Minimal Size MIDGE Vectors Improve Transgene Expression *In Vivo*

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Abstract. *Viral and plasmid vectors may cause immunological side-effects resulting from the expression of therapeutically unwanted genes and from CpG motifs contained in their sequence. A new vector type for minimalistic, immunological-defined gene expression (MIDGE) may overcome these problems. MIDGE is a minimal size gene transfer unit consisting of the expression cassette, including promotor, gene and RNA-stabilizing sequences, flanked by two short hairpin oligonucleotide sequences. DNA not encoding the desired gene is reduced to a minimum. To compare transfection efficiencies in vivo hydrodynamics-based, systemic transfection was performed in BALB/c mice with MIDGE vectors and corresponding plasmids. The transfection efficiencies of the MIDGE vectors as measured by luciferase expression were significantly higher in liver (2.5-fold), lung (3.5-fold), kidneys (3.9-fold) and heart (17-fold) as compared to plasmids. The mean numbers of MIDGE vector molecules per cell as measured by quantitative PCR were also significantly higher. These advantages suggest the preferential use of this new vector type for clinical gene therapy studies.*

Vector systems for gene therapy should offer both, successful transfection and maximum safety for the patient (1). Most clinical gene transfer protocols use viral vectors, such as retroviruses, adenoviruses, adeno-associated viruses and others. Despite effective and stable transduction there are concerns

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about risks, such as recombination with wild-type viruses to produce a deleteriously infectious form (2) or activation of oncogenes (3). Non-viral gene transfer avoids these risks; however, transfection efficiency and duration of transgene expression is usually inferior (1). Furthermore, when either, plasmids or viruses are used as vectors, several genes encoding therapeutically unwanted eu- and prokaryotic proteins (*e.g.* antibiotic resistance genes, viral protein genes) are co-transduced. Prokaryotic promotors are not absolutely silent in eukaryotic cells and targets for the immune system may be expressed (4, 5). Moreover, there is a substantial risk of immunological side-effects including elimination of transfected cells by the hosts immune response (6). In gene therapy protocols considerable immunotoxicity has been observed which was probably at least partially caused by numerous CpG sequences included in the plasmid vectors (7-9).

The construction principle of a new vector type for minimalistic, immunological-defined gene expression (MIDGE) which may overcome these problems has been previously presented (10). MIDGE vectors (created by Wittig and colleagues) are minimal size gene transfer units consisting of the expression cassette, including promotor, gene and RNA-stabilizing sequences, flanked by two short hairpin oligonucleotide sequences (Figure 1). The latter result in a covalent closure of the molecule. It has been demonstrated that MIDGE vectors improve transgene expression *in vitro* in colon carcinoma cells and avoid transfection of undesired DNA (10). Moreover, MIDGE vectors have been used successfully in several studies of DNA vaccines applying local *in vivo* administration, such as intradermal gene gun injection of MIDGE vectors containing the gene for feline immunodeficiency virus (FIV) gp140 in cats (11), intradermal injection of MIDGE vectors containing the gene for bovine herpesvirus-1 glycoprotein D (12) and intramuscular injection or gene gun-mediated intradermal delivery of MIDGE vectors containing the gene for hepatitis B surface antigen (HBsAg)
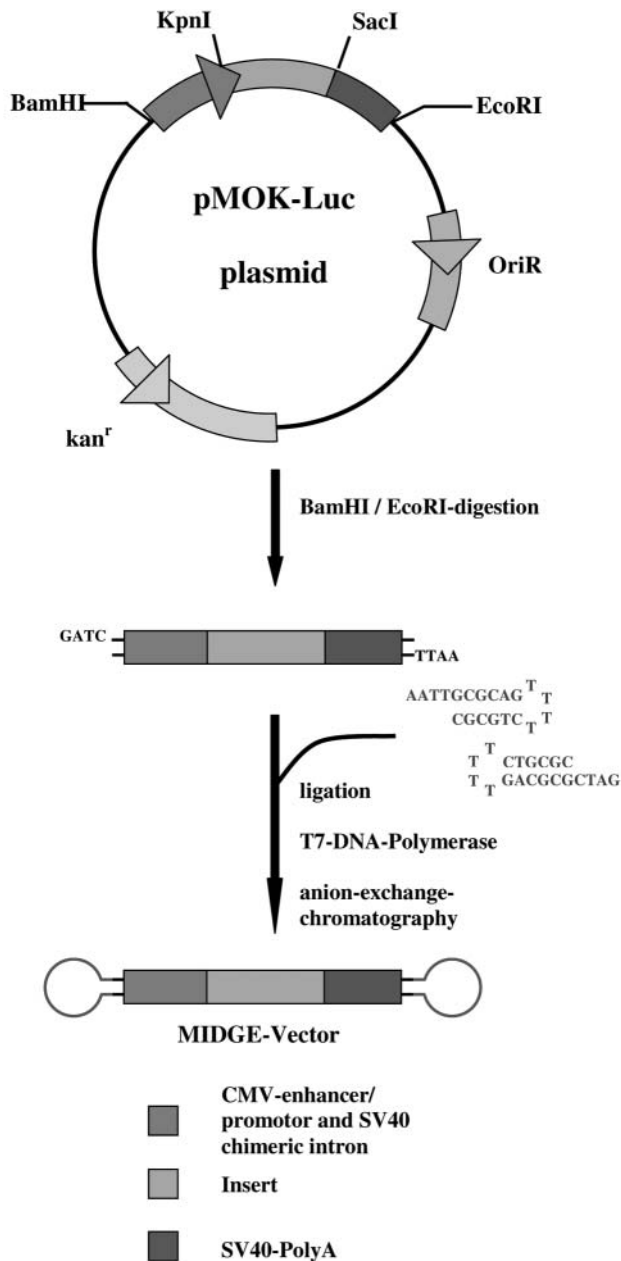
Figure 1. *Construction of the MIDGE vector. The MIDGE-Luc vector was obtained by BamHI/EcoRI digestion of plasmid DNA preparations of pMOK-Luc and ligation of the resulting fragments to 20mer hairpin oligodesoxynucleotides (ODN). Subsequently, all unligated fragments were digested by addition of T7-DNA polymerase. Finally, the MIDGE vectors were purified by anion exchange chromatography. CMV: cytomegalovirus. OriR: Origin of replication.*

