The Cre/loxP recombination system for production of infectious mouse polyomavirus

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Murine polyomavirus mutants are frequently produced for experimental as well as therapy purposes. Commonly used methods for preparation of mutant viral genomes from recombinant vectors are laborious and give variable yields and quality. We describe an efficient and reproducible Cre/loxP-mediated recombination system that generates polyomavirus genomes from recombinant plasmid in vivo. We designed and constructed two variants of recombinant vectors containing the wild-type polyomavirus genome flanked by loxP homologous sites. The loxP sites were introduced either into the intronic region of early genes or between the two poly(A) signal sites of convergent transcriptional units. After cotransfection of the recombinant plasmids with the Cre-expressing vector into mouse 3T6 cells, we obtained infectious virus from the genome variant containing loxP site in the intronic region, but we failed to isolate any infectious virus from the viral genome containing loxP site between poly(A) signals. We show that the Cre/loxP-based method of polyomavirus production is simple, expedient, and reproducible and works with satisfactory efficiency.

1. Introduction

Murine polyomavirus (MPyV) is a small, non-enveloped DNA virus belonging to the family Polyomaviridae and genus Orthopolyomavirus (Fields et al., 2007). Its genome is a 5.3-kbp-long circular dsDNA with complex organization and contains a replication origin (ori) surrounded by non-coding regulatory sequences comprising promoters of so-called early and late genes. Early genes encode viral regulatory oncoproteins: large T antigen (LT), middle T antigen (MT), and small T antigen (ST). These proteins are spliced from one pre-mRNA and act in the early stages of the viral life cycle. Late genes encode the major protein VP1 and two minor proteins (VP2 and VP3), which comprise the viral capsid.

MPyV has served for years as a molecular biology tool uncovering critical cellular processes and has been studied as a potential gene therapy vector (Forstova et al., 1995; Krauzewicz and Griffin, 2000). Detailed studies based on the construction and characterization of MPyV mutants have been conducted to reveal the function of viral early (Benjamin, 1982; Nilsson and Magnusson, 1984) and late genes (Krauzewicz et al., 1990; Mannova et al., 2002; Meluccivigo et al., 1994; Sahli et al., 1993). Moreover, an array of groundwork experiments elucidating the importance of interactions between viral and cellular (onco)proteins has been performed with MPyV mutants (Dilworth, 2002; Freund et al., 1992; Yi et al., 1997). Preparation and selection of cell-specific virus mutants becomes the keystone of the successful use of viruses, including MPyV, in nanomedicine and gene therapy.

All of these applications, however, rely on an effective and reproducible system for viral production and manipulation, which is not always available. MPyV mutants are usually produced in recombinant plasmids, and for characterization of mutant phenotypes, full length viral genomic DNA must be prepared by excision from a plasmid and reconstructed by ligation. Usually, after single restriction enzyme digestion, linear fragments are diluted to a concentration of 3–4 μg/ml or less to increase the probability of intra-molecular ligation (Katinka and Yaniv, 1983). Concentrated conditions favor unwanted inter-molecular ligation events that could lead to head-to-head concatemerization and transcription of cytotoxic double-stranded RNAs causing undesired effects in subsequent assays. This procedure, although widely used, is laborious, gives variable yields, and the quality of viral genome preparations varies from batch-to-batch. This variability prevents the generation of reproducible results of phenotyping analysis of mutants, which are difficult to obtain by other means.

Using Cre/loxP-mediated recombination, we established a highly efficient and reproducible system that generates polyomavirus genomes from recombinant plasmid in vivo, thus eliminating all constrains of in vitro genome ligation.
2. Materials and methods

