzellbank GmbH, Germany). Mononuclear cells (MNC) were prepared from umbilical cord blood and separated by Ficoll-Hypaque density gradient centrifugation as described by Harris *et al.* (16). CD34⁺ cells were further isolated from the initially separated MNC by high-gradient magnetic sorting using Human CD34 MultiSort Kit (MACS, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) following manufacturer's protocol. CD34⁺ cells were grown in X-Vivo 10 (Cambrex Corporation, USA) supplemented with SCF (20 ng/ml; Peprotech Inc, Rocky Hill, NJ, USA), TPO (50 ng/ml; Peprotech Inc, USA), human Flt-3 ligand (50 ng/ml; Peprotech Inc, USA). Human A549, BEAS-2B, HepG2 and HeLa cells were grown in Minimum Essential Medium (Gibco-BRL, Karlsruhe, Germany) containing 10% fetal calf serum (PPA Laboratories, Austria). Jurkat, ED-7R and human primary T cells were grown in RPMI 1640 Medium with Glutamax (Gibco-BRL, Germany) containing 10% fetal calf serum (PPA Laboratories, Austria). All cells were maintained at 37°C in a 5% CO₂ humidified air atmosphere.

Transfections

1×10^6 A549, Jurkat, ED-7R, primary T cells, huHSC and huMSC were transfected with nucleofection (Amaxa Biosystems, Cologne, Germany) following manufacturer's protocols using 5 μ g pDNA in total, respectively. When transfections were performed with Metafectene Pro (Biontex, Planegg, Germany) and Lipofectamin 2000 (Invitrogen, UK), 1×10^5 cells per well were seeded in 24-well plates 24 h prior the transfection. Metafectene Pro was used with a total of 1 μ g pDNA in a ratio 4:1 (v/w) to transfect A549, BEAS-2B, HepG2, HeLa and Jurkat cell lines as recommended by the manufacturer, respectively. Lipofectamin 2000 was used to transfect A549 cells with 1 μ g of pDNA in a ratio 2:1 (v/w).

Luciferase and β -galactosidase assay

Twenty-four or 72 h post-transfection cells were washed twice with PBS followed by the addition of 100 μ l of

lysis buffer (25 mM Tris-HCl, 0.1% Triton X-100, pH = 7.8). Cells were incubated for 10 min at room temperature in the lysis buffer. Ten microlitres of the lysate was used to measure the protein amount by Bio-Rad protein assay (BioRad, Munich, Germany) with a bovine serum albumin standard. Fifty microlitres of the cell lysate was used to measure luciferase activity or β -galactosidase on a Wallac Victor² 1420 Multilabel Counter (Perkin Elmer, Waltham, MA, USA), respectively. For measurement of β -galactosidase activity, β -gal reporter gene assay kit (Roche, Indianapolis, IN, USA) was used following manufacturer's protocol. Values were normalized with respect to the protein concentration.

Western blot analysis

Jurkat and A549 cells were grown in 6-well plates and transfected with either pCMV-Int or pC31-Intopt by nucleofection (as described earlier). Twenty-four hours after transfection, Jurkat cells were washed once with PBS and centrifuged by spinning at 300g for 5 min. Medium was aspirated from A549 cells and the cells were washed with PBS, dislodged from the well bottoms by a rinsing with 200 μ l Trypsin-EDTA (Gibco-BRL, Germany), washed with fresh PBS and Jurkat cell suspensions were pelleted by spinning at 200g for 5 min. Jurkat and A549 cells were then lysed by adding 50 μ l of lysis buffer [0.15 M sodium chloride, 5 mM EDTA, 1% Triton X-100, 100 mM Tris-HCl, 1 M DTT, CompleteTM protease inhibitors (Roche, USA); pH 7.4] After a 15 min incubation on ice, lysates were sonicated with a Digital Sonifier 250 (Branson, Danbury, CT, USA) and cleared by 30 min of centrifugation at 13 400 r.p.m. at 4°C. For western blot analysis, equal amounts of total protein were separated by electrophoresis on 7.5% Tris-HCl gels (BioRad, Germany) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Membranes were incubated either with anti- ϕ C31 integrase rabbit pAb (kindly provided by M. C. Smith, University of Aberdeen, UK) at a 1:2000 dilution or anti-DAXX mouse mAb (Biozol, Eching, Germany) at a dilution of 1:100, respectively. As secondary antibodies, either a goat anti-rabbit horseradish peroxidase (HRP) conjugated antibody (BioRad, Germany) or a goat anti-mouse HRP conjugated antibody (BioRad, Germany) was utilized. Proteins were detected with the ECL chemiluminescence system (Amersham Biosciences, Piscataway, NJ, USA).

Isolation of pDNA and analysis by PCR

Isolation of pDNA from mammalian cells was performed by Qiaprep Spin Miniprep kit (Qiagen, Hilden, Germany) following manufacturer's protocol (News Qiagen Issue No. 2/95). The recombined plasmid product was detected by PCR using Taq DNA polymerase (New England BioLabs, Beverly, MA, USA) and the following primers: FPpuro 5'-GCAAGGGTCTGGGCAGCG-3' and RPpuro 5'-TTGCCGATGTCGAGCCCG-3'. One nanogram of the isolated pDNA was used as template in the reaction. PCR was performed using the following settings: 94°C for 30 s, 30 cycles with 94°C for 30 s, 60°C for 20 s, 72°C with 30 s, followed by 72°C for 5 min.

Isolation of total RNA, reverse transcription and real-time quantitative PCR

Quantitative real-time PCR (qRT-PCR) was performed to quantify recombination products. Ten nanogram of isolated pDNA was used as template. The primers and settings were the same as described earlier.

Total RNA was isolated by RNAeasy Mini Kit (Qiagen, Germany) from Jurkat and A549 cells at specific time-points after transfection, respectively. cDNA was produced from 2 μ g total RNA using Superscript reverse transcriptase (Invitrogen, UK) following the manufacturer's protocol. qRT-PCR reactions were carried out in a volume of 50 μ l. The following primers were used to amplify integrase cDNA after transfection of pCMV-Int and pC31-Intopt, respectively: FPint 5'-ACAAGATATC GTGACCGGGGTGGAC-3', RPint 5'-TCCTCGAGCG CCGCTACGTC-3', FPintopt 5'-ATCACCGGCCTGTG TAAGAG-3', RPintopt 5'-CGTCGGGCTTCTTCTTGT AG-3'. An initial denaturation of 3 min at 95°C was followed by 30 cycles with 95°C for 30 s, 62°C for 20 s and 72°C for 90 s. Two microlitres cDNA template was used per reaction to quantify the integrase mRNA expression.