in mice (13). However, to date it has been unclear whether successful systemic *in vivo* delivery of MIDGE vectors is feasible and whether these vectors remain advantageous *in vivo* as compared to plasmid vectors.

Therefore, in this study the transgene expression achieved by MIDGE vectors was compared with the corresponding plasmids in several organs in mice after tail vein injection. Chemical mediators of transfection, such as cationic lipids or polymers were not used and hydrodynamics-based transfection, which has been established as a simple, convenient and efficient method for studying transgene expression and function *in vivo* (14, 15), was applied.

Materials and Methods

Plasmid construction. The plasmid containing the gene for firefly (*Photinus pyralis*) luciferase (pMOK-Luc) was obtained from Ready Vector (Madrid, Spain). This plasmid served as source plasmid for synthesis of the MOK-Luc MIDGE vector (Ready Vector) and for comparative transfection experiments. The plasmid contained a CMV immediate early enhancer/promotor sequence, a chimeric intron (preceding the coding gene) comprised of the 5'-donor splice site from the first intron of the human β-globin gene and the branch and 3'-acceptor splice site from an intron preceding an immunoglobulin gene heavy chain variable region, a SV40 late protein poly-A site, a self-replication-origin (ori p), and a gene for kanamycin resistance (Figure 1). The plasmid size was 5506 base pairs (bp), the MIDGE vector size was 2864 bp, both including the luciferase gene consisting of 1652 bp.

MIDGE vector construction. The MOK-Luc MIDGE vector (created by Wittig and coworkers) was constructed as described previously (10). In brief, the MIDGE vector consists of the transcription unit (promotor and chimeric intron, coding gene, RNA stabilizing sequence) and two hairpin structures one on each end of the vector generating a covalently closed dumbbell shaped molecule (Figure 1). MIDGE vectors were obtained by BamHI/EcoRI digestion of the plasmid DNA preparations of pMOK-Luc and subsequent ligation (T4 ligase - MBI Fermentas, Vilnius, Lithuania; 25 U/mg) of the resulting fragments to 20mer hairpin oligodeoxynucleotides (ODN) (Sequence: 5'-AATTGCGCAGTTTTCTGCGC-3' and 5'-GATCGCGCAGTT TTCTGCGC-3') synthesized by automated chemical DNA synthesis (TIB MolBiol, Berlin) (Figure 1). Subsequently, all unligated fragments including plasmid backbones were digested by addition of T7-DNA polymerase (150 U/mg) with exonuclease activity. The success of the digestion was checked by agarose gel electrophoresis. MIDGE vectors were further purified by anionic exchange chromatography on an EMD-DMAE resin (Merck KG, Darmstadt, Germany). The MIDGE vector peak was collected and precipitated with isopropanol.

