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The persistence of recombinant adenoviral vectors

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Abbreviations

Ad5 wt	Adenovirus serotype 5 wild type
ADP	Adenovirus death protein
Ad-Pol	Adenoviral polymerase
Ads	Adenoviruses
AdV	Adenoviral vector
ALT	alanine transferase
Amp^R	Resistance against ampicillin
ApoE	Apolipoprotein E
bp	Base pairs
CAR	Coxsackie B and adenovirus receptor
cDNA	Complementary DNA
CIP	Calf intestine phosphatase
dATP	Desoxy adenosine triphosphate
DBP	DNA-binding protein
dCTP	Desoxy cytidine triphosphate
dGTP	Desoxy guanosine triphosphate
DNA	Desoxy ribonucleic acid
dNTPs	Desoxy nucleoside triphosphates (dATP, dCTP, dTTP, dGTP)
dTTP	Desoxy thymidine triphosphate
DMSO	Dimethyl sulfoxide
ds	Double-stranded
DSBR	Double-strand break repair
EDTA	Ethylene diamine tetra acetic acid
FBS	Foetal bovine serum
GD AdV	Gene-deleted adenoviral vector
hAAT	Human alpha-1-antitrypsin
HCR	Hepatic control region
hFIX	Human blood coagulation factor IX
HR	Homologous recombination
HRP	Horseradish peroxidase
ITR	Inverted terminal repeat
Kan^R	Resistance against kanamycin

kb	Kilo base pairs
LB	Luria Bertani
luc	Luciferase
MAR	Matrix attachment region
MCS	Multiple cloning site
MOI	Multiplicity of infection (vp/cell)
mRNA	Messenger RNA
NF	Nuclear factor
NHEJ	Non-homologous end joining
OD_{260}	Optical density at 260 nm
ORF	Open reading frame
ori	Origin of replication
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
p.i.	post infection
pRb	Retinoblastoma tumour suppressor protein
pTP	Preterminal protein
rAdV	Recombinant adenoviral vectors
RNA	Ribonucleic acid
RLU	Relative light units
SDS	Sodium dodecyl sulphate
SV40	Simian virus 40
TAE	Tris-acetate-EDTA
TBS	Tris-buffered saline
TBS-T	TBS-Tween 20
TE	Tris-EDTA
TP	Terminal protein
TRIS	Tris-(hydroxymethyl)-ammonium methane
vp	Viral particles

Summary

Recombinant adenoviral vectors (rAdV) are commonly used as gene transfer vehicles and in gene therapy. Recombinant first generation vectors lack the early genes *E1* and *E3* (*ΔE1/E3*) which makes way for insertion of up to 8.2 kb of foreign DNA. Gene-deleted adenoviral vectors (GD AdV) lacking all viral coding sequences contain only the inverted terminal repeats (ITR) and a packaging signal at its genomic termini. Hence, there is space for the insertion of coding sequences of up to 36 kb of therapeutic transgenes and additional sequences that could stabilise the vector DNA in mitotic cells. Multiple studies for liver-based gene transfer demonstrated that non-integrative rAdV result in long-term phenotopic correction and are maintained life-long in mice and for up to 2 years in rats, dogs and non-human primates. In concordance with these reports, transgene expression in mice was also seen for several months in the present study. Therefore, it is likely that rAdV provide mechanism(s) leading to persistence in mitotic host cells.
However, the mechanisms responsible for vector genome maintenance are unknown. Thus, some of the mechanisms that might mediate the persistence of rAdV genomes *in vitro* and *in vivo* were analysed.

In the present study, episomal replication of GD AdV genomes was tested by a methylase/restriction endonuclease-based system. High-titre preparations of methylated GD AdV were produced and tropism and persistence of transgene expression in murine liver were analysed. It was found that the originally transduced GD AdV genomes are the persistent DNA molecules *in vitro* and in murine liver. Therefore, replication does not influence the persistence of GD AdV genomes.
Furthermore, concatemer as well as circle formation of *ΔE1/E3* and GD AdV genomes were analysed in cell culture experiments and in the liver of mice. For these investigations, pulsed field gel electrophoresis and polymerase chain reaction assays were employed. These experiments showed that - unlike *ΔE4* mutant adenovirus that is characterised by concatemerisation - rAdV genomes are exclusively present as linear monomers. Hence, the persistence of rAdV genomes is independent of concatemeric and circular conformations.
Notably, the molecular analysis of *ΔE4* mutant concatemers showed the diversity of concatemeric junctions. All possibilities of end-junctions were found having various deletions at the connected genomic termini of *ΔE4* virus.

Taken together, the present study provided evidence that GD AdV genomes persist as replication-inactive linear monomers.

Zusammenfassung

Rekombinante adenovirale Vektoren (rAdv) sind verbreitete Gentransfervehikel für die experimentelle Gentherapie. Rekombinanten Vektoren der ersten Generation fehlen die frühen Gene *E1* und *E3* (*ΔE1/E3*), was die Einfügung fremder DNS bis zu einer Größe von 8,2 kb ermöglicht. Gendeletierte adenovirale Vektoren (GD AdV) besitzen keinerlei virale DNS-Sequenzen außer den invertierten terminalen Wiederholungssequenzen (ITR) und ein DNS-Verpackungssignal. Deshalb können in das Genom von GD AdV therapeutische Transgene und zusätzliche Genom-stabilisierende DNS-Elemente von bis zu 36 kb eingebaut werden. In zahlreichen Studien wurde gezeigt, dass ein Transgen von solchen nicht integrierenden rAdV längere Zeit exprimiert wird und dass die DNS von rAdV in Ratten, Hunden und Affen bis zu zwei Jahre lang persistiert. In Übereinstimmung mit diesen Studien konnte auch in der vorliegenden Arbeit eine Transgenexpression von rAdV für mehrere Monate beobachtet werden. All diese Daten deuten darauf hin, dass rAdV Mechanismen aufweisen, die zur Persistenz des Vektorgenoms in sich teilenden Wirtszellen führen. Solche Mechanismen, die für die Aufrechterhaltung des Vektorgenoms verantwortlich zeichnen, sind bisher allerdings nicht beschrieben worden. Deshalb wurden einige dieser möglichen Mechanismen *in vitro* und *in vivo* untersucht.

