Improvements in adenovirus technology : aiming at replication specificity and vector integration

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CHAPTER 5

Towards integrating vectors for gene therapy: expression of functional bacteriophage MuA and MuB proteins in mammalian cells

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Towards integrating vectors for gene therapy: expression of functional bacteriophage MuA and MuB proteins in mammalian cells

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Bacteriophage Mu has one of the best studied, most efficient and largest transposition machineries of the prokaryotic world. To harness this attractive integration machinery for use in mammalian cells, we cloned the coding sequences of the phage factors MuA and MuB in a eukaryotic expression cassette and fused them to a FLAG epitope and a SV40-derived nuclear localization signal. We demonstrate that these N-terminal extensions were sufficient to target the Mu proteins to the nucleus, while their function in *Escherichia coli* was not impeded. *In vivo* transposition in mammalian cells was analysed by co-transfection of the MuA and MuB expression vectors with a donor construct, which contained a miniMu transposon carrying a Hygromycin-resistance marker (Hyg^R). In all co-transfections, a significant but moderate (up to 2.7-fold) increase in Hyg^R colonies was obtained if compared with control experiments in which the MuA vector was omitted. To study whether the increased efficiency was the result of bona fide Mu transposition, integrated vector copies were cloned from 43 monoclonal and one polyclonal cell lines. However, in none of these clones, the junction between the vector and the chromosomal DNA was localized precisely at the border of the Att sites. From our data we conclude that expression of MuA and MuB increases the integration of miniMu vectors in mammalian cells, but that this increase is not the result of bona fide Mu-induced transposition.

For gene therapy of hereditary diseases, long-term expression of the transgene is essential. Integration of the transgene into the DNA of the target cells is required for persistence of the transgene upon cell division. Recent studies on transposable elements have revealed their feasibility as tools in mammalian genetic research and gene therapy [1-4]. Although several prokaryotic systems have been applied successfully in mammalian cells [5,6], the applicability of a prokaryotic transposase was never reported.

In the prokaryotic field, bacteriophage Mu has one of the best characterized transposition machineries, which is unequalled in its efficiency [7]. In addition, the 37-kb bacteriophage genome is one of the largest transposable elements which should provide ample space for exogenous sequences. Mu transposition requires the transposase MuA, a phage-encoded 75-kDa protein which, as a monomer, binds DNA in a sequence-specific manner [8,9]. The DNA strand cleavage and joining reactions are established by a higher-order protein–DNA complex, the so-called Mu transpososome. This complex consists of a tetramer of MuA proteins, which binds to the two ends of the Mu genome, the so-called L and R attachment sites (L att and R att, respectively). An internal activation

sequence (IAS) is involved in the initial formation of this complex as well, and enhances transposition nearly 100-fold [10]. The active transpososome performs a single-stranded cleavage of the Mu DNA just outside the L and R att sites in such way that 3′-OH groups of the transposable element are exposed. These 3′-ends are subsequently joined to the 5′-ends of the target DNA, which has been cleaved by a 5-bp staggered cut. The resulting DNA structure is a common intermediate for the two transposition products: co-integration and simple insertion [11]. In each case, the staggered cut will cause a duplication of 5 bp of the target DNA enclosing the transposed element.

For efficient transposition a second Mu-encoded protein, MuB, is required. This 35-kDa protein forms a protein–protein complex with the transposase and stimulates the assembly of the transpososome [12], the cleavage of Mu DNA and the intermolecular strand transfer into the target site [13 15]. These stimulatory features cause a 100-fold increase in transposition efficiency [16]. Besides MuA, MuB and a proper DNA template, efficient Mu transposition also depends on the host-related factor Hu and on the presence of ATP and Mg^{2+} [17]. Although Hu is absent in mammalian cells, it has been suggested that the eukaryotic proteins HMG-1 and HMG-2 can replace Hu in the assembly of the Mu transposition complex [18,19]. Moreover, recent studies on *in vitro* Mu transposition showed that integration can take place in the absence of host-encoded elements and depends solely on the MuA transposase and appropriate DNA substrates [20,21]. This reduced *trans*-requirement for transposition, together with its large transposon length and its high efficiency, makes the Mu transposition machinery an appealing system to be explored for chromosomal integration of therapeutic genes after gene transfer with non-integrating adenoviral or non-viral vectors.