In vivo gene expression. Groups (n=4) of BALB/c mice (6-8 week, Charles River, Sulzfeld, Germany) were transfected with pMOK-Luc or MIDGE-Luc vector *via* the tail vein by hydrodynamics-based transfection. 8.25 pmol of each vector were injected in a total volume of 1.2 ml within 5 seconds. Mice injected with PBS (phosphate-buffered saline) served as controls. The transgene expression was assessed 8 hours after transfection. Liver, spleen, lung, heart, and kidneys were isolated from the sacrified animals by standard surgical procedures. The organs were homogenized for 15 sec with the tissue tearor ($\sim$20,000 rpm) in 5 ml of ice-cold lysis buffer (25 mM Tris-HCL, 2 mM dithiotreitol (DTT), 2 mM EDTA, 10% glycerol, 1% Triton X-100, pH=8.0). For liver, 10 ml lysis buffer was used. After

Table I. *Luciferase expression in various organs after the hydrodynamics-based transfection of MIDGE-vector and pMOK-Luc-plasmid. Groups (n=4) of BALB/c mice were compared.*

Organ	Luciferase expression [RLU/mg protein]		*p*
	MIDGE-vector	pMOK-Luc-plasmid	
Liver	$7.94 \pm 3.30 \times 10^7$	$3.12 \pm 0.50 \times 10^7$	0.025
Lung	$1.24 \pm 0.21 \times 10^5$	$0.36 \pm 0.30 \times 10^5$	0.002
Kidney	$1.69 \pm 0.52 \times 10^5$	$0.43 \pm 0.35 \times 10^5$	0.001
Heart	$1.03 \pm 0.14 \times 10^5$	$0.06 \pm 0.06 \times 10^5$	0.001
Spleen	$1.13 \pm 0.67 \times 10^5$	$0.90 \pm 0.46 \times 10^5$	0.460

Table II. *Determination of vector copies per 1000 cells after hydrodynamics-based transfection of BALB/c mice and following DNA-isolation of different organs.*

Organ	Vector copies per 1,000 cells		*p*
	MIDGE-vector	pMOK-Luc-plasmid	
Liver	7.3 ± 2.8	4.2 ± 0.5	0.010
Lung	9.1 ± 0.6	5.1 ± 0.2	0.001
Kidney	4.7 ± 0.3	1.8 ± 0.3	0.001
Heart	11.3 ± 1.2	1.5 ± 0.6	0.001
Spleen	6.3 ± 0.9	6.8 ± 0.7	0.450

centrifugation (15,800 xg, 10 min, 4°C) 100 μl of luciferase assay reagent (Promega, Mannheim, Germany) was added to 100 μl of supernatant and luciferase activity was measured in a luminometer (Lumat LB 9507, Berthold, Bundoora, Australia) for 10 sec. The total protein concentrations of each tissue extract were determined by a standard protein assay (Bradford reagent, BioRad, Munich, Germany). The luciferase activity was normalized to present the relative light units (RLU) per mg of extracted protein.

Isolation of complete DNA. DNA was isolated from the different organs by phenol/chloroform extraction. After homogenization of the organs an aliquot was incubated with 200 μg/ml proteinase K PCR grade (Roche, Mannheim, Germany) over night. For DNA precipitation 1/10 volume of 3 M NaAc (pH=5.6) and two volumes of 95% ethanol were added and incubated for two hours at room temperature. Subsequently, the solution was centrifuged (13,000 rpm, 10 min) and washed with 75% ethanol. The DNA was air dried and resuspended in a suitable volume of TE buffer (10 mM Tris·Cl, pH 8.0, 1 mM EDTA). The isolated DNA was quantified photometrically at 260/280 nm. The number of cells in the tissue specimens was determined by division of the complete DNA mass by 6 pg (known amount of DNA/mouse cell).