In der vorliegenden Arbeit wurde die episomale Replikation von GD AdV mittels eines Methylase/Restriktionsendonuklease-basierten Ansatzes getestet. Hochtitrige Stammlösungen eines methylierten GD AdV wurden hergestellt und sowohl Tropismus als auch persistente Transgenexpression wurden in der Mausleber untersucht. Es konnte gezeigt werden, dass die ursprünglich transduzierten Vektorgenome ohne Replikation *in vitro* und in der Mausleber persistieren. Die Persistenz GD AdV wird deshalb nicht durch Replikation vermittelt.
Außerdem wurde die Bildung von konkatemeren und zirkulären *ΔE1/E3* und GD AdV Genomen in Zellkultur und in der Mausleber untersucht. Dafür wurden Pulsfeldgelelektrophorese (PFGE) und Polymerasekettenreaktionen (PCR) eingesetzt. Diese Experimente zeigten, dass – im Gegensatz zur *ΔE4* Virusmutante, die starke Konkatemerbildung ausweist – rAdV Genome als lineare Monomere in der Wirtszelle vorliegen. Deshalb ist die Persistenz rAdV Genome unabhängig von zirkulären und konkatemeren DNS Konformationen.
Ferner zeigte die molekulare Analyse der Konkatemere von *ΔE4* Virusmutanten, dass die Verknüpfungspunkte zwischen den Konkatemeren vielfältige Deletionen aufweisen.

Zusammenfassend hat die vorliegende Studie gezeigt, dass rAdV Genome als nicht replizierende, lineare Monomere persistieren.

A Introduction

1.1 Properties of human adenoviruses and adenoviral infections

Adenovirus was first discovered in adenoids of a patient with persistent infection in 1953 (Rowe *et al.* 1953). Adenoviruses (Ads) are non-enveloped, icosahedral viruses with a capsid of 70 to 90 nm in diameter. The viral capsid consists of three major proteins: hexons, pentons and knobbed fibers [**figure 1**]. The linear double-stranded DNA (dsDNA) of Ad varies between 25 and 40 kb (Stewart *et al.* 1993, Russell 2000). Covalently bound to both 5' ends is a terminal protein (TP) that plays a unique role in viral DNA replication (Rekosh *et al.* 1977). Besides Ads that are pathogenic for humans, Ads can also infect animal hosts like mammals and birds. Currently, 51 human Ad serotypes are identified by immunological means and classified into 6 subgroups (A – F) (De Jong *et al.* 1999). Subgroup C adenoviruses include Ad serotype 5 (Ad5) that is the most extensively characterised serotype.

Ad5-derived vectors are widely used as gene therapeutic vehicles in gene therapy. Due to their tropism Ads predominantly infect epithelial cells of the respiratory and gastrointestinal tracts and the conjunctiva. In natural infections subgroup C Ads (e.g. Ad5) enter their host cells by receptor mediated endocytosis. The fiber proteins of the capsid bind to coxsackie B and adenovirus receptors (CAR) on the cell surface and bring the penton proteins close to integrins in the plasma membrane. The internalised viral particle escapes from the endosome and is subsequently transported to the nucleus via the cytoskeleton. Having reached the nucleus, the viral genome is released from the capsid and amplification of progeny virus is started. The newly generated virus is released by lysis of the host cell (Shenk 1996).

In immunocompetent patients, Ads typically cause acute, self-limiting and non-persistent diseases like rhinitis, pharyngitis, gastroenteritis, diarrhoea and conjunctivitis. In patients with compromised immune status, however, primary adenoviral infections may lead to disseminated, life-threatening complications (Horwitz 1996; Cichocki *et al.* 2008). Moreover, the high incidence of adenoviral infections in organ transplant recipients (kidney, bone marrow and liver) and AIDS patients implies that these onsets very likely represent reactivations of latent/persistent adenoviral infections. If the infection is not yet controlled by the host immune system, Adenoviruses are able to persist for years in adenoids and tonsils or are excreted via the faeces for several months (Humar *et al.* 2006; Lee *et al.* 2008; Michaels *et al.* 1992; McGrath *et al.* 1998; Hierholzer 1992).