In this study, we describe the adaptation of the bacteriophage Mu integration machinery from its prokaryotic host to mammalian cells, in order to evaluate its feasibility as integrating entity for gene-therapy purposes

Materials and Methods

Bacterial strains and plasmids. The bacterial strains used were Stable 2 (Life Technologies, Breda, The Netherlands), INVαF' (Invitrogen, Groningen, The Netherlands), MC1061 (Stratagene Europe, Amsterdam, The Netherlands), KMBL 1164 [Δlac-proXIII, thi209, $SupE^+$](16) and KMBL 1001 [$SupE^-$], derived from W1485 [22].

Plasmid pmMu876 was derived from pGP876 [23] by removal of the genetic code for MuA and MuB by partial EcoRI digestion and re-circularization.

The construction of plasmids pSC-NLS-MuA and pSC-NLS-MuB, which contain the transposase, MuA and transposition stimulator, MuB, respectively, was performed as described below. To isolate the Mu-A coding sequence, pGP876 was digested with the restriction enzymes EcoRV and PmeI, a fragment of 2018 bp was isolated and blunted by Klenow polymerase filling-in of the sticky-ends. Subsequently, this fragment was ligated into the eukaryotic expression vector pSuperCatch-NLS [24], a derivative of pCatch-NLS, which had been digested with BamHI and filled-in with Klenow polymerase. The construct, which contained MuA under control of the cytomegalovirus promoter (pCMV), was called pSC-NLS-MuA (Fig. 1A).

The coding sequence for MuB was obtained by digestion of pGP876 with SspI and DraIII. A fragment of 1231 bp was isolated and the DraIII-originated 3′overhang was removed by exonuclease 3′→5′. This blunt-ended fragment was cloned into the BamHI-digested and Klenow polymerase-blunted pSuperCatch-NLS. The construct pSC-NLS-MuB (Fig. 1A) contained the MuB gene driven by the CMV promoter. DNA sequence analyses confirmed the integrity of the modified MuA and MuB genes in plasmids pSC-NLS-MuA and pSC-NLS-MuB.

Three donor constructs, plasmids containing a miniMu transposon, have been used in the in vivo transposition experiments (Fig. 1B). The first construct, pmMuHyg was constructed by digestion of plasmid pCep4 (Invitrogen) with NruI and partially with SalI, isolation of the 3.2-kb fragment and cloning it into SmaI-and SalI-digested pmMu876. The second donor construct, pmMuHyg-GFP, is identical to the first one, except that a GFP-marker is added outside the miniMu transposon. First, the GFP-marker was cloned into pmMu876. To that end, plasmid phGFP-S56T (Clontech Laboratories, Palo Alto, CA) was digested with MluI and BamHI, the resulting 2.3-kb fragment, which carries the complete GFP-expression cassette, was isolated and blunted with Mung Bean nuclease. This fragment was cloned into pmMu876, which had been linearized with ClaI and

blunted with Mung Bean nuclease. The Hygromycin-resistance marker (Hyg^R) was subsequently cloned into the resulting construct in the same way as described for pmMuHyg. The last donor construct, pmMuNeo was made as follows. Plasmid pmMu876 was digested with AatII and SalI and a 5927-bp fragment was isolated. In this we cloned the 3707-bp AatII-and SalI-digested fragment from the retroviral vector pBAG [25].

Immunofluorescence. The detection of the MuA and MuB proteins, both fused to a Flag epitope, was performed with a 400-fold dilution (3% BSA in PBS) of a mouse monoclonal antibody against the Flag epitope (mαFlag M2; Kodak, New Haven, CT). Fluorescein-isothiocyanate-labeled goat anti-mouse antibody (GαMFitc; Jackson Immunoresearch Laboratories, WestGrove, PA) was used as second antibody. Nuclear DNA was stained with 1 µg/ml 2,4-diamino-2-phenylindole (DAPI), 2% 1,4 diazabicyclo[2,2,2]-octane and 0.1 M Tris–HCl pH 8.0 in glycerol.