Specific primers were used to quantify DAXX: FP-DAXX 5'-GGACCCACAAATGCAAACACTGC-3' and RP-DAXX 5'-AGGGGATGCGCTCTATGACA CG-3'. Here, an initial denaturation of 3 min at 95°C was followed by 30 cycles with 95°C for 30 s, 68°C for 20 s and 72°C for 30 s.

Of the cDNA template, 0.5 μ l was used per reaction to quantify the DAXX mRNA expression, respectively. Specificity of the PCR products was assessed by a melting curve analysis and agarose electrophoresis.

Statistical methods

Results of multiple experiments are reported as means \pm S.D. Data were analysed using a two-tailed paired Student's *t*-test. Probability values $P < 0.05$ (marked as asterisk) designated significant differences between test points.

RESULTS

Long-term gene expression after transfection of haematopoietic T cell lines

Jurkat T cells were co-transfected with different constructs coding for the ϕ C31 integrase and p Δ lCpG-Luc-*attB*, containing the *attB* sequence and a luciferase gene in a CpG dinucleotide reduced backbone. The following integrase constructs were tested: pCMV-Int, p Δ lCpG-Int, pVAX1-Int, pPGK-Int(NLS) and pCAG-Int(NLS) (Figure 1). p Δ lCpG was used as a negative control for the integrase plasmids. These plasmids were co-transfected with p Δ lCpG-Luc-*attB* in a ratio of 4:1 (w/w) using nucleofection, which is an electroporation-based transfection method and has previously been shown to efficiently transfect suspension cells (17–19). Co-transfection of pDNA coding for the ϕ C31 integrase compared to the negative control p Δ lCpG (Figure 2A) or using p Δ lCpG-Luc lacking *attB* could not significantly enhance

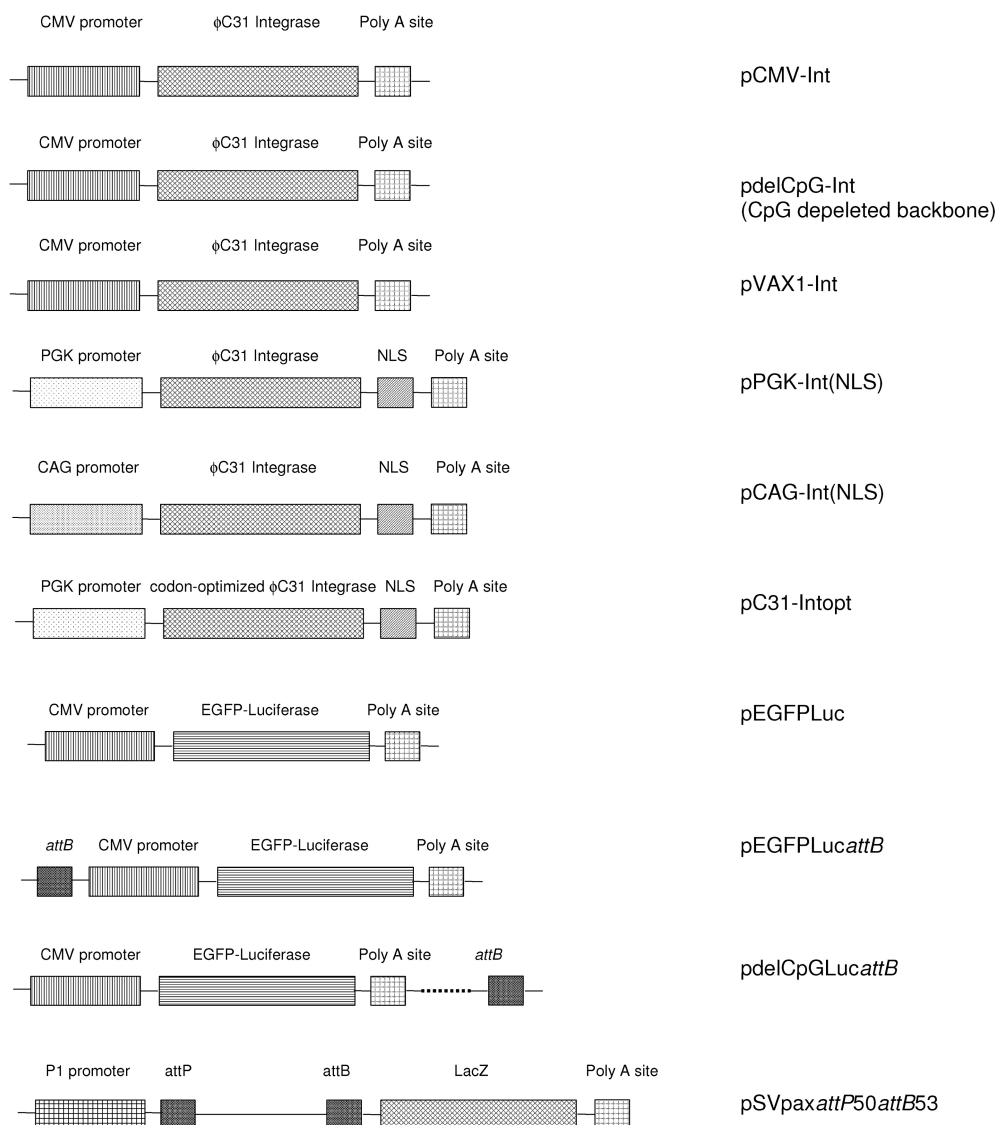


Figure 1. Schematic representation of the pDNA constructs used in the present study.

long-term luciferase expression in Jurkat cells (data not shown). One of the reasons for the lack of long-term gene expression may have been low integrase expression levels. As it has previously been shown that mRNA transfection in haematopoietic monocytes is more efficient than pDNA transfections (20), additional experiments were performed using ϕ C31 integrase mRNA instead of pDNA. Using nucleofection, 0.5, 1.0 or 2.0 μ g of integrase mRNA was co-transfected with 1 μ g pdelCpG-Luc-*attB*. pdelCpG vector was used as negative control in these experiments. With integrase mRNA, significantly enhanced long-term gene expression, compared to the negative control, could be observed up to day 33 post-transfection when a high amount (2 μ g) of integrase mRNA was used (Figure 2B). We repeated these experiments in another T cell line ED-7R, but in contrast to Jurkat cells, transgene expression was not significantly different from pdelCpG control transfections (Figure 2C). Similar results were obtained when these long-term

expression experiments were repeated using electroporation instead of nucleofection as gene delivery method, using pEGFPLuc*attB* instead of pdelCpG-Luc-*attB* or using different ratios of integrase plasmids to luc-*attB* plasmid (data not shown).