Quantitative PCR. Quantitative PCR for quantification of vectors within the complete DNA isolated from the tissues was performed using the light cycler system (Roche Diagnostics, Mannheim, Germany). A standard curve was created by amplifying 1×10^4 to 1×10^{11} copies of pMOK-Luc. Five hundred ng isolated DNA, 6 μl DNA Master SYBR green (2-{2-[(3-dimethylamino-propyl)-propylamino]-1-phenyl-1*H*-chinolin-4-ylidenmethyl}-3-methyl-benzothiazol-3-ium-Kation), 1 μl 25 mM MgCl$_2$, 1 μl primer mix (each 0.3 μM) was filled up with PCR grade water to a final PCR volume of 10 μl and transferred to light cycler capillaries. The PCR conditions for luciferase-vector determination were: initial denaturation at 95°C 1 min, then 40 cycles: 95°C 1 sec, 58°C 3 sec, 72°C 20 sec. The primer sequences for luciferase vectors were: Sense 5'-CCC CAT TGA CGT CAA TGG GAG-3', antisense 5'-CTT ACC TGC TCG AGC ACT GAC-3'.

Statistical analysis. Paired *t*-tests were used to analyze for statistical significance. The differences were considered statistically significant when the *p*-value was less than 0.05. Data are expressed as mean±standard error of the mean. Analyses were carried out using Excel software (Microsoft Corp., Redmond, WA, USA).

Table III. *Comparison of factors of increase of transgene expression and factors of increase of vector copy numbers between MIDGE and plasmid vector .*

Organ	Ratios MIDGE / plasmid	
	Luciferase expression	Vector copies
Liver	2.50	1.70
Lung	3.50	1.80
Kidney	3.90	2.60
Heart	17.00	7.50
Spleen	1.23	0.93

Results

Transgene expression of MIDGE vectors and corresponding plasmids in various organs. Luciferase activity could be detected in all the organs of the transfected mice examined but varied considerably between organs in a range of four orders of magnitude. By far the highest levels of reporter transgene expression were observed in the liver (Figure 2).

Transfection efficiencies of the MIDGE vectors as measured quantitatively by luciferase protein expression were significantly higher in the liver (2.5-fold; *p*=0.025), lung (3.5-fold; *p*=0.002), kidneys (3.9-fold; *p*=0.001) and heart (17-fold; *p*=0.001) compared to the corresponding plasmid vector. In the spleen, the mean transfection efficiencies with the MIDGE vectors and the plasmid vectors were not significantly different (*p*=0.46). Details are shown in Table I and Figure 2.