Protein analysis by western blotting. The hybrid protein products from pSC-NLS-MuA and pSC-NLS-MuB have been produced in vitro with the TNT T7 Coupled Reticulocyte Lysate System (Promega, Leiden, The Netherlands), in accordance with the manufacturer's protocol. Stably transfected 911 cells provided the hybrid proteins after in vivo expression. Cell lysate was obtained by scraping the cells in RIPA lysis buffer (25mMTris–HCl pH7.4,50mM NaCl, 0.5% Doc, 2% NP-40, 0.2% SDS). After a 10 min incubation at room temperature, the lysates were cleared by centrifugation and the protein concentration of the supernatant was measured by the Bradford protein assay.
Cell-lysate and in-vitro transcription translation samples were fractionated on a 10% SDS–PAGE gel. Proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA) and incubated with mαFlag (Kodak) as first antibody. The second antibody was a horseradish peroxidase-labeled goat anti-mouse antibody (Brunschwig Chemie, Amsterdam, The Netherlands). The resulting protein–antibody complexes were visualized by enhanced chemiluminescence.

Complementation of MuA am and MuB am phages. Cultures of the Escherichia coli strains KMBL 1001 [Sup E^-] transformed with pSC-NLS-MuA, KMBL 1001 [Sup E^-]transformed with pSC-NLS-MuB and KMBL 1164 [Sup E^-]were diluted to an OD_{650}=0.1 (10^8 cells/ml). At this concentration, 1 ml culture samples were infected by wild-type bacteriophage Mu, MuA am or MuB am at multiplicities of infection (m.o.i.) of 2–3. After phage addition, the samples were incubated at 37°C for 20 min, without perturbation. Then, the samples were diluted 1000-fold (5 µl:5 ml) and grown at 37°C. After 1 h incubation, 50 µl chloroform was added, the samples were centrifuged for 10 min at 800 g and the phage-containing supernatant was collected.
The phage titre of the supernatant was determined on

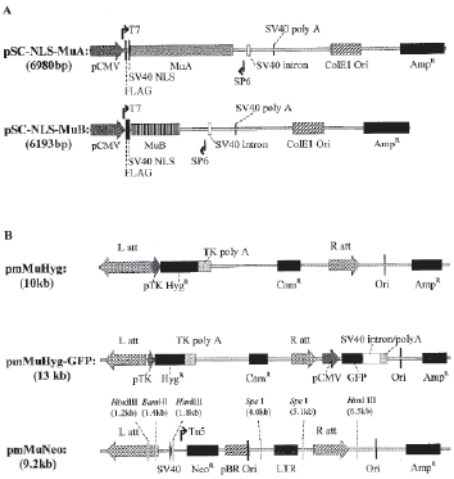

Figure 1. *Constructs used in the analysis of Mu transposition in mammalian cells.* **(A)** *Plasmid pSC-NLS-MuA contains the eukaryotic expression cassette encoding the Mu transposase, MuA, fused to a Flag epitope and SV40 NLS. The T7 RNA-polymerase transcription initiation site enables prokaryotic expression of this fusion protein. Plasmid pSC-NLS-MuB contains the eukaryotic expression cassette encoding the transposition stimulator, MuB, similar to the MuA construct.* **(B)** *The donor plasmids are characterized by the presence of a miniMu transposon, delimited by the L and R attachment domains (L att and R att). These att domains are depicted as arrows pointing to the location of the MuA-induced nick sites. The L att domain also includes the LAS region. The miniMu transposon of pmMuHyg contains a Hyg^R marker driven by a TK promoter. Donor construct pmMuHyg-GFP contains the previously mentioned miniMu transposon with Hyg^R marker and a CMV-driven GFP marker, which is located just outside this transposon. The miniMu transposon from pmMuNeo contains a Neo^R marker driven by the SV40 promoter for eukaryotic expression and by the Tn5 promoter for prokaryotic expression. This element also includes a pBR322-derived origin of replication (pBR Ori). pCMV, immediate early promoter from cytomegalovirus; pSV40, promoter from Simian Virus 40; PolyA, polyadenylation site; ColE1, origin of replication; NLS, nuclear-localization signal; Amp^R, Ampicilin-resistance marker; T7 and Sp6, RNA-polymerase transcription-initiation sites; pTK, thymidine-kinase promoter; Hyg^R, Hygromycin-resistance marker; Cam^R, Chloramphenicol-resistance marker; GFP, green fluorescence protein; LTR, long terminal repeat; Tn5, Tn5 Neo promoter. Unless indicated otherwise, the plasmids were used in supercoiled form.*