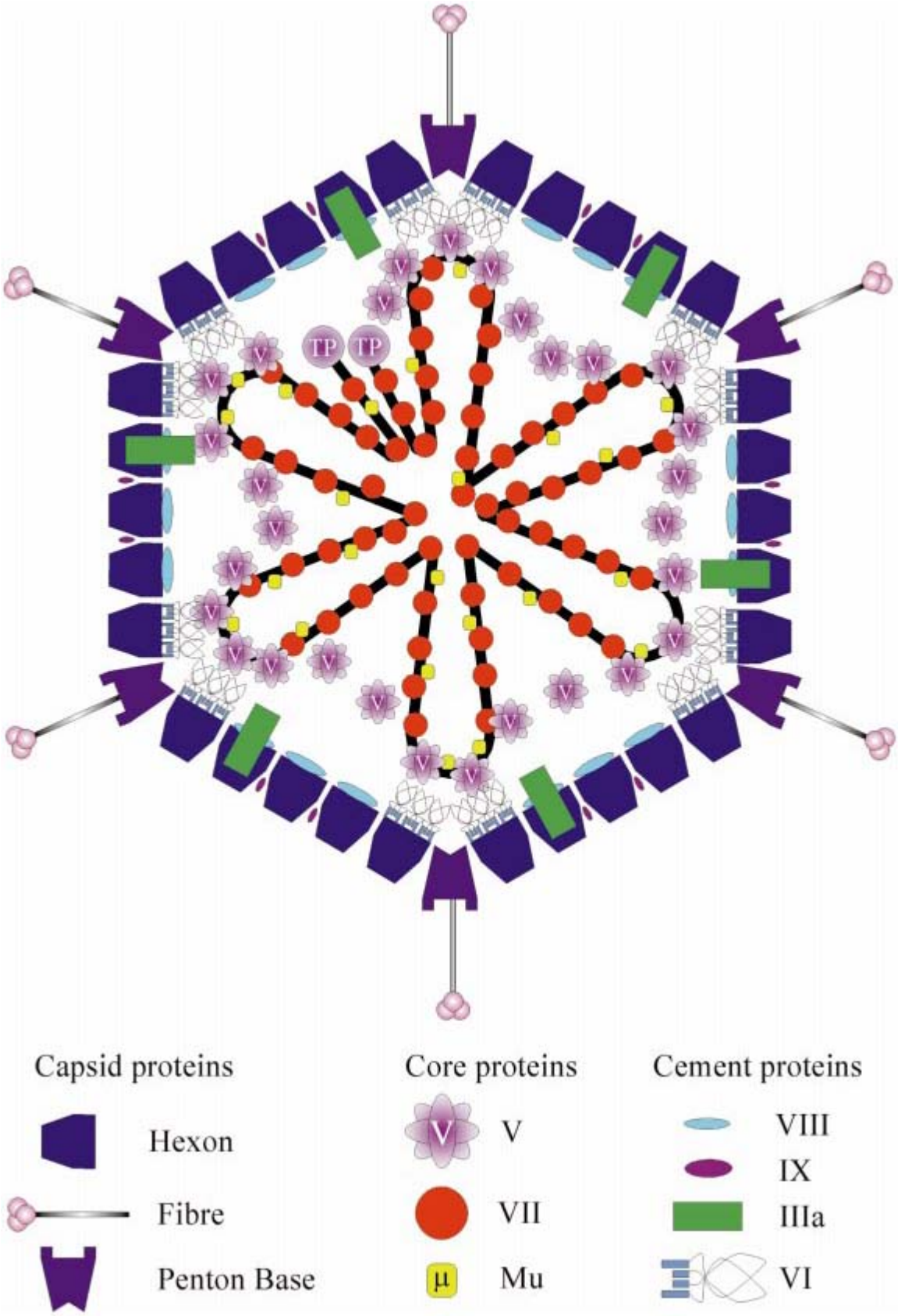


Figure 1: Adenoviral structure. The capsid proteins represent the main structural proteins; the core proteins are DNA-binding proteins stabilising and packaging the viral genome within the capsid shell. The cement proteins reinforce the virion's structure and tie the viral DNA to the capsid sheath. TP = terminal protein. See text for details. Reprinted from Russell 2000.

1.2 Adenoviral genome organisation and replication

The double-stranded linear genome of adenoviruses is an example for efficient genomic organisation within limited space. Open reading frames (ORFs) coding for proteins that play a specific role during the viral replication cycle are grouped together. The adenoviral genome can be divided into early transcription units (*E1*, *E2*, *E3* and *E4*) and late transcription units (*L1*, *L2*, *L3*, *L4* and *L5*) **[figure 2A]**. The early ORFs encode effector enzymes that promote viral replication, the late ORFs code for structural proteins that are assembled to the capsid. The proteins of early transcription units (I) establish a beneficial environment for viral replication (*E1*), (II) start and accomplish viral DNA replication (*E2*), (III) protect against host cell defence (*E3*) and (IV) shut down cellular protein expression and promote viral growth (*E4*). E1 forces the host cell from G_1 to S phase by binding to the retinoblastoma tumour suppressor (pRb) and p53. E1 is also a transactivator of other viral genes and is involved in viral mRNA transport and inhibits apoptosis (Lewin 1991; Steegenga *et al.* 1998; Chinnadurai 1998; With 1998; Perez and Withe 1998). The E2 proteins are necessary for viral genome replication: for example, the preterminal protein (pTP) acts as a primer for the adenoviral DNA polymerase (Ad-Pol)(Hay *et al.* 1995; Ramachandra and Padmanabhan 1995; Van der Vliet 1995). E3 prevents the transport of major histocompatibility complex I (MHC-I) molecules to the cell surface (Wold *et al.* 1995; Wold *et al.* 1999). E4, whose promoter activity is enhanced by E1, inhibits cellular protein expression and prevents concatemer formation by interacing with the double strand break repair system (DSBR) of the host cell (Akusjärvi 1993; Dobbelstein *et al.* 1997; Krätzer *et al.* 2000; Horridge and Leppard 1998; Gabler *et al.* 1998). At the end of the adenoviral replication cycle the genome is packaged into the capsid and the newly generated virus is released by lysis of the host cell (Tollefson *et al.* 1996).