Determination of mean vector copy number per cell. The mean numbers of MIDGE vector molecules per cell were significantly higher in the liver (1.7-fold; *p*=0.01), lung (1.8-fold; *p*=0.001), kidney (2.6-fold; *p*=0.001) and heart (7.5-fold; *p*=0.001) compared to the mean numbers of the corresponding plasmid vector molecules. In the spleen, the mean numbers of MIDGE vectors and plasmid vectors per
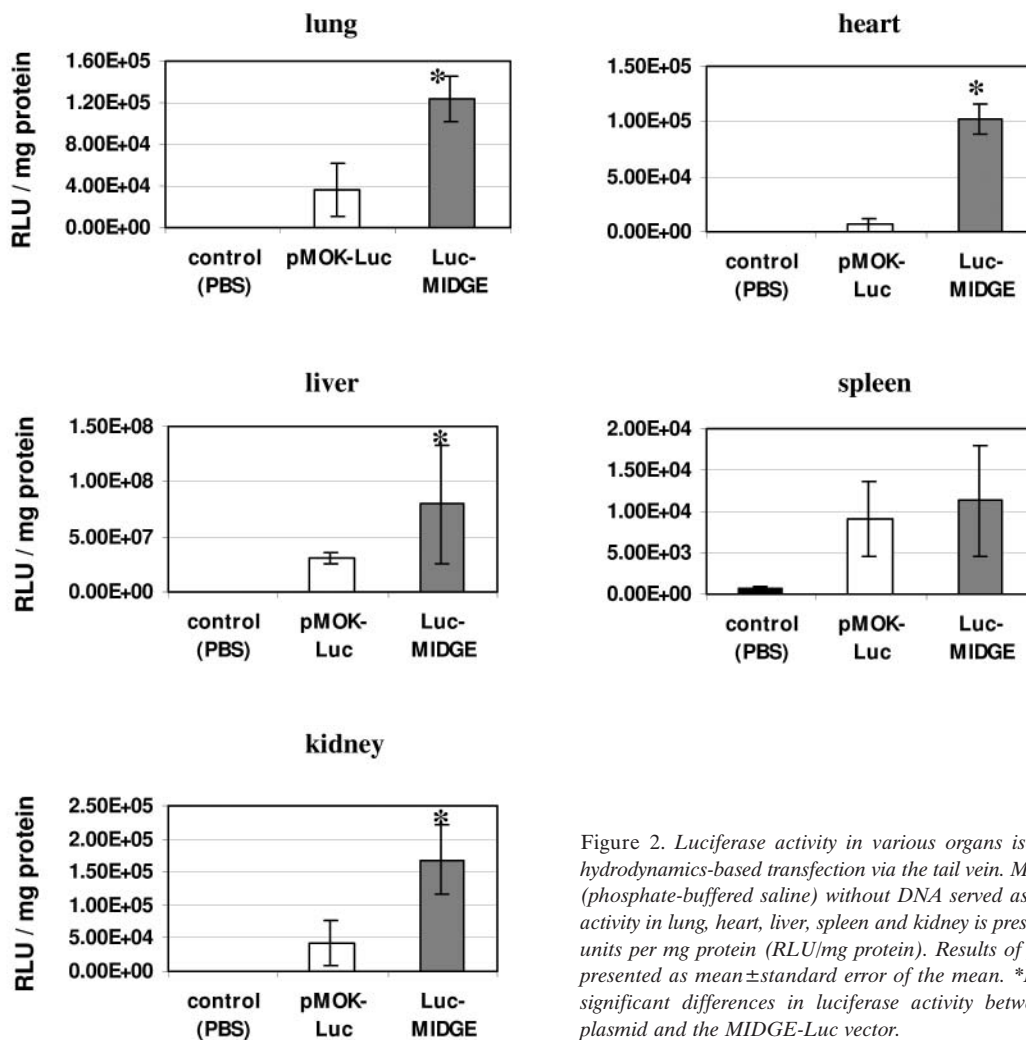
Figure 2. *Luciferase activity in various organs isolated 8 hours after hydrodynamics-based transfection via the tail vein. Mice injected with PBS (phosphate-buffered saline) without DNA served as controls. Luciferase activity in lung, heart, liver, spleen and kidney is presented as relative light units per mg protein (RLU/mg protein). Results of four experiments are presented as mean±standard error of the mean. *Indicates statistically significant differences in luciferase activity between the pMOK-Luc plasmid and the MIDGE-Luc vector.*

cell were not significantly different. Details are depicted in Figure 3 and Table II. The extent of the increase in transgene expression and of vector copy numbers with the MIDGE vectors in comparison with the corresponding plasmid vectors is shown in Table III.

Discussion

The safety and toxicity of human gene therapy is justly and increasingly discussed in the scientific community and by the public. Considerable effort should be devoted to the optimization of vector safety for the patient. The new minimal-size MIDGE vectors consist solely of the transcription unit (promotor, coding gene, RNA stabilizing sequence) with hairpin structures on both ends and thus abolish the transfer of therapeutically not required or even detrimental sequences. Therefore, the MIDGE vectors offer

a maximum of biological safety for the patient. This study has revealed that the MIDGE vectors are *in vivo* several-fold more effective (or concerning the spleen at least equally effective) compared to the corresponding plasmids when hydrodynamics-based transfection *via* tail vein injection in mice is used. These results confirm and extend our previous results from *in vitro* investigations (10) which showed that in colon carcinoma cell lines the expression of MIDGE vector transfected genes was at least equivalent and in several experiments approximately two to four-fold higher compared to the respective source plasmids.