strains KMBL 1164 and KMBL 1001. The supernatant was diluted 10-or 1000-fold and 100 µl of these dilutions were added to a 1-ml culture of KMBL 1164 or 1001, respectively. The mixture was incubated for 20 min at 37°C, without perturbation. Hereafter, 3 ml top agar (1:1, agar versus LB) was added and immediately transferred to LB plates. After overnight incubation

at 37°C the plaques were counted and the titre was calculated.

Cell culture, transfection and selection. The Ad5E1-transformed human embryonic retina cell line 911 [26] and the osteosarcoma cell line U2OS were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), antibiotics and 3 g/l glucose in a 5% CO_2 atmosphere at 37°C. Transfection of 911 cells was performed by the calcium-phosphate technique [27]. U2OS cells were transfected with Fugene (Boehringer Mannheim, Almere, The Netherlands) in accordance with the manufacturer's protocol. Approximately 48 h post-transfection, selection of transfected cells was started by the addition of 150 µg/ml Hygromycin (Boehringer Mannheim) or 450 µg/ml G418 (Life Technologies) to the medium. One week post-transfection, all mock-transfected cells died and Hygromycin or G418-resistant colonies appeared. Selection pressure was then reduced to 50 µg/ml Hygromycin or 200 µg/ml G418. Solitary colonies were picked, cell lines established and chromosomal DNA isolated.

Analysis of chromosomal DNA for transposition events. Chromosomal DNA was isolated from G418-resistant colonies obtained after co-transfection of 911 or U2OS cells with the donor construct pmMuNeo, pSC-NLS-MuA and with or without pSC-NLS-MuB. To check for Mu-catalyzed integrations, the DNA flanking the att-sites was analyzed by the following procedure. For the analysis of the DNA flanking the L att site, 1 µg chromosomal DNA was digested overnight with 3 U of SpeI (Fig. 1B). After heat inactivation of the restriction enzyme, the samples were diluted to 4 ng/µl and the fragments circularised. After overnight incubation, DNA was precipitated and used for electroporation of E.coli strain Stable 2. Trans-formants containing the Neomycin-resistance marker (Neo^R) were selected by growth on Kanamycin-containing LB plates at 30°C. The resulting colonies were checked for Mu-related origin by contra-selection on Ampicilin (Amp). The Amp-sensitive colonies were further analyzed by SpeI and HindIII restriction analysis, PCR based on primers surrounding the Mu-induced nick site and, finally sequence analysis.
The same analyses were performed to study the DNA flanking the R att site, but in this case BamHI was used to digest the chromosomal DNA.

RESULTS

Evaluation of the constructs pGP876, pSC-NLS-MuA and pSC-NLS-MuB

The construct pGP876 provides all the vital elements for Mu transposition, since it contains the MuA and MuB genes and a miniMu transposon, consisting of a Chloramphenicol-resistance marker (Cam^R) delimited by the L and R attachment sites. The functionality of these elements was confirmed by Mu transposition in *E.coli* (data not shown), in a conjugation experiment [16,28].