1.3 Adenoviral vectors for gene therapy

The ability of adenoviruses to transduce a broad range of cell types, dividing and non-dividing and the episomal nature of its dsDNA genome make adenoviruses and its vectors an attractive tool for gene therapeutic applications. Moreover, high titres of adenoviral vectors can be produced. To re-engineer adenovirus to adenoviral vectors some genes of the adenoviral genome must be removed. This removal makes way for the insertion of therapeutic transgene expression cassettes. Recombinant adenoviral vectors (rAdV) have been used in gene therapeutic trials treating diseases of liver, lung, eye, muscle and brain (Alba *et al.* 2005,

Jager and Ehrhardt 2007). To date, most clinical trials for gene therapy involved adenoviral vectors (n=342) (www.wiley.co.uk /genmed/clinical).

1.3.1 First generation adenoviral vectors

The 1st generation of adenoviral vectors lack the *E1* and *E3* sequences *(ΔE1/E3*) [**figure 2B**] rendering these vectors replication deficient in non-cancer cells due to the missing *E1A* expression, and reduced in transactivation of other early gene clusters for example *E4*. The lack of the immune-modulating E3 proteins does not affect vector propagation and experiments *in vitro*. *In vivo*, however, 1st generation vectors significantly elicited the host cell immune system by *de novo* viral protein synthesis, causing innate, cell-mediated and humoral immune responses. Despite the high transduction and transgene expression rates, long-term vector genome maintenance was prevented by clearance of transduced cells. Furthermore, an efficient re-administration of the vector was difficult due to the development of memory cells (Benihoud *et al.* 1999; Zhang 1999). However, transgene expression and persistence of 1st generation AdV can be detected in certain mouse strains for several months (Ehrhardt and Kay 2002; Wadsworth *et al.* 1997).

1.3.2 Gene-deleted adenoviral vectors

The latest development in vector design was the construction of adenoviral vectors lacking all early and late viral coding sequences [**figure 2C**]. These so-called gene-deleted adenoviral vectors (GD AdV) only retain the ITRs flanking the vector genome at its termini and a packaging signal (Ψ) as the prerequisites for initiation of vector DNA amplification and encapsidation (Fisher *et al.* 1996; Kochanek *et al.* 1996; Hearing *et al.* 1987; Gräble and Hearing 1990, 1992). The production of GD AdV is realised by co-infection with a helper virus providing all wild type viral genes *in trans* excluding E1 (for more details see the chapter on GD AdV production in the section material and methods). The only viral proteins that are introduced into the host cell by GD AdV, are the capsid proteins, the TP covalently attached to the 5' termini and DNA-binding proteins pVII and μ (Steward *et al.* 1993; Russell 2000). There is no viral *de novo* gene expression.

There are some advantages of GD AdV for gene therapy of inherited deseases. Despite their improved toxicity profile regarding immune responses, GD AdVs have a large capacity of incorporating foreign DNA and transgene sequences. In contrast to 1st generation vectors with insert size limitation of 8.2 kb, therapeutic and other advantageous DNA sequences of up to

36 kb can be introduced into the GD AdV genome. For the treatment of Duchenne muscular dystrophy for example, this offers the possibility to incorporate the large transgene expression cassette of the human dystrophin gene (Matecki *et al.* 2004; Dudley *et al.* 2004). Furthermore, it is feasible to construct vectors with additional elements leading to vector genome persistence by providing DNA-maintaining functions. However, to date it is not clear which molecular mechanisms are responsible for the persistence of gene-deleted vector genomes.

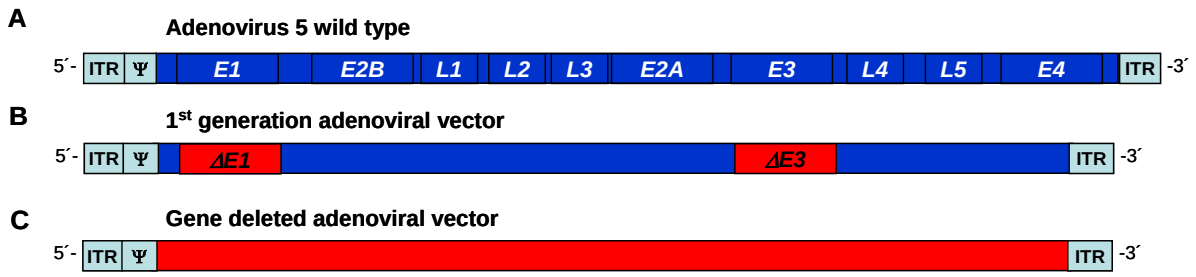


Figure 2: Schematic overview over the viral and vector genomes. A: Adenovirus wild type; the early (*E1-4*) and late (*L1-5*) genes are indicated. B: first generation vector lacking *E1* and *E3*; C: gene-deleted adenoviral vector. Elements shown in red represent deletions. The resulting space can be used for the insertion of transgenes and other DNA sequences. The single elements depicted are not drawn to scale.