Expression of ϕ C31 integrase protein

The expression studies in Jurkat and ED-7R cells suggested either inefficient integrase expression or low recombination efficiency of the ϕ C31 integrase to achieve long-term gene expression in T cell lines. To confirm the expression of the integrase protein in the experimental settings, Jurkat cells were transfected with either pCMV-Int or the codon-optimized pC31-Intopt and western blot analysis for the integrase protein was performed 24 h post-transfection. As positive control for the integrase expression constructs, A549 lung cells were transfected with the same plasmids, a cell line in which it has previously been

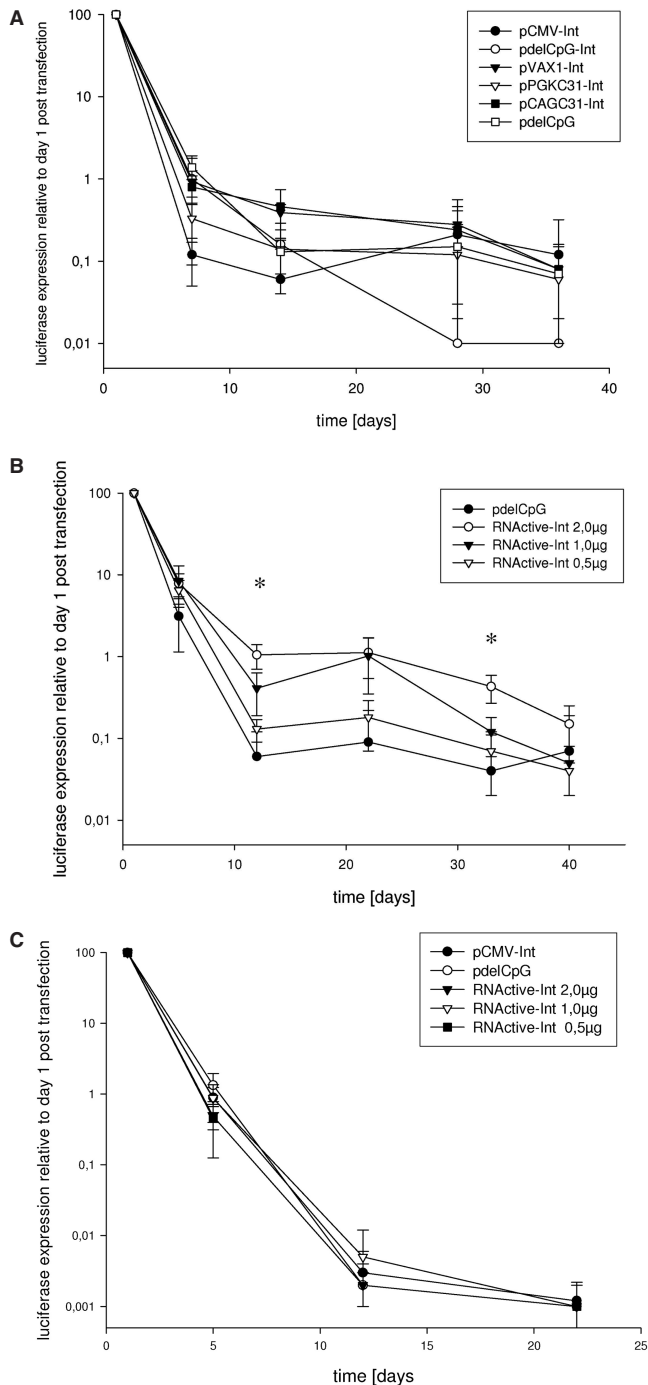


Figure 2. (A) Co-transfections of 1 µg pdelCpG-Luc-*attB* and 4 µg integrase/control plasmids, respectively, were performed in Jurkat cell line. No significant long-term enhancement of luciferase activity could be detected for the integrase constructs above pdelCpG control. (B) Co-transfections of 1 µg pdelCpG-Luc-*attB* and various amounts of mRNA coding for ϕ C31 integrase, respectively, were performed in Jurkat cell line. Significant enhancement of luciferase activity above control could be detected at two time points for 2 µg of integrase mRNA. (C) Co-transfections of 1 µg pdelCpG-Luc-*attB* and various amounts of mRNA or different plasmids coding for ϕ C31 integrase, respectively, were performed in ED-7R cell line. No luciferase activity could be detected for any construct after day 10. Nucleofection was used for all transfections. Luciferase values are relative to day 1 values. All experiments were performed in five replicates ($n = 5$).

shown that the integrase mediates long-term gene expression (10). Integrase specific bands for both constructs were detected in A549 and Jurkat T cells, respectively, and expression levels (either native or codon optimized) were equivalent in each cell line (Figure 3A). Further, integrase mRNA was quantified for pCMV-Int and pC31-Intopt using qRT-PCR in both cell lines, respectively. The pCMV-Int integrase mRNA level was significantly 5.8-fold higher in A549 cells than in Jurkat cells (Figure 3B), whereas no significant difference was detected for pC31-Intopt mRNA (Figure 3C).

Analysis of the ϕ C31 integrase function by measurement of reporter gene activity of episomal recombination products in different cell types

To specify whether the lack of integrase-mediated long-term transgene expression in T cells lines was only due to detection limits of luciferase or due to other yet unknown cell specific reasons, we set up an episomal recombination assay to investigate the ϕ C31 integrase activity in more detail. Various cell types were co-transfected with pSVpaxattP50-*attB53* and pdelCpG-Int or pCAG-Int together with an internal transfection control pEGFP_{Luc}. The 7.2 kb pSVpaxattP50-*attB53* comprises an 'expression-blocking' sequence flanked by *attB* and *attP* sites between the P1 promoter and the *lacZ* cDNA. This 'blocking' sequence prohibits expression of β -galactosidase. The ϕ C31 integrase mediates the excision of the 'blocking' sequence and brings the promoter in frame with the *lacZ* gene. Successful recombination results in a 6.1 kb recombined *plasmid A*, comprising the P1 promoter in frame with the *lacZ* cDNA, which results in β -galactosidase expression after transcription, and in a 1.1 kb recombined *plasmid B*, comprising the 'blocking' sequence. For all experiments a negative control was performed using pUC21 instead of the integrase plasmid. By subtracting the pUC21 negative control background from the β -galactosidase activity of the replicates, which were transfected with the integrase pDNA, it is possible to calculate the relative recombination efficiency of the ϕ C31 integrase in different cell types in terms of active transcription of the recombination product (*plasmid A*). Luciferase activity in all experiments confirmed successful transfections. β -galactosidase activity derived from *plasmid A* in all non-haematopoietic cell lines indicated functionality of the ϕ C31 integrase, whereas no β -galactosidase activity was measured in any of the haematopoietic cell types (Figure 4A). The β -galactosidase expression was highest in bronchial BEAS-2B cells, intermediate in liver HepG2 and HeLa cervix carcinoma cells and lowest in alveolar type II A549 and huMSC cells, which suggests a cell type-specific activity of the ϕ C31 integrase. To exclude effects due to variations of the transfection efficiency, different transfection methods and integrase constructs were investigated. A549 cells were co-transfected with pSVpaxattP50-*attB53*, pEGFP_{Luc} and pdelCpG-Int, pCAG-Int(NLS) or pUC21, respectively, using either nucleofection, Metafectene Pro or Lipofectamin 2000. Although the transfection methods resulted in variations of luciferase expressions ranging from low to high levels,