2.1. Plasmids

pBS-PyA [carrying the MPyV genome interrupted by plasmid backbone sequence between polyadenylation signal sites (PAS) and flanked by loxP sites] and pBS-PyI (carrying the MPyV genome interrupted in the intronic region of early genes and flanked by loxP sites) plasmids were constructed as follows. Similar to the approach established for HPV (Wang et al., 2009), a double-stranded DNA fragment containing two loxP sequences separated by Xhol, NotI and Sall restriction sites was prepared from two single-stranded oligonucleotides modified by 5’ phosphorylation (Integrated DNA Technologies, IA) [ATACCTGCATAGCATACATTACAGATCTCGAGCCGGCGGGTCGACATAACCTGTTACATTACATTACGAAGTTAT (loxP sites underlined)]. Specifically, 300 pmol of each oligonucleotide was dissolved in 30 μl of HD buffer (70 mM Tris–HCl, 10 mM MgCl2, 5 mM DTT, 50 mM NaCl, pH 7.6) and hybridized for 5 h at continuously decreasing temperature from 99 °C to 25 °C preceded by a 5-min incubation at 99 °C. The complete sequence of the pMJG plasmid (Krauzewicz et al., 1990) containing the MPyV genome, strain BC, (inserted into a unique EcoRI restriction site) was amplified by PCR using primers (synthesized by Integrated DNA Technologies, IA) complementary to either early gene intronic sequence (Py-I-fw: TTCTTACGGGCTCTCCCCCTTAG, Py-I-rv: CTTACTTGATGATACAGGGCTCT) or PAS sequence (Py-A-fw: GAATATACATTAATTCACGG, Py-A-iv: CAAAATCTGTATTCAGC) for pBS-PyI construction or to the PAS sequence (Py-A-fw: GAATATACATTAATTCACGG, Py-A-iv: CAAAATCTGTATTCAGC) for pBS-PyA construction. The ligation reaction was performed by blunt-end ligation. The resulting construct was separately digested by EcoRI, Xhol or EcoRI, and NotI restriction enzymes, which led to excision of two MPyV genome fragments, each flanked by a single loxP site. Both DNA fragments were simultaneously ligated into the BlueScript-SK plasmid (Agilent Technologies, CA) linearized by NotI and Xhol enzymes. This method resulted in creation of pBS-PyI or pBS-PyA, both carrying intact MPyV genome sequence flanked by loxP sites. Constructs were verified by DNA sequencing.

The Puro.Cre empty vector [Addgene plasmid 17408, (Kumar et al., 2008)] was used as a Cre recombinase expressing plasmid throughout the study.

2.2. Cells and viruses

3T6 Swiss albino mouse fibroblasts (DSMZ, Germany) were grown in a humidified incubator with 5% CO2 at 37 °C. Cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma–Aldrich, MO) supplemented with 10% fetal bovine serum and 2 mM GlutaMAX™-1 Supplement (Gibco, CA).

Wild-type viral genome used for transfection was prepared from the pMJG plasmid. pMJG was digested by EcoRI enzyme, heat inactivated, and the resultant linear fragments were diluted to a concentration of 4 μg/ml in ligation buffer (400 mM Tris–HCl, 100 mM MgCl2, 100 mM DTT, 5 mM ATP, pH 7.8). Digested DNA was ligated by 1 U/ml T4 DNA ligase (Thermo Fisher Scientific, IL) overnight at 16 °C. The ligated DNA was ethanol precipitated, and ligation mixtures were transfected into the cells. The viral genome variants containing loxP sequences (Py-I, Py-A) were prepared by the same procedure from pBS-PyI and pBS-PyA plasmids digested with Xhol and Sall restriction enzymes, creating mutually cohesives termini.

2.3. DNA transfection

Cells were seeded 24 h before transfection, and 4 × 10⁶ cells were transfected with a total amount of 6 μg DNA using the Amaxa® Cell Line Nucleofector® Kit V (Lonza, Switzerland). Transfection was performed according to the manufacturer’s instructions. To ensure expression of Cre recombinase in the cells, 3 μg of Puro.Cre empty plasmid was cotransfected with the appropriate ligation mixture or plasmid (as indicated in Table 1). Plasmid DNA for transfection experiments was prepared using an EndoFree maxi kit (Qiagen, Germany).

2.4. Virus preparation and cell infection

3T6 cells transfected with viral DNA were collected in culture medium 6 days post transfection, and virus was liberated from cell suspension by three rounds of freeze-thaw cycles followed by pelleting of cellular debris. The supernatant (cell-free extract) was used as a viral inoculum for infection. For verification of genome encapsidation, the extract was supplemented with magnesium chloride (final concentration 10 mM), treated with 0.1 mg/ml DNase I (Roche, Switzerland) for 30 min in 25 °C and used for infection. One day before infection, cells were seeded onto coverslips in 24-well dishes at a concentration of 3 × 10⁴ cells per well. Cells were washed with serum-free medium and incubated with 100 μl of viral inoculum for 1 h at 37 °C. One milliliter of complete medium was added to each well, and the cells were further incubated in CO2 at 37 °C.