Progress in the field of non-viral gene therapy is hampered by our very limited knowledge of the steps and kinetics between administration of the transgene to the organism or cell culture and measurement of the transgene product. In particular, information about the causes of the differences in transfection efficiencies between the vector
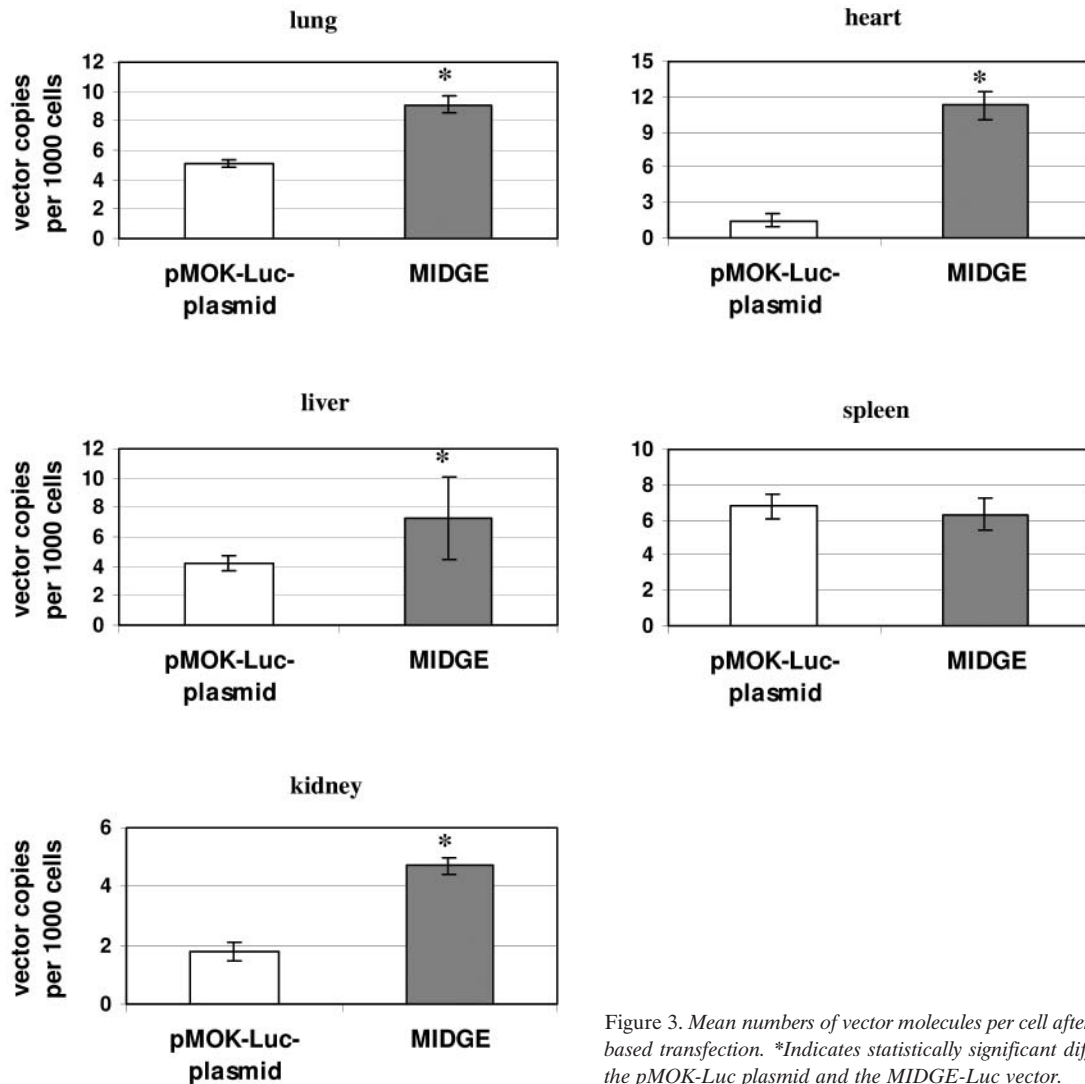
Figure 3. *Mean numbers of vector molecules per cell after hydrodynamics-based transfection. *Indicates statistically significant differences between the pMOK-Luc plasmid and the MIDGE-Luc vector.*

types is essential for achieving further improvement. However, in most studies only data on quantification of the transgene product are provided. An important but rarely investigated parameter is the number of vector copies per cell. We have determined mean numbers of vector molecules per cell by quantitative PCR. The mean numbers of MIDGE vector molecules per cell were significantly higher in the liver, lung, kidneys and heart compared to mean numbers of the corresponding plasmid vector. However, the extent of the increase of the average vector molecule numbers per cell by the use of MIDGE vectors was not as high as the extent of increase of transgene expression (Table III). From this comparison, we conclude that the majority of the increase of transgene expression mediated by the MIDGE vectors was caused by an improved delivery of vector molecules into the cell. This

may be explained by the fact that the MIDGE vectors are markedly smaller compared to the corresponding plasmids (*e.g.* an approximately two-fold size reduction for luciferase vectors) and that nuclear uptake of DNA is considered to be limited by vector size (16, 17). However, other mechanisms such as enhanced transcription of the single vector molecule effected by an increased activity of RNA polymerase may also be involved since in all organs except the spleen the factor of increase of transgene expression by the use of the MIDGE vectors was higher compared to the factor of increase of vector molecule numbers per cell.