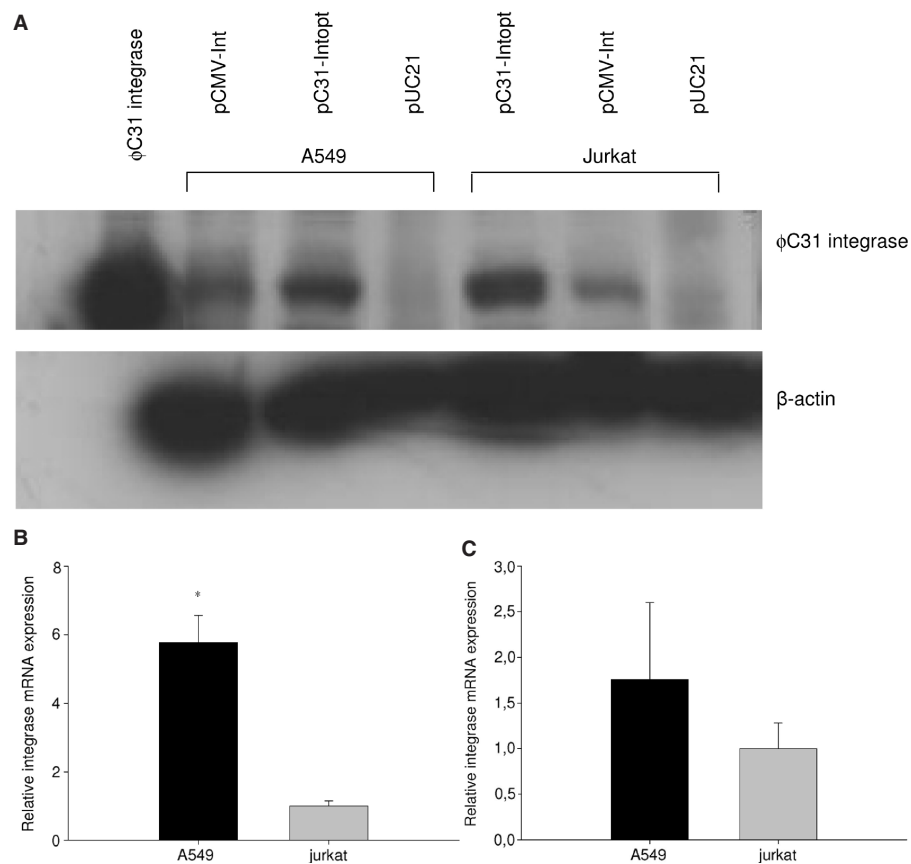


Figure 3. (A) Cell lysates of either pCMV-Int transfected A549 and Jurkat cells or pC31-Intopt transfected A549 and Jurkat cells were analysed by western blotting. Cell lysates of untreated A549 and Jurkat cells are represented. Detection of the integrase protein positive control is shown. (B) Total RNA was isolated from A549 and Jurkat cells 24 h post-transfection with pSVpaxattP50-attB53 and either pCMV-Int or (C) pC31-Intopt. Total cDNA was produced by reverse transcriptase and integrase cDNA was quantified by qRT-PCR. The values represent the relative mRNA integrase expression normalized to pCMV-Int in Jurkat cells 24 h post-transfection.

β -galactosidase activity derived from *plasmid A* could be measured for p δ elCpG-Int and pCAG-Int(NLS) and correlated with luciferase expression, in contrast to pUC21 control (data not shown). Therefore, the assay is independent of the transfection method and the used integrase construct.

Analysis of the ϕ C31 integrase function by PCR detection of the episomal recombination products in different cell types

To investigate whether the observed lack of episomal recombination in haematopoietic Jurkat T cells is due to limitations of β -galactosidase activity detection of *plasmid A* in the above used assay, or low activity of the ϕ C31 integrase in haematopoietic Jurkat T cells, a PCR-based assay was established to directly visualize the recombination product (*plasmid B*). Primers were designed, which bind within the blocking sequence next to each other and are amplified in opposite directions. This would allow amplification of a 0.94 kb fragment of the smaller recombination product (*plasmid B*) with a standard Taq polymerase. In contrast, neither the large 6.1 kb recombination product *plasmid A* nor the 7.2 kb parental plasmid could be amplified by a standard Taq polymerase because

of size constraints (>5 kb). In case of successful recombination mediated by the ϕ C31 integrase, a PCR fragment of 0.94 kb is expected. Whereas clear bands of the expected 0.94 kb size could be detected in A549, BEAS-2B, HepG2 and HeLa cells transfected with the integrase construct (p δ elCpG-Int), only a very weak band was detected for Jurkat cells (Figure 4B). As expected no bands could be detected in the pUC21 negative controls in all samples. The weak band indicated low activity of the ϕ C31 integrase in Jurkat cells.

Quantification of the episomal recombination efficacy of the ϕ C31 integrase in haematopoietic Jurkat T cells and A549 lung cells

A549 and Jurkat cells were chosen as model cell lines to quantify the activity of the ϕ C31 integrase in cells from representative tissues. To achieve optimal integrase activity, pC31-Intopt, which is a highly efficient integrase construct due to a codon-optimized cDNA and a NLS (21), was examined in comparison with pCMV-Int, which has been used in most previous studies (8,13,22,23), and pUC21 as a negative control. Metafectene Pro was used as transfection reagent, which is capable of transfecting, both A549 and Jurkat cells.