2.5. Purification of virions

Cells (6 × 10⁶) were infected by DNase I treated cell-free extract from transfected cells. Infected cells were harvested 12 days post infection. Virions were isolated as described for polyomavirus (Türlinger and Beard, 1985), by pelleting through a 10% sucrose cushion (Beckman SW 28 rotor, 25,000 r.p.m., 3 h, 4 °C), resuspended in B buffer [150 mM NaCl, 10 mM Tris–HCl (pH 7.4), 0.01 mM CaCl₂] and purified by CsCl gradient ultracentrifugation (Beckman SW 55 Ti rotor, 35,000 r.p.m., 24 h, 18 °C). Gradient fractions were collected by bottom puncture and assayed for presence of VP1 protein by dot-blot analysis. The fractions containing a peak amount of VP1 (1.33–1.29 g/cm³) were dialyzed against B buffer, concentrated by pelleting through a 10% sucrose cushion (Beckman SW 55 Ti rotor, 35,000 r.p.m., 3 h, 4 °C), resuspended in 200 μl of B buffer and subjected to the electron microscopic examination.

| Table 1 | Summary of transfection experiments. |
|----------------|-----------------|-----------------|-----------------|-----------------|
| Name        | DNA type        | Amount          | Cre (3 μg)      | Outcome after transfection |
| Py-wt       | Ligation mixture| 3 μg            | +               | Wild type genome    |
| Py-I        | Ligation mixture| 3 μg            | +               | Genome with double loxP in intron |
| Py-A        | Ligation mixture| 3 μg            | +               | Genome with double loxP in PAS region |
| pBS-PyA     | Plasmid         | 3 μg            | +               | Genome with single loxP in PAS region |
| pBS-PyI     | Plasmid         | 3 μg            | +               | Original plasmid, Fig. 2a. |
|             |                 | 6 μg            | –               | Original plasmid, Fig. 2b. |

* Co-transfection with Cre-expressing vector (Puro.Cre empty vector).
2.6. Electron microscopy

Virions were visualized by negative staining. Parlodion-carbon coated grids, activated by glow discharge were floated on the top of a 10 μl drop of sample for 5 min, washed twice on the drop of filtered distilled water, incubated on a drop of 2% phosphotungstic acid (pH 7.3) for 1 min and then dried. Electron micrographs were recorded in a JEM-1011 electron microscope (JEOL) operating at 80 kV.

2.7. DNA extraction from virions and PCR

Purified virions (20 μl) were incubated in the presence of 20 mM Tris–HCl, pH 8.0, freshly prepared 20 mM DTT (Sigma–Aldrich, MO), 20 mM EDTA, 0.2% SDS, and 40 μg/ml Proteinase K (Qia-gen, USA) for 15 min at 56 °C. DNA was extracted by High Pure PCR Cleanup Micro Kit (Roche, Switzerland) and used (5 ng) as template for PCR amplification. The forward primers (synthesised by Integrated DNA Technologies, IA) Py-intron-loxP (GTAAAGAATCTTGTATGACATAC) or Py-intron-wt (TAAGTGATCAAAGGGCGGGTG) and common reverse primer 3PyV-1113-Rw (TCTGATAGAGATTCTGG) were used for verification of intronic region identity. The PCR products (382 bp) were analyzed on Gel Red (Biotium Inc., USA) stained 1% agarose gel.

2.8. Western blotting

Cells were harvested 48 h post transfection, washed with phosphate-buffered saline (PBS; Lonza, Switzerland) and resuspended in pre-chilled RIPA buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris–HCl, pH 7.4, 0.05% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with a protease inhibitor cocktail [Complete Mini EDTA free (Roche, Switzerland)]. The suspension was incubated for 20 min on ice to ensure cell lysis. Cell debris was removed by a 5-min centrifugation at 14,000 × g. Proteins in cell lysates were resolved by 10% SDS-PAGE, electrotransferred onto a nitrocellulose–NC45 membrane (Serva, Germany) and immunostained with antibodies as described in (Mannova et al., 2002). Membranes were developed with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, IL) and exposed to CP-BU NEW X-ray film (Agfa, Belgium).