An important advantage of the design of the MIDGE vectors is the relatively low content of unmethylated CpG sequences. The plasmid backbone (which is lacking in the MIDGE vectors) usually contains the majority of these sequences (18, 19) because in eukaryotic DNA, such as most

of the transgenes, CpG motifs are usually repressed (6). CpG motifs are considered to be responsible for at least some of the immunotoxicity observed in gene therapy protocols (7). For example, in a randomized study patients with cystic fibrosis who received aerosolized cationic lipid/pDNA complexes developed inflammatory symptoms like fever, myalgia and a reduction of pulmonary function to a more severe degree and with a higher frequency compared to the control group who received cationic liposomes alone (20). Furthermore, high level production of TNF-α, IFN-γ, IL-6 and IL-12 and other toxic effects such as elevated serum concentrations of transaminases have been shown to be triggered by intravenous administration of plasmid DNA complexed with cationic lipid vectors (8, 21).

Two other groups (22, 23) have reported alternative approaches to reduce plasmid-associated problems in systemic *in vivo* gene transfer. Yew *et al.* cloned out 270 out of 526 CpG dinucleotides from their reporter plasmid. This modification did not affect reporter gene expression, but their strategy requires a considerable expense (23). In contrast, construction of MIDGE-vectors provides a far more simple way of eliminating CpG motifs and offers several additional advantages such as improved transfection efficiency and further reduction of therapeutically unnecessary DNA. Hofman *et al.* amplified a 3.1 kb region of the plasmid CMV-Luc that contained the CMV promotor, the luciferase gene and a polyadenylation signal by PCR (22). This "non covalently-closed" fragment also showed higher levels of luciferase activity than plasmid DNA following hydrodynamics-based transfection and similar levels of gene expression after intravenous administration of cationic lipid–protamine DNA complexes (22). The use of synthetic DNA is an interesting approach to reduce CpG-related adverse events. However, it has to be considered, that the error rates of DNA polymerases used in PCR in the range of 10^{-4} to 10^{-6} per nucleotide per generation (24, 25) are several orders of magnitude higher compared to conventional amplification of plasmids in bacteria where error rates occur in the range of 10^{-9} to 10^{-10} (26). Therefore, an increased risk of mutated transgene products and thus occurrence of unexpected side effects should be considered with the use of synthetic genes compared to the MIDGE vectors or plasmids.

Hydrodynamics-based transfection of naked DNA was selected instead of cationic lipid-mediated gene transfer for the investigation of the *in vivo* transfection properties of the MIDGE vector in this study, in order to avoid a specific methodical problem of cationic lipid-mediated transfer: When cationic complexes are used for comparative transfection of vectors with different sizes, it is impossible to maintain constant equimolar vector concentration, constant transfection reagent concentration and constant N/P ratio (number of nitrogen residues of cationic lipid complex per DNA anionic phosphate). Apart from the vector-related results our experiments have confirmed that hydrodynamics-based transfection can be used to introduce and express genes and is especially useful for the comparison of different vector types carrying the same transgene.

The expression rates of the reporter gene were two to three orders of magnitude higher in the liver compared to the other organs after transfection with both MIDGE vectors and plasmids. This is in accordance with the literature where the liver has been repeatedly observed to be the organ with the highest expression rates following hydrodynamics-based transfection (27). It has been speculated that this effect is due to a large portion of the DNA solution being forced into the liver *via* the inferior vena cava in a direction that is opposite to the regular circulation (14). Strikingly, the vector molecule numbers per cell in the liver of both the MIDGE vector and the plasmid ranged in the same order of magnitude as in the other internal organs investigated. This observation indicates not that vector uptake was more successful in the liver cells but that much more intensive CMV promotor driven expression caused the high levels of transgene product in the liver in our experiments.

In summary, our results demonstrate that systemic administration of MIDGE vectors improves transgene expression *in vivo* compared to the corresponding plasmid vectors. This advantage combined with the optimized biological safety of MIDGE vectors due to a maximum reduction of therapeutically detrimental sequences encourage further development and the preferrential use of this new vector type for clinical gene therapy studies.

Acknowledgements

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