For the expression of the MuA and MuB proteins in mammalian cells we cloned their coding regions in the pSuper-Catch-NLS vector (Fig. 1A). *In vitro* transcription and translation of the constructs enabled by the T7 promoter demonstrated the presence of products of the expected size that were recognized by the FLAG antibody. (Fig. 2). Besides some minor products, that were also obtained from the empty vector (pSuperCatch-NLS; lane2), pSC-NLS-MuA and pSC-NLS-MuB encode proteins of ~80 (lane 3) and 40 kDa (lane 4), respectively. In lysates of 911 cells (lane 1), stably expressing the modified MuA and MuB genes, products of identical sizes can be seen. These products were absent in the cell lysate of the unmodified parental 911 cells (data not shown).
The nuclear localization of both fusion proteins, a prerequisite for transposition

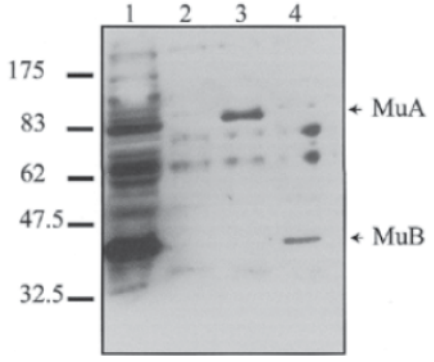

Figure 2. *Western analysis of in vitro and in vivo expression of pSC-NLS-MuA and pSC-NLS-MuB. Lane 1, total cell lysate (40 µg protein) of a 911-derived cell line, stably transfected with pSC-NLS-MuA and pSC-NLS-MuB; lanes 2, 3 and 4, the in vitro transcription translation products of pSuperCatch-NLS, pSC-NLS-MuA and pSC-NLS-MuB, respectively. Arrows, location of the fusion proteins, MuA and MuB.*

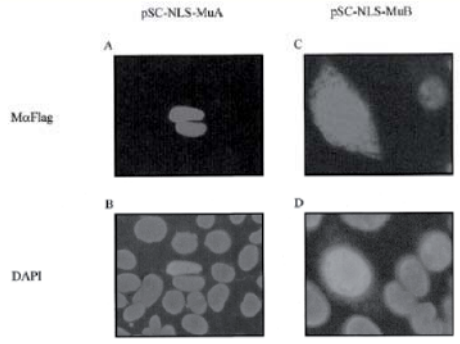

Figure 3. *Immunofluorescence of 911 cells transiently transfected with pSCNLS-MuA or pSC-NLS-MuB. (***A** *and* **C***) Visualize the presence of the MuA- and MuB-fusions, respectively, after staining with MαFlag and GαMFitc antibodies.(***B** *and* **D***) Nuclei of the same cells by DAPI staining.*

in mammalian cells, was evaluated by immunofluorescence. After a transient transfection of 911 cells with either pSC-NLS-MuA or pSC-NLS-MuB, it was clearly shown that both fusion proteins located predominantly in the nucleus (Fig. 3).

Functional analysis of the modified Mu proteins in *E.coli*

To establish the functionality of the MuA and MuB proteins fused to the NLS and the FLAG epitope, they were analyzed in a complementation assay in *E.coli*. In this assay pSC-NLS-MuA and pSC-NLS-MuB were used to complement the deficiency in Mu phages carrying MuA amber and MuB amber mutations, respectively. The complementation was determined by phage replication on KMBL 1001, transformed or not transformed with pSC-NLS-MuA or pSC-NLS-MuB.

The expression of MuA and MuB in KMBL 1001 transformants was confirmed by western analysis (data not shown). Furthermore, the replication of the MuA or MuB amber phages on KMBL 1164, containing the amber suppressor Sup E was evaluated (Fig. 4). As expected, the MuA amber and MuB amber phages replicated normally in KMBL 1164. The titre of the harvests, as determined on KMBL1164 was 2.1×10^6 and 3.7×10^7 p.f.u./ml, respectively. As a control, the same harvests were also titrated on KMBL 1001. Due to the lack of Sup E, no plaques were formed ($<10^2$ p.f.u./ml), confirming the presence of the amber mutations. If, however the MuA *am* and MuB *am* phages were grown on KMBL 1001, which was transformed with pSC-NLS-MuA and pSC-NLS-MuB, respectively, efficient propagation of the phages was observed, as determined by titration of each harvest on KMBL 1164. (Fig. 4). When these phage