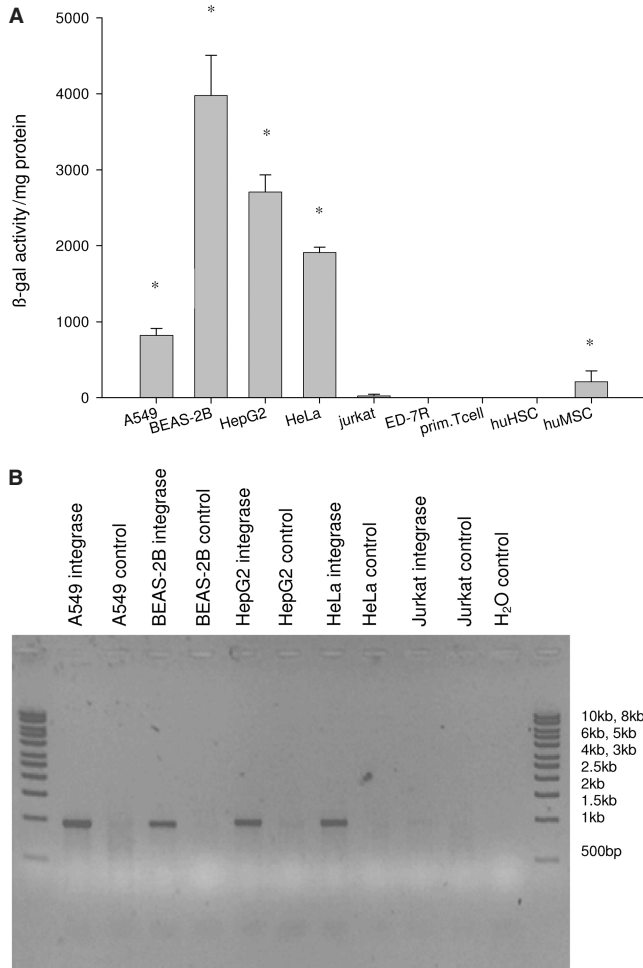


Figure 4. (A) Triple transfections of pSVpaxattP50-attB53, an integrase plasmid and pEGFP_{Luc} were performed in various cell types. Activity of ϕ C31 integrase is shown as β -galactosidase activity per milligram protein. The mean \pm standard deviation of ratio of β -gal per protein ($n = 5$) is shown at 24h post-transfection. (B) pDNA was isolated from cells transfected with pSVpaxattP50-attB53 and pdelCpG-Int or pUC21 control 24h post-transfection. Amplification of a 0.94 kb fragment of the 1.1 kb recombinant plasmid mediated by the functional ϕ C31 integrase was carried out by PCR. A549, BEAS-2B, HepG2, HeLa, Jurkat cells co-transfected with pdelCpG-Int, respectively; A549, BEAS-2B, HepG2, HeLa, Jurkat cells co-transfected with negative control pUC21, respectively; H₂O. Strong bands could be detected for all non-haematopoietic cell types transfected with the integrase plasmid by gel electrophoresis, whereas only a very weak band could be detected for Jurkat cell line. pUC21 negative controls show no bands in any cell type.

Twenty-four and 72h after transfection with pC31-Intopt, β -galactosidase activity from *plasmid A* was 45- and 265-fold higher in A549 than in Jurkat cells, respectively. After transfection of A549 cells with pCMV-Int, β -galactosidase activity was 3- and 34-fold lower than after transfection with pC31-Intopt 24 and 72h post-transfection, respectively. Transfection of Jurkat cells with pCMV-Int did not result in measurable β -galactosidase activity from *plasmid A* (Figure 5A). No β -galactosidase activity was observed in the negative pUC21 controls. These observations demonstrate that episomal recombination can be principally observed by measurement of active

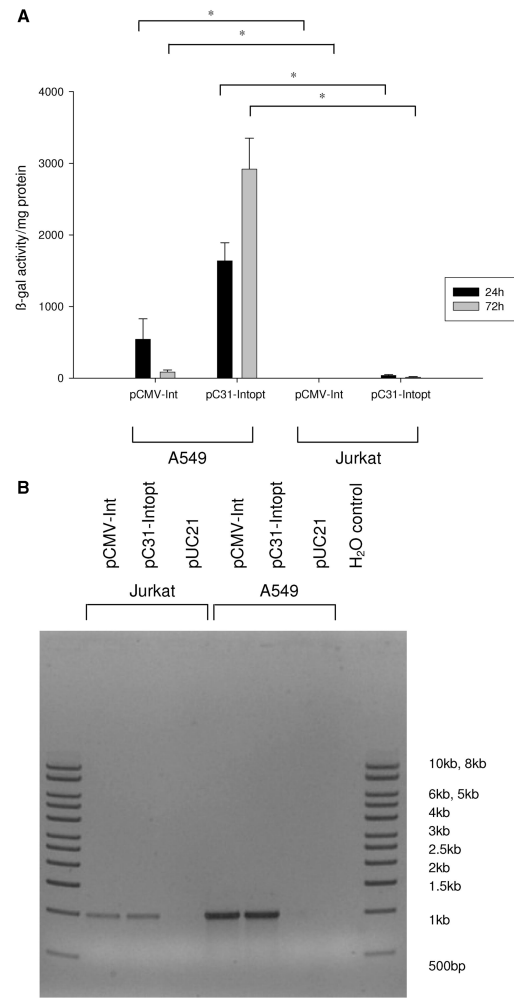


Figure 5. (A) Triple transfections of pSVpaxattP50-attB53, pCMV-Int or pC31-Intopt and pEGFP_{Luc} were performed in A549 and Jurkat cell lines. Activity of ϕ C31 integrase is expressed as β -galactosidase activity per milligram protein. The mean \pm standard deviation of ratio of β -gal per protein ($n = 5$) is shown at 24 and 72h post-transfection. (B) pDNA was isolated from A549 and Jurkat cells transfected with pSVpaxattP50-attB53 and pCMV-Int, pC31-Intopt or pUC21 control 24h post-transfection. Amplification of a 0.94 kb fragment of the 1.1 kb recombinant plasmid mediated by the functional ϕ C31 integrase was carried out by PCR. Jurkat cells co-transfected with pCMV-Int, pC31-Intopt, pUC21, respectively; A549 cells co-transfected with pCMV-Int, pC31-Intopt, pUC21, respectively; H₂O. Strong bands could be detected for A549 cells by gel electrophoresis, whereas only weak bands could be detected for Jurkat cell line transfected with the integrase plasmids. pUC21 negative controls show no bands in any cell type.

transcription of the recombination products in haematopoietic Jurkat cells by using a codon-optimized ϕ C31 integrase.