2.9. Immunofluorescence

The cells grown on glass slides were fixed in 3% paraformaldehyde (Sigma–Aldrich, MO) in PBS for 30 min, permeabilized with 0.5% Triton X-100 (Serva, Germany) in PBS for 5 min, rinsed 3 times with PBS and blocked with PBS containing 0.25% bovine serum albumin (Sigma–Aldrich, MO) and 0.25% porcine skin gelatin (Sigma–Aldrich, MO) for 30 min. Immunostaining with primary and secondary antibodies was carried out for 1 h and 30 min, respectively, with extensive washing with PBS after each incubation step. Slides were mounted in 50% glycerol with DAPI and examined with an Olympus Microscope System. Micrographs were taken using an Olympus U-CMAD-2 camera and processed using Lucia G Ver. 5.10 software. As standards, Auto-Exposure and Auto-White-Balance were used (auto-exposure mode). In some cases constant exposure mode was used to compare the signal intensities in samples.

2.10. Antibodies

Primary antibodies included the following: rabbit anti-GAPDH polyclonal antibody (Abcam, UK), mouse anti-MPyV VP1 monoclonal IgG (αPyVP1-A; immunoreactive by Western blot) (Forstova et al., 1993), mouse anti-MPyV VP1 monoclonal antibody (D4; prepared in our laboratory, recognizes native epitope), and rat anti-MPyV LT monoclonal IgGs designated anti-PyLT1, anti-PyC1 and anti-PyC4 (Dilworth and Griffin, 1982). Anti–PyC1 and anti-PyC4 antibodies react with all three T-antigens (immunoreactive by Western blot), whereas anti-PyLT1 reacts only with native large T antigen.

Secondary antibodies included the following: donkey anti-mouse IgG conjugated to Alexa Flour 488 (Invitrogen, CA), goat anti-rat IgG conjugated to Alexa Flour 546 (Invitrogen, CA), goat anti-rabbit and goat anti-mouse IgGs conjugated to HR peroxidase (BioRad, CA), and goat anti-rat IgG conjugated to HR peroxidase (Sigma–Aldrich, MO).

3. Results

3.1. Design and construction of the Cre/loxP system for murine polyomavirus production

We intended to use the Cre/loxP recombination strategy for production of viral mutants in vivo, as shown in Fig. 1a. We
decided to place two co-aligned loxP sites flanking the viral genome sequence in the recombinant plasmid. After transfection into Cre-expressing permissive cells, Cre recombination would liberate the viral genome by excision and circularization of the DNA between the loxP sites. This action would lead to production of viral progeny in transfected cells. A potential limitation of this strategy is the incorporation of the 34-bp loxP site into the viral genome after recombination. We strove to use a region of the viral genome that would be least likely to alter the normal viral life cycle. We did not attempt to modify the non-coding MPyV regulatory region because, in our experience, insertions in this region have a dramatic effect on viral genome replication (Spanielova, 2002).

Working from findings in the literature, we selected two possible sites: the site right between the polyadenylation [poly (A)] signals (PAS) of early and late viral genes (Gu et al., 2009) and an intronic region common to all three early genes (Huang and Carmichael, 2009) as shown in Fig. 1c and Fig. 1b, respectively. For viral production, we prepared two variants of recombinant plasmids: pBS-PyA, carrying the MPyV genome interrupted by the plasmid backbone sequence between the PAS sites and flanked by loxP sites; and pBS-PyL, carrying the MPyV genome interrupted in the intronic region of early genes and flanked by loxP sites (Fig. 2a and b). Both plasmids mentioned together are designated as pBS-PyL/A.