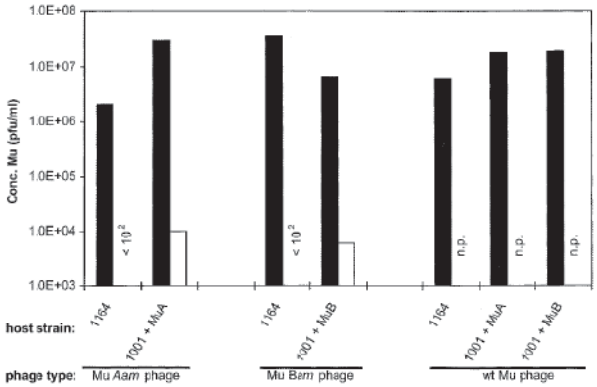

Figure 4. *Complementation of MuA amber and MuB amber phages by pSC-NLS-MuA and pSC-NLS-MuB, respectively. Indicated bacteriophage types were replicated on KMBL 1164 (1164), KMBL 1001 + pSC-NLS-MuA (1001 + MuA) or KMBL 1001 + pSC-NLS-MuB (1001 + MuB). The resulting harvests were titrated on KMBL 1164 (black bars) or on KMBL 1001 (white bars). n.p., not performed; KMBL 1164, [SupE$^+$], E.coli strain with suppressor of amber mutation; KMBL 1001, [SupE$^-$], E.coli without suppressor of amber mutation.*

titres were assayed on KMBL 1001, a severe reduction in titre was seen again. However, the titres obtained (1×10^4 p.f.u./ml for MuA *am* phages and 6×10^3 p.f.u./ml for MuB *am*) were higher than expected. This can be explained by homologous recombination between the wild-type MuA and MuB genes on the plasmids, and the mutated genes in the phages.

The obtained titres, as determined on KMBL 1164, were in accordance with those from wild-type bacteriophage-Mu harvests. Replication of wild-type Mu on KMBL 1164, KMBL 1001 transformed with pSC-NLS-MuA or KMBL 1001 transformed with pSC-NLS-MuB resulted in titres of 5.9×10^6, 1.8×10^7 and 1.9×10^7 p.f.u./ml, respectively. From these data we conclude that the N-terminal extensions of the MuA and MuB genes do not impede their functions.

Transposition in mammalian cells

To test the feasibility of the use of the Mu transposition machinery in mammalian cells, we studied the number of colonies obtained after transfer of the donor construct, pmMuHyg-GFP, with or without the expression cassettes for MuA and MuB. As can be seen in Table 1, the introduction of MuA increased the number of Hyg^R colonies with not more than 17%. The combined introduction of MuA and MuB, however, gave rise to an 81% increase in colony number. The positive effect of MuA on the number of stable transfectants was also obtained in transfections with other donor constructs (data not shown). Since the GFP marker of pmMuHyg-GFP was located outside the miniMu transposon, the absence of GFP-expression in the Hyg^R colonies could be an indication for bona fide Mu transposition via simple insertion. Although co-transfection of pmMuHyg-GFP and pSC-NLS-MuA in the absence or the presence of pSC-NLS-MuB resulted in 2 and 11% GFP-negative colonies, respectively, it only partially explained the raise in Hyg^R colonies.