However, in order to directly investigate episomal recombination, the recombination products were further analysed by PCR and qRT-PCR. A strong band of the expected 0.94 kb fragment of the recombination product *plasmid B* was detected in A549 cells after transfection with pCMV-Int and pC31-Intopt, whereas only a weak band was observed in Jurkat cells 24h post-transfection (Figure 5B). Twenty-four hours after transfection, qRT-PCR resulted in statistically significant 100- and 29-fold

higher amounts of recombination product *plasmid B* in A549 than in Jurkat cells for pCMV-Int and pC31-Intopt, respectively. After 72 h, 3- and 120-fold higher amounts of recombination products were detected in A549 than in Jurkat cells for pCMV-Int and pC31-Intopt, respectively (Figure 6). Together with the measured mRNA levels after transfection with pCMV-Int or pC31-Intopt (Figure 3B and C), the ϕ C31 integrase recombination efficacy can be normalized to the integrase mRNA levels for each of the cell lines by dividing the amount of recombination product *plasmid B* by the amount of integrase mRNA (Table 1). In independent experiments with either pCMV-Int or pC31-Intopt, a 17- and 16-fold higher normalized ϕ C31 integrase activity was observed in A549 than in Jurkat cells 24 h post-transfection, respectively (Table 2). These data demonstrate

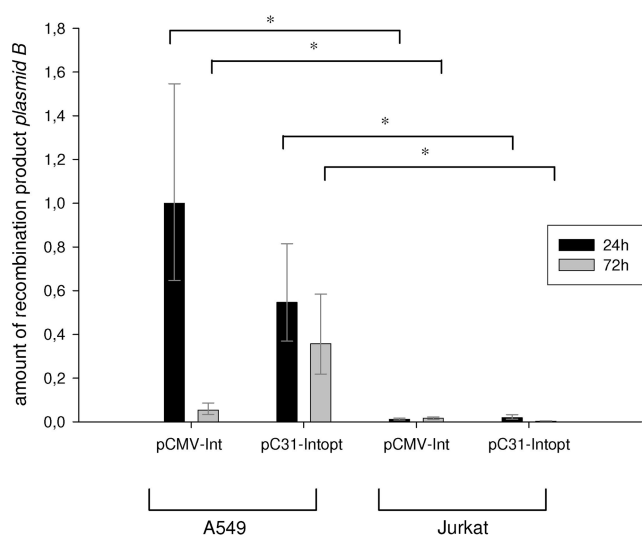


Figure 6. pDNA was isolated from A549 and Jurkat cells transfected with pSVpaxattP50-attB53 and pCMV-Int, pC31-Intopt or pUC21 control 24 and 72 h post-transfection was amplified and quantified by qRT-PCR. The amount of recombination product *Plasmid B* of the ϕ C31 integrase is shown for A549 and Jurkat cells 24 and 72 h post-transfection. The values represent the amounts of recombination products normalized to pCMV-Int in Jurkat cells 24 h post-transfection.

Table 1. ϕ C31 integrase activity in Jurkat T and lung A549 cells

Cell type	Time point (h)	β -galactosidase activity ^a		Amount of recombination product <i>plasmid B</i> ^b		Relative integrase mRNA expression ^c	
		pCMV-Int	pC31-Intopt	pCMV-Int	pC31-Intopt	pCMV-Int	pC31-Intopt
Jurkat	24	<i>n.a.</i>	3	1	2	1	1
	72	<i>n.a.</i>	1	1	0.3	<i>n.a.</i>	0.2
A549	24	49	149	100	55	5.8	1.7
	72	8	265	5	36	1	2

^aThe values represent the β -galactosidase activity normalized to pC31-Intopt in Jurkat cells 72 h post-transfection.

^bThe values represent the amount of recombination product normalized to pCMV-Int in Jurkat cells 72 h post-transfection.

^cThe values represent the integrase mRNA expression normalized to pCMV-Int in Jurkat cells 24 h post-transfection.

that the ϕ C31 integrase recombination efficacy is markedly reduced in haematopoietic Jurkat T cells compared with lung cells.

Quantification of DAXX protein in haematopoietic Jurkat T cells and A549 lung cells

DAXX protein has been previously shown to interact with ϕ C31 integrase and inhibit its recombination efficiency (14). To investigate if cell type specific differences in the amount of DAXX protein could be a likely reason for the reduced activity of the ϕ C31 integrase in T cells, western blot analysis of DAXX protein in Jurkat and A549 cells was carried out. Indeed, higher amounts of DAXX protein were observed in Jurkat than in A549 cells by semi-quantification of the western blot analysis (Figure 7A).

Table 2. Comparison of normalized ϕ C31 integrase activity in Jurkat T and lung A549 cells

Time point (h)	pCMV-Int ^a	pC31-Intopt ^b
24	17	16
72	<i>n.a.</i>	12

^aThe values represent the ratio of the native integrase efficacy (amount of recombination product *plasmid B*/relative integrase expression) between A549 and Jurkat-cells.

^bThe values represent the ratio of the codon-optimized integrase efficacy (amount of recombination product *plasmid B*/relative integrase mRNA expression) between A549 and Jurkat cells.

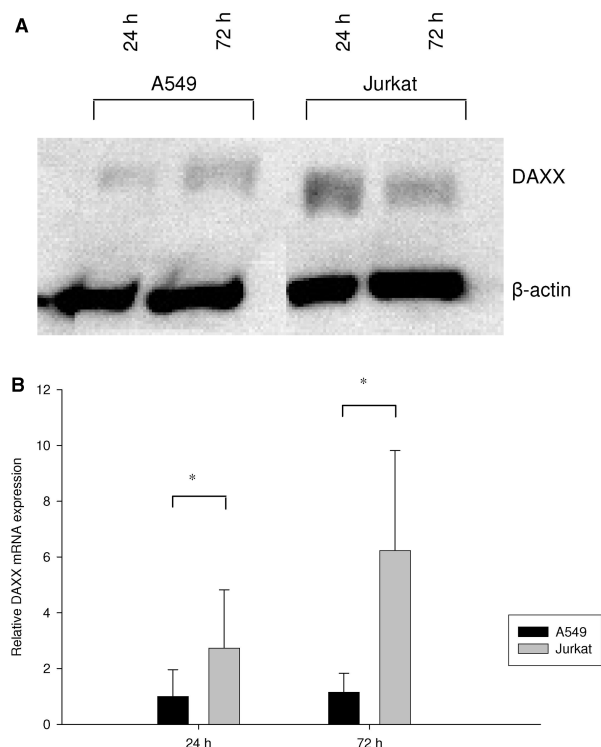


Figure 7. (A) DAXX protein was detected 24 and 72 h post-transfections of CMV-Int in A549 and Jurkat cells by western blotting, respectively. (B) Total RNA was isolated from A549 and Jurkat cells 24 and 72 h post-transfection with pSVpaxattP50-attB53 and pCMV-Int. Total cDNA was produced by reverse transcriptase and DAXX cDNA was quantified by qRT-PCR.

Further, qRT-PCR was performed to quantify DAXX on mRNA level in A549 and Jurkat cells. The amounts of DAXX were significantly 2.7- and 5.4-fold higher in Jurkat than in A549 cells 24 and 72 h post-transfection, respectively (Figure 7B).