1.3.2.1 Immune response against gene-deleted adenoviral vectors

The acute or chronic hepatotoxicity caused by viral protein expression that was observed in *in vivo* experiments using 1st generation vectors, was not detectable after GD AdV administration (Kim *et al.* 2001; Morral *et al.* 1998; Schiedner *et al.* 1998; Chuah *et al.* 2003). However, the incoming adenoviral capsid proteins elicit an immune response and reactivate existing anti-adenoviral capsid memory cells. This can lead to a life-threatening anaphylactic shock in a dose dependent manner. Brunetti-Pierri and colleagues showed that baboons injected with GD AdV at a dose of $5.6x10^{12}$ viral particles (vp)/kg reacted only with a moderate immune response, which subsided after 24 h. Upon injection of a higher viral dose ($1.1x10^{13}$ vp/kg), however, the animal suffered from severe toxicity (Brunetti-Pierri 2004). A clinical trial aiming at the treatment of ornithine transcarbamylase deficiency with a 2nd generation vector resulted in the death of one of the patients enrolled (Raper *et al.* 2003). This adverse event was caused by a fatal systemic inflammatory response against structural adenoviral proteins. Therefore, a successful gene replacement gene therapy for inherited diseases needs to meet the following objectives: Limited immunogenicity and insertional

mutagenesis, long-term transgene expression and vector genome persistence after one single vector administration.

1.3.2.2 Long-term persistence of recombinant adenoviral vectors

Persistence and long-term transgene expression of 1st generation AdV for up to one year was shown in rodents (Ehrhardt and Kay 2002; Barr *et al.* 1995). Also GD AdV persist long-term in small and large animal models with negligible induction of the immune system. For example, human α-1-antitrypsin was expressed longer than one year in baboons after GD AdV administration (Morral *et al.* 1999). Sustained transgene expression of α-fetoprotein was detectable for about two years in baboons and apolipoprotein E (ApoE) expression was even maintained life-long (2.5 years) in ApoE-deficient mice (Brunetti-Pierri *et al.* 2006; Kim *et al.* 2001). Moreover, transgene expression was persistent for 413 days in non-human primates after administering GD AdV via a minimally invasive liver-directed infusion method (Brunetti-Pierri *et al.* 2007). Phenotopic correction of haemophilia B was achieved in a dog model for about two years with GD AdVs (Brunetti-Pierri *et al.* 2005). Furthermore, Toietta *et al.* reported life-long correction of hyperbilirubinemia in rats with GD AdVs (Toietta *et al.* 2005). These examples show that host cells of different organs maintain the vector genome and that recombinant AdV genomes can persist long-term within a transduced cell. However, the molecular mechanisms involved in episomal persistence of recombinant AdV are unknown.

1.3.2.3 Previous work on the persistence of recombinant adenoviral vectors

The *in vivo* persistence of rAdV is influenced by the DNA sequences enclosed in the adenoviral vector genome, the transgene expression cassette and its methylation status (Cristiano *et al.* 1993; Parks *et al.* 1999; Grave *et al.* 2000; Brooks *et al.* 2004; Van Linthout 2002). Moreover, the persistence is influenced by the vector dosage, the mouse strain or experimental animal, the innate immediate immune response as well as cell-mediated and humoral immune reactions (Mills *et al.* 2000; Barr *et al.* 1995; Schowalter et al 1999; Ehrhardt and Kay 2002; Lieber *et al.* 1997a; Zhang *et al.* 2001).

However, anti-viral immune responses are not the primary reason for the progressive loss of rAdV genomes, since a similar duration of transgene expression was observed in immuno-compromised and immuno-competent mice infected with GD AdV or 1st generation AdV (Ehrhardt *et al.* 2003; Barr *et al.* 1995; Lusky *et al.* 1998). Ehrhardt *et al.* (2003) reported that

slowly declining transgene expression levels of human blood coagulation factor IX (hFIX) were detectable in C57Bl/6 and immuno-compromised C57Bl/6-SCID mice. Furthermore, no decreased transgene expression was found in mice that received anti-adenoviral cytotoxic T-cell lymphocytes (Wadsworth *et al.* 1997). All these reports suggest that the progressive loss of adenoviral vector genomes can only be in part dependent on host immune reactions.

Cell cycling of transduced host cells was proposed as another possible mechanism accounting for the continuous loss of vector genomes over time. In quiescent or slowly proliferating host cells vector genome maintenance could be prolonged (Carpenter and Stevens 1996; Wagner and Bloom 1997). In fast replicating host cells, on the other hand, loss of vector genomes might be accelerated, if there is no intrinsic feature of the vector mediating its persistence. This question was addressed by Ehrhardt and co-workers by inducing rapid liver cell proliferation in mice after transduction with a GD AdV (Ehrhardt *et al.* 2003). GD AdV genomes were maintained to a much higher degree than a non-viral plasmid-derived control vector. From these findings the authors concluded that host cell division alone is not the primary cause for declining transgene expression.

Furthermore, the possibility of rAdV genome integration into the host genome as mechanism of persistence was investigated (Harui *et al.* 1999; Stephen and Kochanek 2007). 1st generation AdV integrates at a rate of 10^{-3} to 10^{-5} *in vitro* (Harui *et al.* 1999). Stephen and Kochanek found that the *in vivo* integration frequency of the preferentially episomal GD adenoviral vectors is low in murine liver. Since the murine liver consists of approximately $1x10^{8}$ hepatocytes, it is expected that integration occurred only in 10 to 1000 hepatic cells.

Therefore, it is likely that other mechanisms mediate sustained persistence of GD AdV genomes in quiescent as well as in proliferating host cells.

1.3.2.4 Potential mechanisms of persistence and molecular conformations of recombinant adenoviral vectors

How is the adenoviral vector genome maintained in the host cell during cell cycling *in vivo*? Currently, there are several potential mechanisms and molecular conformations under discussion that also might mutually interact (Jager and Ehrhardt 2007; Ehrhardt *et al.* 2003). (I) Either the adenoviral vector genome replicates episomally or it (II) forms concatemers or (III) circularises. The vector DNA may contain areas that possess (IV) centromeric function or mediate (V) nuclear retention [**figure 3**].