To establish the contribution of co-integration to the observed raise in Hyg^R colonies, we linearized the donor construct in the subsequent co-transfection series. In this way, co-integration will lead to chromosomal breakage, leaving simple insertion as the only option for the production of a viable Mu-induced stable transfectant. The effect of the linearized donor template in a series of 1–0.1 µg on the number of Hyg^R colonies is shown in Table 2. In all cases the combined introduction of pSC-NLS-MuA and pSC-NLS-MuB resulted in more Hyg^R colonies than the control in which pSC-NLS-MuA was omitted. This effect was not seen when the donor construct was replaced by pCMVNeo. Furthermore, we found a pronounced effect of the linearization of the donor construct. The elimination of co-integration reduced the formation of Hyg^R colonies from 181 (Table 1) to 124%, in relation to their control without MuA. This reduction could be abolished when the amount of donor template was optimized. Compared to the number of colonies obtained in co-transfections without pSC-NLS-MuA, we found an increase to 124% for 1 µg template, 208% for 0.3 µg template and 266% for 0.1 µg template in combination with pSC-NLS-MuA.

Since direct evidence for Mu-mediated transposition can only be found at the molecular level, we analyzed the chromosomal DNA of G418-resistant colonies that were obtained after co-transfection of pSC-NLS-MuB and pSC-NLS-MuA with the donor construct pmMuNeo (Fig. 1B). From an experiment in 911 cells, 20 G418-resistant monoclonal cell lines were established. In parallel, from an experiment in U2OS cells we established 23 G418-resistant monoclonal cell lines and one polyclonal. The miniMu transposon of pmMuNeo contains both a eukaryotic and a prokaryotic expression cassette for the Neo^R marker in combination with a pBR322-derived origin-of-replication. This facilitated recovery and molecular cloning of the DNA flanking the inserted

Table 1. *In vivo analysis of the Mu transposition machinery by co-transfection into 911 cells*

Co-transfection				HygR colonies			
				Total		GFP–	
pmMuHyg-GFP	pSC-NLS-MuA	pSC-NLS-MuB	ssDNA	Number	%	Number	% of total
1 µg	-	2 µg	7 µg	72	100	0	0
1 µg	2 µg	-	7 µg	84	117	2	2
1 µg	2 µg	2 µg	5 µg	130	181	14	11

The effect of the indicated co-transfections of the circular donor construct pmMuHyg-GFP with pSC-NLS-MuA and/or pSC-NLS-MuB was measured by the amount of resulting HygR colonies. The GFP status was used here as indication for possible Mu-induced integration, where the absence of GFP expression (GFP–) suggested Mu transposition via a simple insertion of the miniMu transposon.

Table 2. *Effect of linearization and quantity of the donor construct, pmMuHyg, on the formation of Hyg^R colonies by the Mu transposition machinery*

Co-transfection					HygR colonies		
pmMuHyg (lin.)	pCMVNeo (lin.)	pSC-NLS-MuA	pSC-NLS-MuB	ssDNA	Exp. 1.	Exp. 2.	Average MuA effect
1 µg	-	-	2 µg	7 µg	152	50	100%
1 µg	-	2 µg	2 µg	5 µg	174	67	124%
0.3 µg	-	-	2 µg	7.7 µg	82	17	100%
0.3 µg	-	2 µg	2 µg	5.7 µg	119	46	208%
0.1 µg	-	-	2 µg	7.9 µg	14	19	100%
0.1 µg	-	2 µg	2 µg	5.9 µg	48	36	266%
-	0.1 µg	-	2 µg	7.9 µg	98	125	100%
-	0.1 µg	2 µg	2 µg	5.9 µg	71	89	72%

Indicated amounts of linearized donor construct were co-transfected with pSC-NLS-MuA and/or pSC-NLS-MuB into 911 cells and the resulting number of Hyg^R colonies was counted. The effect of MuA on the efficiency to form stable transfectants is shown in the last column. The results obtained without pSC-NLS-MuA are regarded as 100%. All transfections were performed with 10 µg DNA. Lin, linearized.

transgenes. To this end, chromosomal DNA of the monoclonal and polyclonal cell lines was isolated, digested with *Spe*I (to clone the left-hand border) or *Bam*HI (to clone the right-hand border), re-circularized by ligation and used to transform *E.coli*. The Kan^R transformants were subsequently tested for Amp resistance, restriction analysis and PCR analysis. In total we analyzed 37 right-hand borders and 143 left-hand borders (Table 3). In none of these we found a junction at the att sites, which would have been used in case of a bona fide Mu integration. To verify the efficiency of the procedure, we sequenced some of the junctions and confirmed that the junction between donor and target DNA was not at the att sites. Thus we could not find the 5 bp duplication of host DNA, which is characteristic for a Mu-insertion event.