3.2. Gene expression from viral genomes containing the loxP site

Cre recombination of the pBS-Py/A plasmids leads to generation of an intact polyomaviral genome containing one loxP sequence in the appropriate location. To ascertain whether our strategy would allow the correct expression of viral genes, we cotransfected pBS-PyL or pBS-PyA with a Cre-expressing vector into permissive 3T6 cells and performed immunofluorescence staining for LT and VP1 viral proteins 24 h post transfection (Fig. 3). We also performed parallel cotransfections of in vitro religated viral genomes containing loxP sites in the intronic and PAS regions (designated Py-L and Py-A, respectively) and religated genome of wild-type virus (Py-wt) with a Cre-expressing plasmid as the control. The summary of the transfection experiments performed in this study is shown in Table 1. Whereas expression of LT antigen driven by an early polyomaviral promoter is easily achieved under various conditions, transcription of late genes driven from the natural promoter placed in a recombinant vector is usually negligible. During infection, replication of viral DNA induced by LT is a prerequisite for the generation of stable mature VP1 mRNA. High levels of VP1 protein in our experiment therefore indicate that functional LT protein is generated in the cells, serving as a marker of a fully transcriptionally active viral genome.

Analysis of immunofluorescence data (Fig. 3a) revealed that all in vitro and in vivo generated genomes produced LT and VP1 antigens, but the intensity of the signal was rather low for the Py-A sample (not apparent from Fig. 3a due to the auto-exposure mode). Similar results were observed after cotransfection of pBS-PyA with the Cre-expressing vector. When pBS-PyL and pBS-PyA were transfected into the 3T6 cells without a Cre-expressing vector in control reactions, detectable levels of LT antigen and VP1 protein were observed (Fig. 3a). This result is especially interesting for pBS-PyL because an antibody specifically reacting with the distal part of LT (encoded by the second exon) was used for immunodetection. This observation indicates that the splicing of the 2.5 kb vector backbone introduced in the early gene intron region in pBS-PyL proceeds, at least in part, correctly.

To quantify the data, we determined the number of positive cells relative to the total cell number counted (proportion) in each sample and these values were normalized to those obtained after transfection of the in vitro prepared wild-type viral genome (Py-wt) (Fig. 3b). Transfection of cells with Py-wt resulted in a similar relative proportion of VP1-positive and LT-positive cells as transfection with Py-wt. Importantly, approximately 70% of that value has been reached after cotransfection of pBS-PyL with the Cre-expressing vector. Conversely, the percentage of LT- and VP1-positive cells was markedly lower in Py-A-transfected samples, and we observed disproportionate numbers of VP1-positive and LT positive cells in this sample. Cotransfection of pBS-PyA with the Cre-expressing vector followed a similar pattern.

These results suggest that loxP insertion into the intronic region of early genes does not negatively influence production of proteins from the viral genome and that Cre recombinase is capable of generating an intact viral genome from pBS-PyL that results in restitution of viral protein production. On the contrary, loxP insertion between PAS seems to adversely affect viral gene expression.

To verify the quantitative data and to confirm that full-length viral proteins are generated from modified viral genomes, we performed Western blot analysis. Transfected cells (Table 1)

**Fig. 2.** Illustration of plasmids designed for Cre recombination-mediated viral production. Plasmids are composed of the pBluescript-SK bacterial vector carrying the MPyV genome flanked by loxP sites. SalI and XhoI restriction sites are introduced next to each loxP site (red rectangles). Origin of replication (Ori) and genes coding LT and VP1 proteins are indicated in the viral genome. (a) pBS-PyA containing viral genomic DNA interrupted between polyadenylation signals; (b) pBS-Py containing viral genomic DNA interrupted in the site of the intronic region common in all three early genes.
were harvested two days post transfection, and cell extracts were subjected to SDS-PAGE and immunoblotting with antibodies against LT (Fig. 4a) and VP1 (Fig. 4b). As expected, we observed normal production of intact LT and VP1 proteins in cells transfected with Py-1. Viral protein levels were slightly lower in cells cotransfected with pBS-Pyl and the Cre-expressing vector, where the viral genome is produced by recombination. However, the amounts of LT and VP1 were markedly lower in cells transfected with the viral genome carrying loxP insertion between PAS (Py-A), and reduced production of VP1 and LT was even more obvious when the viral genome was generated from plasmid pBS-PyA by Cre recombination (pBS-PyA + Cre). When pBS-PyA was transfected
3.3. The Cre/loxP-based system generates infectious virus