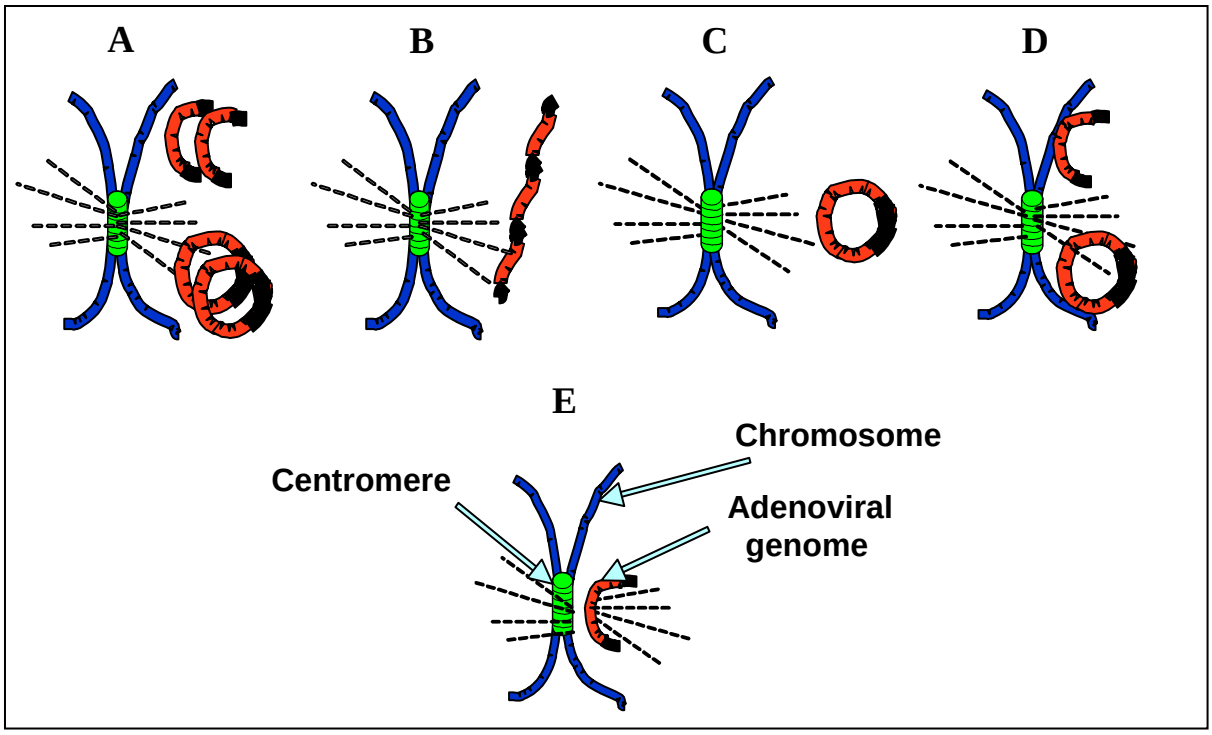


Figure 3: Potential mechanisms of persistence and molecular conformations of gene-deleted adenoviral vector genomes during the cell cycle *in vivo*. A: Episomal replication; B: Concatemer formation; C: Circularisation; D: Nuclear Retention; E: Centromeric function.

In the present study it was analysed whether GD AdV genomes replicate episomally and form concatemers or circles.

(I) Episomal replication of a circular DNA molecule is responsible for persistence of Epstein-Barr-Virus in lymphocytes using an origin of replication and Epstein-Barr nuclear antigen 1 (EBNA1) (Hammerschmidt and Sugden 2004, Ritzi *et al.* 2003; Aiyar *et al.* 1998). Furthermore, high amounts of vector DNA after a surgical two-thirds partial hepatectomy (Ehrhardt *et al.* 2003) and the prolonged persistence of GD AdV for up to 2 years in rats, dogs and non-human primates (Brunetti-Pierri *et al.* 2007; Ehrhardt and Kay 2002; McCromack *et al.* 2006), led to the hypothesis that vector DNA replication may contribute to episomal persistence. Replication of the GD AdV vector used in the present study might rely on activation of putative mammalian origins of replication located within the alphoid repeat DNA of this vector [**figure 4**]. Replication of GD AdV vector genomes would occur completely independent of viral E1 and E2 proteins. Moreover, replication of GD AdVs could be responsible for keeping up an equilibrium between decrease and increase of vector DNA during mitosis. Even a combination of several mechanisms is imaginable.

(II) Concatemer formation takes place in other gene transfer systems based on transfection of linear naked DNA (Chen *et al.* 2003) or transduction of target cells with recombinant adeno-associated viral (AAV) vectors (Miao *et al.* 1998). Weiden and Ginsberg

have previously shown that deletions in the early region 4 (E4) of the adenoviral genome produce DNA head-to-head, tail-to-tail and head-to-tail concatemers (Weiden and Ginsberg 1994). These large chains of DNA consist of multiple viral genomes connected to each other. The connection itself is carried out by double-strand break repair (DSBR) proteins of the host cell (Boyer *et al.* 1999; Stracker *et al.* 2002). We speculated that concatemeric vector DNA could be more persistent during host cell mitosis due to combination and multiplication of stabilising effects of one monomer (centromeric region, matrix attachment region, nuclear retention signals) within one large multimeric molecule. Additionally, it could be that host enzymes leading to degradation of vector DNA do not recognise multimeric vector genomes as efficiently as smaller molecules.