DISCUSSION

A precisely defined integration as performed by transposons holds great promise for gene therapy. Especially, since in mice the integration efficiency of the Tc1-like transposase, Sleeping Beauty, was shown to equal the efficiency of lentivirus and rAAV [4]. This encouraged us to evaluate the transposition mechanism of bacteriophage Mu, one of the best studied and most efficient transposition machineries, for its applicability in mammalian cells. The transfer of the Mu system from prokaryotic

cells to mammalian cells required several adaptations, such as the addition of a Flag-epitope and an NLS. These extensions did not hamper their original functionality, as was confirmed by a complementation assay in *E.coli*. The expression of MuA and MuB from their mammalian expression vectors was confirmed in *in vitro* and *in vivo* analyses, which revealed their correct size and translocation to the nucleus.

In vivo analysis of the functionality of the Mu transposition machinery was performed by transfection of 911 or U2OS cells. Although less efficiently integrated than the circular donor templates, the linear donor construct in combination with MuA and MuB increased the number of stable transfectants to maximally 266%. In contrast to the effect of MuA and MuB, the use of ClpX, an *E.coli* protein which facilitates the disassembly of the transpososome [29], was less favourable in co-transfection. Although the positive effect of MuA and MuB was still observed, presence of ClpX showed a dramatic reduction in Hyg^R colonies, which suggests a cytotoxic effect in the tested mammalian cells (data not shown). Molecular cloning of 43 monoclonal and one polyclonal integrants and subsequent characterization of the junction fragments failed to provide evidence for bona fide Mu-mediated transposition. This suggests that the transfectants originate from non-homologous recombination, a process that is apparently positively affected by the presence of the MuA and MuB proteins in combination with a donor construct. It is tempting to speculate that binding of the nucleus-targeted Mu proteins to the att sites facilitates the transport of the donor construct into the nucleus [30,31].

The Mu-defined transposition was considered to be an ordered stepwise mechanism, until recently when new transpososome intermediates were described, which suggested alternative reaction pathways [13,32]. This implies that, even without the transposase's functionality of strand cleavage and joining, the DNA-binding capacity of MuA and MuB can induce the formation of a so-called type-0 target capture (TC-0) complex. This complex consists of MuA and MuB proteins, which bring the donor and target DNA in close proximity. Since in our situation the cleavage/joining reactions are not observed, this might favor non-homologous recombination. This hypothesis is supported by the observed relative increase in Hyg^R colonies, which was dependent on which Mu proteins were provided. When the co-transfection consisted of the donor construct and the MuA and MuB expression vectors, we observed an increase to 181%. This was reduced to 117%, when MuB was omitted from the co-transfection (Table 1). Moreover, when the donor construct was omitted, the positive effect of MuA and MuB on the amount of stable transfectants was completely lost (Table 2).

Obviously, the processes which underlie the increase in stable transfectants and the absence of detected Mu-specific integrations need to be elucidated further. It is however questionable whether an active Mu transposase can be established in mammalian cells and whether this will increase the efficiency of

Table 3. *Analysis of the chromosomal DNA from stable transfectants established after co-transfection of the indicated mammalian cell line with pSC-NLS-MuA, pSC-NLS-MuB and pmMuNeo*

Transfection		Analysis L att border		Analysis R att border	
Cell line	Number of Neo^R cell lines	mc/pc	Kan^R clones	mc/pc	Kan^R clones
911	20 mc	18	61	2	20
U2OS	23 mc	23	77	6	17
U2OS	1 pc	1	5	–	–

The Kan^R clones obtained from Mu-originated transfectants, would harbor the Neo^R marker in combination with the L att or R att domain and the flanking chromosomal DNA. mc, monoclonal cell lines; pc, polyclonal cell line.

integration to such extent that it can compete with existing integration systems [4].

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