To prove that our Cre/loxP-based system is able to produce infectious virus, we again performed transfection experiments as indicated in Table 1. We collected cells with media six days post transfection, subjected them to three freeze/thaw cycles to liberate the viruses and pelleted the cellular debris. We then used the supernatant to infect 3T6 cells and detected LT and VP1 viral protein by immunofluorescence. The proportions of positive cells, which reflect the amount of infectious virus, were normalized to Py-wt sample and plotted in Fig. 5. Surprisingly, the yield of virus isolated from Py-I-transfected cells was substantially higher than that obtained from cells transfected with Py-wt (Fig. 5, Py-I). When cells were infected by virus produced from pBS-Pyl using Cre recombination, we observed approximately 60% LT/VP1-positive cells in comparison with wild-type virus (Fig. 5, pBS-Pyl + Cre). No virus was generated in the absence of Cre recombinase (Fig. 5, pBS-Pyl).

Importantly, we did not detect any infectious virus produced either from in vitro prepared genome Py-A (Fig. 5, Py-A) or from pBS-PyA in the presence of Cre recombinase (Fig. 5, pBS-PyA + Cre).

In order to verify that intact virions are generated from pBS-Pyl vector after Cre recombination and exclude the possibility that non-encapsidated DNA caused transduction of viral genes, we performed an additional experiment. We either transfected 3T6 cells with Py-wt or cotransfected the same cells with pBS-Pyl and Cre-expressing plasmid. Six days post transfection the cell-free extract was prepared and examined under the electron microscope by negative staining technique. The compact virions were observed (Fig. 6a) in both samples. Further, the same extracts were either treated or non-treated with DNase I and used for subsequent infection of 3T6 cells. Two days post infection, the cells were fixed and LT-expressing cells were detected by immunofluorescence. As shown in Fig. 6b, the DNase I treatment did not change the number of infected cells by wild type virus as well as by virus generated from viral genomes containing the loxP site in intronic region. To assure that virus arising in vivo from pBS-Pyl can sustain the infectivity in the additional rounds of infection and produce a high titer stock, we performed a medium scale infection. The 3T6 cells \((6 \times 10^6)\) were infected by cell free extract from transfected cells treated with DNase I. Cells were harvested twelve days post infection and subjected to regular virus purification procedure (Türler

Fig. 5. Efficiency of infection with virus generated by Cre/loxP system. Transfections were performed as indicated in Table 1. Virus was isolated 6 days post transfection and used for infection of 3T6 cells grown on coverslips. Cells were fixed for immunofluorescence staining 48 h post infection. Antibodies against LT (αPyLT1) and against VP1 (D4) were used for immunodetection of viral proteins. LT-expressing or VP1-expressing cells were scored as positive by immunofluorescence microscopy. The number of positive cells relative to the total cell number counted (proportion) was determined for each of 10 scored microscopic field. The relative proportion of positive cells refers to the mean proportion of positive cells determined in each sample normalized to the mean value obtained after infection with Py-wt (represented by value 1 on the y-axis in the graph). Two independent experiments are shown in the graph. Value 1 on the y-axis corresponds to an average 38% or 23% (experiment I) and 24% or 22% (experiment II) of LT-expressing or VP1-expressing cells, respectively. At least 1000 cells were counted in each sample. The standard deviation of all counted frames in each sample is represented by the error bar. NC – mock-infected cells.
and Beard, 1985). As shown in Fig. 6c the wild type virus as well as virus generated by Cre recombination was produced in ample amounts. To rule out the possibility of contamination with wild-type virus, the presence of loxP site in the genome of the virus generated by Cre recombination from pBS-Pyl vector was verified by PCR (Fig. 6c).

Altogether, these data confirm that loxP site situated in the intronic region of early genes have no negative effect on viral gene expression, and we are able to produce infectious virions from the pBS-Pyl plasmid. Conversely, integration of a loxP site between the PAS has a dramatic negative effect on viral gene expression and consequent virion production.

4. Discussion

Using Cre/loxP-mediated recombination, we established a highly efficient and reproducible system for direct generation of polyomaviral genomes from recombinant vectors in vivo. Our approach requires only a high efficiency of cotransfection of supercoiled plasmids carrying the viral genome and the Cre-recombinase gene into the permissive cell line. However, the Cre/loxP system unavoidably generates a 34-bp loxP site insertion in the viral genome, and its location must be carefully selected.