(III) Circular DNA molecules persist within the nucleus of the host. This was shown for numerous DNA viruses like members of the *Herpesviridae*, Hepadnavirus and non-viral vectors like pEPI (Roizman and Sears 1996; Ganem 1996; Stehle *et al.* 2003, Ehrhardt *et al.* 2008). Therefore, circularisation seems to favour genome persistence through, apart from replication of circular genomes, yet unknown mechanisms. Furthermore, circularisation of vector genomes could play an important role for episomally replicating vectors because the difficulties associated with replication of linear DNA would be circumvented by a rolling circle or theta-replication mode. A rolling circle and theta-replication was already demonstrated for Epstein-Barr-Virus and simian virus 40 (Gussander and Adams 1984; Pfüller and Hammerschmidt 1996; Li and Kelly 1984). Moreover, Kreppel and Kochanek reported circularisation of one of their GD AdVs (Kreppel and Kochanek 2004).

Aims of this work

Recombinant adenoviral vectors (rAdV) that are administered as gene therapeutic vehicles contain a double-stranded DNA genome. The vector genomes are introduced into the host cell as linear monomers and persist as episomes. First generation adenoviral vectors lack the early genes *E1* and *E3* (*ΔE1/E3*) whereas gene-deleted adenoviral vectors (GD AdV) lack all viral coding sequences. In multiple studies it was demonstrated that rAdV genomes are maintained for several months and up to years in small and large animal models resulting in long-term phenotopic correction of genetic disease. However, the mechanism of vector genome persistence and the molecular status of rAdV DNA are unknown. Therefore, it was the goal of the present study to analyse three potential mechanisms of genome persistence and molecular conformations of rAdV DNA: Episomal replication, concatemer formation and circle formation. These possibilities of genome maintenance have also been discussed for members of the *Herpesviridae* and recombinant adeno-associated virus.

Within the present study, tropism and stability of transgene expression of GD AdV were analysed *in vivo*. To analyse episomal replication of GD AdV, it was the plan to establish a methylase/restriction endonuclease-based system. After transduction of eukaryotic cell lines and murine liver, this system was used to investigate the replicative state of GD AdV by endonuclease digestion.

Additionally, we aimed at investigating the possibility of concatemer and circle formation of rAdV *in vitro* by pulsed field gel electrophoresis. Moreover, a polymerase chain reaction specific for concatemeric and circular rAdV genome conformations was established analysing concatemerisation and circularisation of *ΔE1/E3* and GD AdV in quiescent and proliferating murine liver.

B Material and Methods

1. Material

1.1 Plastic ware

All sterile plastic ware was supplied by Peske and Falcon.

1.2 Chemicals and enzymes

All chemicals were obtained from Roth or Sigma, respectively. All enzymes were purchased from New England Biolabs, if not stated otherwise.

1.3 Equipment

Bench top centrifuge	Biofuge fresco, Heraeus
Clinical centrifuge	Rotanta 460, Hettich
Electroporator	Gene Pulser II, Biorad
ELISA reader	Sunrise, Tecan
Luminometer	Microlumat Plus LB 96V, Berthold
Luciferase live imager	IVIS Imaging System 200 Series, Xenogen
Light-Cycler	Light Cycler 2.0, Roche
Light microscope	Axiovert 25, Zeiss
PCR cycler	T professional basic, Biometra
Phosphoimager	FLA-3000, Fujifilm
Pulsed Field Gel Elecrophoresis	CHEF-DR III, Biorad
Rotor for ultracentrifuge	SW 41, Beckman Coulter
Spectrophotometer	Ultrospec 3000, Pharmacia
Taqman-Cycler	7500 Fast Real-time PCR System, Applied Biosystems
Ultracentrifuge	Beckman Coulter LE 80K
UV cross-linker	UV Stratalinker 1800, Stratagene

2. Viruses, viral vectors, oligonucleotides, plasmids and bacteria

2.1 Viruses and viral vectors

Table 1: Overview over viruses and viral vectors used.

Virus/vector	Relevant characteristics	Reference	Figure
Adenovirus serotype 5 wild type (Ad5 wt)	Natural virus occurring in the human population	Garnett *et al.* 2002	**1 and 2A**
ΔE4 mutant virus (H5dl1004)	Ad5-based, deletion in *E4* coding sequence (*ORF2-ORF7*)	Sandler and Ketner 1989; Weiden and Ginsberg 1994	**24A**
Helper virus (AdNG163R-2)	Ad5-based, inverted packaging signal Ψ is flanked by loxP-sites; promotes replication of GD AdV	Palmer and Ng 2003	**5**
1st generation vector (*ΔE1/E3*)	Ad5-based, deleted for E1 and E3 regions; transgene hFIX is driven by SV40 promoter	Ehrhardt and Kay 2002	**2B**
Gene-deleted adenoviral vector ApoE HCR hAAT hFIX (GD AdV)	Ad5-based, no viral coding sequences, transgene hFIX is regulated liver-specifically	Ehrhardt and Kay 2002	**4**
Gene-deleted adenoviral vector ApoE HCR hAAT luc (GD AdV luc)	Ad5-based, no viral coding sequences, transgene luciferase is regulated by liver-specific promoter/enhancer	This study	**14**
Gene-deleted adenoviral vector SV40 luc (GD AdV SV40 luc)	Ad5-based, no viral coding sequences, transgene luciferase is regulated by SV40 promoter	This study	

2.2 List of oligonucleotides

Table 2: Overview over the oligonucleotides used in this study. All oligonucleotides were obtained from operon. Oligonucleotides were stored as 10 µM stock solution. The working concentration was 200 nM, if not stated otherwise.