DISCUSSION

It has previously been shown that the ϕ C31 integrase from the *Streptomyces* bacteriophage mediates integration of transfected pDNA carrying *attB* sites into pseudo *attP* sites of mammalian genomes. This process is relatively site specific. Whereas other recombinases as *Cre* or *Flp* also mediate excision (24), the recombination of the ϕ C31 is unidirectional resulting in only genomic integration (25). These properties give the ϕ C31 integrase a great potential for safe and long-term transgene expression. The ϕ C31 integrase has been shown to function in several cell types and tissues, e.g. murine liver (9), murine and human lung cells (10), human myoblasts (23) and human T cell lines (13). Its functionality has been confirmed by analysis of genomic integration and by observation of long-term expression of transgenes in the investigated cell types. It has recently been shown that the integration specificity of the ϕ C31 integrase is cell line dependent (26). To investigate if the recombination efficiency of the ϕ C31 integrase is also cell type specific, we directly compared the efficacy of the ϕ C31 integrase in cells from different tissues and could demonstrate cell-dependent integrase activity.

We assessed the feasibility of the ϕ C31 integrase to mediate long-term gene expression in two human T cell lines without application of selection pressure. We could observe long-term luciferase expression above controls in Jurkat cells only when using integrase mRNA instead of pDNA. In a second T cell line, ED-7R no enhanced long-term transgene expression could be observed. To investigate, if these findings were due to a lack of integrase protein expression, western blot analysis was performed that could clearly detect the integrase protein in Jurkat cells after transfection of integrase pDNA. To further exclude insufficient sensitivity of the luciferase detection method, we tested several cell types from different tissues under identical conditions in a more sensitive episomal recombination assay. Successful episomal recombination results in active transcription of a β -galactosidase reporter plasmid, which allows assessing recombination efficiency by β -galactosidase measurement. Using this assay β -galactosidase activity could be clearly detected in A549 alveolar type II, BEAS-2B bronchial epithelial, HepG2 hepatocytes and HeLa epithelial cervix carcinoma cell lines, as well as, in primary human mesenchymal stem cells, although the β -galactosidase values varied up to 20-fold between huMSC and BEAS-2B cells. However, neither Jurkat, ED-7R, primary T lymphocytes nor primary human CD34⁺ haematopoietic progenitor cells showed any β -galactosidase activity in recombination assays. To exclude variations due to P1 promoter dependent β -galactosidase expression, we directly analysed ϕ C31-mediated episomal recombination by PCR amplification of the recombination product. In each of the transfected cells a PCR band of the expected fragment could be observed, also in Jurkat T cells.

To investigate this effect in further detail, we performed additional experiments in A549 lung and Jurkat T cells. In these experiments, in addition to pCMV-Int, we used pC31-Intopt, a highly efficient codon-optimized plasmid coding for the ϕ C31 integrase, which carries a C-terminal NLS. As observed in the previous experiments, β -galactosidase expression was not detected in Jurkat T cells for pCMV-Int, but low β -galactosidase activity was found with the highly efficient pC31-Intopt in Jurkat cells, although this was 50-fold lower than in A549 cells 24 h post-transfection. These observations demonstrate that in principal the ϕ C31 integrase is active in haematopoietic cells but suggests that high levels of integrase are necessary for successful recombination in these cells.

Unexpectedly, the amount of recombination product *plasmid B* for pCMV-Int was 1.8-fold higher than for pC31-Intopt in A549 cells 24 h after transfection. The lower amount of recombined pDNA for pC31-Intopt compared to pCMV-Int may be explained by the NLS in pC31-Intopt. The NLS apparently enhanced ϕ C31 integrase translocation into the nucleus, whereas a large fraction of the pDNA remains in the cytoplasm, because the nuclear membrane represents a limiting barrier for pDNA to enter the nucleus (27). This may cause an adverse ratio of cytoplasmic pDNA to integrase protein in case of pC31-Intopt compared to pCMV-Int, resulting in lower amounts of initial recombination products. In contrast, at the 72 h time point after transfection 7.2-fold higher levels of recombination products were found for pC31-Intopt than pCMV-Int. This observation may be explained by the prolonged integrase expression from the codon-optimized pC31-Intopt.

Furthermore, it has to be considered that the optimized episomal plasmid-based assay used in this study does not reflect influences of the chromatin structure and the reduced homology of the pseudo *attP* sites in the mammalian genome compared with wild-type *attP* site. Therefore, the assay may overestimate the true recombination efficacy in the *in vivo* situation.

To further exclude hidden effects due to low ϕ C31 integrase expression in Jurkat cells, we analysed the ϕ C31 integrase mRNA levels in both cell lines after transfection. The integrase mRNA levels were used to normalize the integrase recombination activity. For this purpose, the ratio of recombination product *plasmid B* to integrase mRNA was calculated. These calculations revealed a 16- and 17-fold higher recombination efficacy in A549 than in Jurkat cells for, both pCMV-Int and pC31-Intopt, respectively. Together these observations indicate that the ϕ C31 integrase activity is cell type specific.

It has previously been published that the cellular protein DAXX interacts with ϕ C31 integrase and inhibits its recombination efficiency (14). We could detect higher DAXX levels in Jurkat than in A549 cells by western blot analysis, which suggests stronger integrase inhibition in Jurkat cells and, therefore, a relative lower activity. To strengthen this suggestion, we further quantified DAXX on mRNA levels in both cell lines and could observe clear differences 24 and 72 h post-transfection. The significantly 3- to 5-fold higher DAXX levels in Jurkat cells than in A549 cells might cause the reduced activity due to interaction of

DAXX with the ϕ C31 integrase. However, other factors are likely to be additionally involved in the interaction with the ϕ C31 integrase because the increase in DAXX levels did not proportionally correlate with the reduction of the normalized ϕ C31 integrase efficacy. It has to be further investigated, which factors may be involved in this interaction to explain cell type specificity of the ϕ C31 integrase and how to overcome any of these interactions in the haematopoietic T cells.

However, to successfully apply the ϕ C31 integrase for site-specific recombination in haematopoietic T cells without the utilization of selection pressure, it may be suggested that expression levels have to be largely increased. It may be questioned if this can be achieved with non-viral gene delivery systems without further fundamental improvements. A conceivable future strategy could be the use of either non-integrating lenti- and retro-viral vectors, which transfect haematopoietic cells with high efficiencies, or co-delivery of the recombinant ϕ C31 integrase itself. In particular, the later may be an attractive alternative for the reason that it is independent of the intracellular transcription and translation machinery and may, therefore, allow to achieve high intranuclear ϕ C31 integrase levels. However, difficulties with successful protein transduction will remain a challenging task which has to be carefully addressed for this purpose.

FUNDING

BMBF BioFuture program (FKZ 0311898); 'Magnetoselectofection' (FP6 LSH-PL-019038). Funding for open access charge: BMBF BioFuture program (FKZ 0311898); 'Magnetoselectofection' (FP6 LSH-PL-019038).

Conflict of interest statement. None declared.