In the pBS-PyA construct, we have chosen to place the loxP site right between early and late PAS. The PAS region seemed to be
the most appropriate site for manipulation because viable mutant viruses with altered late PAS have been described (Gu et al., 2009). Specifically, the viral mutant containing a synthetic PAS instead of the normal viral late PAS preceded by a six-nucleotide-long restriction site has been shown to grow similar to the wild-type virus, with a normal early-to-late switch (Gu et al., 2006). To our surprise, the IoxP insertion between PAS exerted a dramatic effect on the expression of major early and late viral genes and we failed to isolate an infectious virus. This effect is unambiguously attributable to insertion of the IoxP sequence in this particular site because the in vitro prepared genome of Py-A displayed the same defect as the Cre-mediated viral variant. Although the expression of both LT and VP1 genes was reduced, the disproportional production of VP1 protein vs. LT antigen (Fig. 3b) suggests that regulation of late gene transcription may be primarily affected.

On the other hand, incorporation of a IoxP sequence in the early gene intron region seemed to be less promising in the beginning. The introns of the mRNAs for middle T and small T are very short (62 nt and 48 nt, respectively, Fig. 1b), and the distance between the branchpoint and the 3' splice site is one of the shortest in eukaryotic systems (Ge et al., 1990). Moreover, ST splicing requires a functional 3' splice site used for MT splicing, which lays 14 nucleotides downstream of the ST 3' splice site. This leaves only a very short space for IoxP introduction, and we strove to minimize the changes in splicing. Therefore, during the pBS-Pyl construction, the IoxP site replaced the short ST intron in such a way that all relevant nucleotides (Ge et al., 1990) appeared in the appropriate positions (Fig. 1b). This strategy proved to be successful, and our data indicate that IoxP insertion in this particular location likely did not adversely affect the function of the virus. Infectious wild-type and Py-L viruses were generated in comparable amounts after transfection of in vitro prepared genomes. The slightly higher virus yield generated from the Py-L genome compared to the wild-type genome can, in fact, be related to the different quality of the prepared ligation mixture. Importantly, Cre/IoxP recombination of pBS-Pyl in vivo led to the generation of infectious virus representing approximately 60% of yield obtained after transfection of in vitro prepared viral genome. This result is more than satisfactory when actual cotransfection and/or coexpression rates of parental and Cre-expressing vectors are taken into account. As shown in detail for human embryonic kidney cells, coexpression efficiencies among transfected cells are variable and a nonnegligible number of transfected cells express only a single target protein (Ma et al., 2007). Therefore, we believe that a currently prepared permissive cell line with stable expression of Cre recombinase can significantly improve the virus yield. Moreover, after transfection, an excision of genomes from the parental plasmids is dependent on Cre recombinase expression, and we therefore noticed that the production of viral proteins and virions is slightly delayed compared to wild-type virus; prolonged cultivation of transfected cells improved the yield (data not shown).

In summary, the strategy we describe offers opportunities to produce virus mutants that cannot be propagated in cell lines using standard methods. A similar approach was used successfully for the production of human papillomaviruses (Chow et al., 2009; Lee et al., 2004; Wang et al., 2009), which are particularly difficult to cultivate. The Cre/IoxP-based method of papillomavirus production is simple, expedient and reproducible and works with satisfactory efficiency. We are currently employing the system for papillomavirus mutants with deletions in genes for minor structural proteins, which are not viable (Mannova et al., 2002).

The complex organization of genomes of all papillomaviruses limits the genetic manipulation of viral DNA for production of fully viable virus. Our work demonstrates that the intronic region of early genes seems to be especially suitable for manipulation. It should be noted that during preparation of the Py-L genome by religation of the genome in vitro, two IoxP sites appear in the genome of the virus, and thus, the length of the intronic region increases. However, this manipulation had little effect on virus production, and we are currently determining the upper length limits for sequences that can be safely inserted in this location. Any functionally silent modification (besides IoxP insertion) in this particular site in the genome can significantly broaden the methods used for virus research and may be of value in developing therapeutic options. Furthermore, the similar organization of viral genomes in the Polyomaviridae family justifies the presumption that analogous approaches can be used for other papillomaviruses.

Disclosure statement
No competing financial interest exists.

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