REFERENCES

- Cavazzana-Calvo, M., Hacein-Bey, S., de Saint Basile, G., Gross, F., Yvon, E., Nusbaum, P., Selz, F., Hue, C., Certain, S., Casanova, J.L. *et al.* (2000) Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science*, **288**, 669–672.
- Gaspar, H.B., Parsley, K.L., Howe, S., King, D., Gilmour, K.C., Sinclair, J., Brouns, G., Schmidt, M., Von Kalle, C., Barington, T. *et al.* (2004) Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. *Lancet*, **364**, 2181–2187.
- Cavazzana-Calvo, M. and Fischer, A. (2007) Gene therapy for severe combined immunodeficiency: are we there yet? *J. Clin. Invest.*, **117**, 1456–1465.
- Cole, A. (2008) Child in gene therapy programme develops leukaemia. *Br. Med. J.*, **336**, 13.
- Hacein-Bey-Abina, S., von Kalle, C., Schmidt, M., Le Deist, F., Wulffraat, N., McIntyre, E., Radford, I., Villeval, J.L., Fraser, C.C., Cavazzana-Calvo, M. *et al.* (2003) A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N. Engl. J. Med.*, **348**, 255–256.
- Hacein-Bey-Abina, S., Von Kalle, C., Schmidt, M., McCormack, M.P., Wulffraat, N., Leboulch, P., Lim, A., Osborne, C.S., Pawliuk, R., Morillon, E. *et al.* (2003) LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science*, **302**, 415–419.
- 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.
- Thyagarajan, B., Olivares, E.C., Hollis, R.P., Ginsburg, D.S. and Calos, M.P. (2001) Site-specific genomic integration in mammalian cells mediated by phage ϕ C31 integrase. *Mol. Cell Biol.*, **21**, 3926–3934.
- Olivares, E.C., Hollis, R.P., Chalberg, T.W., Meuse, L., Kay, M.A. and Calos, M.P. (2002) Site-specific genomic integration produces therapeutic Factor IX levels in mice. *Nat. Biotechnol.*, **20**, 1124–1128.
- Aneja, M.K., Imker, R. and Rudolph, C. (2007) Phage ϕ C31 integrase-mediated genomic integration and long-term gene expression in the lung after nonviral gene delivery. *J. Gene Med.*, **9**, 967–975.
- Chalberg, T.W., Genise, H.L., Vollrath, D. and Calos, M.P. (2005) ϕ C31 integrase confers genomic integration and long-term transgene expression in rat retina. *Invest. Ophthalmol. Vis. Sci.*, **46**, 2140–2146.
- Ortiz-Urda, S., Thyagarajan, B., Keene, D.R., Lin, Q., Fang, M., Calos, M.P. and Khavari, P.A. (2002) Stable nonviral genetic correction of inherited human skin disease. *Nat. Med.*, **8**, 1166–1170.
- Ishikawa, Y., Tanaka, N., Murakami, K., Uchiyama, T., Kumaki, S., Tsuchiya, S., Kugoh, H., Oshimura, M., Calos, M.P. and Sugamura, K. (2006) Phage ϕ C31 integrase-mediated genomic integration of the common cytokine receptor gamma chain in human T-cell lines. *J. Gene Med.*, **8**, 646–653.
- Chen, J.Z., Ji, C.N., Xu, G.L., Pang, R.Y., Yao, J.H., Zhu, H.Z., Xue, J.L. and Jia, W. (2006) DAXX interacts with phage ϕ C31 integrase and inhibits recombination. *Nucleic Acids Res.*, **34**, 6298–6304.
- Kumaki, S., Ishii, N., Minegishi, M., Tsuchiya, S., Cosman, D., Sugamura, K. and Konno, T. (1999) Functional role of interleukin-4 (IL-4) and IL-7 in the development of X-linked severe combined immunodeficiency. *Blood*, **93**, 607–612.
- Harris, D.T., Schumacher, M.J., Rychlik, S., Booth, A., Acevedo, A., Rubinstein, P., Bard, J. and Boyse, E.A. (1994) Collection, separation and cryopreservation of umbilical cord blood for use in transplantation. *Bone Marrow Transplant.*, **13**, 135–143.
- Landi, A., Babiuk, L.A. and van Drunen Littel-van den Hurk, S. (2007) High transfection efficiency, gene expression, and viability of monocyte-derived human dendritic cells after nonviral gene transfer. *J. Leukoc. Biol.*, **82**, 849–860.
- Schakowski, F., Buttgerit, P., Mazur, M., Marten, A., Schottker, B., Gorschluter, M. and Schmidt-Wolf, I.G. (2004) Novel non-viral method for transfection of primary leukemia cells and cell lines. *Genet. Vaccines Ther.*, **2**, 1.
- von Levetzow, G., Spanholtz, J., Beckmann, J., Fischer, J., Kogler, G., Wernet, P., Punzel, M. and Giebel, B. (2006) Nucleofection, an efficient nonviral method to transfer genes into human hematopoietic stem and progenitor cells. *Stem Cells Dev.*, **15**, 278–285.
- Martinet, W., Schrijvers, D.M. and Kockx, M.M. (2003) Nucleofection as an efficient nonviral transfection method for human monocytic cells. *Biotechnol. Lett.*, **25**, 1025–1029.
- Raymond, C.S. and Soriano, P. (2007) High-efficiency FLP and ϕ C31 site-specific recombination in mammalian cells. *PLoS ONE*, **2**, e162.
- Chalberg, T.W., Portlock, J.L., Olivares, E.C., Thyagarajan, B., Kirby, P.J., Hillman, R.T., Hoelters, J. and Calos, M.P. (2006) Integration specificity of phage ϕ C31 integrase in the human genome. *J. Mol. Biol.*, **357**, 28–48.
- Quenneville, S.P., Chapdelaine, P., Rousseau, J., Beaulieu, J., Caron, N.J., Skuk, D., Mills, P., Olivares, E.C., Calos, M.P. and Tremblay, J.P. (2004) Nucleofection of muscle-derived stem cells and myoblasts with ϕ C31 integrase: stable expression of a full-length dystrophin fusion gene by human myoblasts. *Mol. Ther.*, **10**, 679–687.
- Sauer, B. (1994) Site-specific recombination: developments and applications. *Curr. Opin. Biotechnol.*, **5**, 521–527.
- Groth, A.C., Olivares, E.C., Thyagarajan, B. and Calos, M.P. (2000) A phage integrase directs efficient site-specific integration in human cells. *Proc. Natl Acad. Sci. USA*, **97**, 5995–6000.
- Ehrhardt, A., Engler, J.A., Xu, H., Cherry, A.M. and Kay, M.A. (2006) Molecular analysis of chromosomal rearrangements in mammalian cells after ϕ C31-mediated integration. *Hum. Gene Ther.*, **17**, 1077–1094.
- Rolland, A. (2006) Nuclear gene delivery: the Trojan horse approach. *Expert Opin. Drug Deliv.*, **3**, 1–10.