Target/Name	Orientation	Sequence 5' to 3'	Restriction recognition
GAPDH	Forward	TGC CTC CTG CAC CAC CAA CT	
GAPDH	Reverse	CGC CTG CTT CAC CAC CTT	
CircleRevHDhFIX436 Rev436	Reverse	ACG CCA CTT TGA CCC GGA ACG	
CircleForHDhFIX5781 For-1700	Forward	TCA GGC CAA GCT TAT CGA AAT TCC	

Table 2 continued			
Target/Name	**Orientation**	**Sequence 5' to 3'**	Restriction recognition
Ad5ITRfw1-24	For/Rev	CAT CAT CAA TAA TAT ACC TTA TTT	
H5dl1004For35585Circ	Forward	CAC CAG CTC AAT CAG TCA CAG TG	
U6Rev775	Reverse	AGA TCT CGA GCT AGC GGC CTA AC	
32591Forfibercircle	Forward	TCA GTC AAG TTT ACT TAA ACG GAG	
Ad5ForITREnde35836	Forward	CTA CGT CAC CCG CCC CGT TCC	
TaqFor	Forward	TCT GAG GCG GAA AGA ACC A	
TaqRev	Reverse	AAC AGC CTT GTA TCG TAT ATG CAA A	
SondeRep		[6-FAM] TTC ACC GAG GGC CTA TTT CCC ATG AT [TAMRA]	
L3 forward	Forward	AGA AGC TTA GCA TCC GTT ACT CGA GTT GG	
L3 reverse	Reverse	ATA AGC TTG CAT GTT GGT ATG CAG GAT GG	
L3-specific probe		[6-FAM] CCA CCC GTG TGT ACC TGG TGG ACA [TAMRA]	
LeberProForSacI	Forward	AGA GCT CAC TAG TCT GCA GGC TCA GAG	*Sac*I
LeberProRevNheI	Reverse	AGC TAG CAT CGA TAC CGT CGA GGC CGC	*Nhe*I
PaeR7Rev	Reverse	TTGCCGTGCAGCTTCACGGTTG	
ForPaeR7Methylase	Forward	ACGATTGACCGTATTACCCCGG	
RevpIRESpuro2	Reverse	CAAAGGGTCGCTACAGACGTTG	
PMTSequenzierung	Forward	TACGGGCTGAGCCACGAAGAAC	

2.3 Plasmids

Table 3: Plasmids used for cloning and production of GD AdV.

Plasmid name	Characteristics	Reference
pGL3	Luciferase expression, SV40 promoter, Amp^R	Promega
pGL3 ΔSV40	pGL3 w/o SV40 promoter, MCS w/o *Xho*I, *Bgl*II and *Hin*dIII sites, Amp^R	
pIRESpuro2	5.2kb, Amp^R	Clontech
pIRESpuro2 PMT	*Pae*R7 methyl transferase as PCR-amplified *Bam*HI fragment, Amp^R	This study
pCEP-PMT	Based on pCEP4, 10.2 kb, PaeR7 methyl transferase, Amp^R	Nelson and Kay 1997
pFTC ApoE HCR hAAT luc	Based on pAdFTC, ApoE-HCR-hAAT promoter/enhancer and luciferase gene as I-*Ceu*I/PI-*Sce*I fragment, Amp^R	This study

Table 3 continued		
Plasmid name	**Characteristics**	**Reference**
pFTC SV40 luc	Based on pAdFTC, SV40 promoter drives luciferase, cloned as I-*Ceu*I/PI-*Sce*I fragment, Amp^R	This study
pGL3ΔSV40 ApoE HCR hAAT luc	w/o SV40 promoter, contains liver-specific ApoE-HCR-hAAT enhancer/promoter and luciferase gene, Amp^R	This study
pHM5	Shuttle vector, Kan^R	Mizuguchi and Kay 1999
pAdFTC	Ad5 cloning plasmid, Amp^R	Ehrhardt and Kay 2002
pHM5 SV40 luc	SV40 promoter and luciferase, as *Sal*I/*Sac*I fragment from pGL3, Kan^R	This study
pHM5 ApoE HCR hAAT luc	Liver-specific enhancer/promoter and luciferase, as *Sal*I/*Sac*I fragment from pGL3ΔSV40 ApoE HCR hAAT, Kan^R,	This study
pAAV-EF1a-hFIX	human elongation factor 1a [EF1a] gene enhancer/promoter-driven human coagulation factor IX [hFIX] expression cassette	Nakai *et al.* 2001
pCRBluntIITopo	Cloning of PCR fragments, Kan^R	Invitrogen

2.4 Bacteria

Table 4: Bacterial strains.

***E. coli* strain**	**Characteristics**	**Reference**
DH10B	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ- *rps*L *nup*G	Invitrogen
DH5α	*end*A1 *hsd*R17(rk-mk+) *supE44 thi*-1 *recA1 gyrA relA1* Δ(*lacZYAargF*) U169 (φ80*lacZ*Δ*M15*)	Hanahan 1983
Sure	e14– (McrA–) Δ(mcrCB-hsdSMR-mrr)171 endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC::Tn5 (Kan^R) uvrC [F' proAB $lacI^qZ$ΔM15 Tn10 (Tet^R)]	Stratagene
Stabl-2	F- endA1 glnV44 thi-1 recA1 gyrA96 relA1 Δ(lac-proAB) mcrA Δ(mcrBC-hsdRMS-mrr) λ^-	Invitrogen

3. Bacterial media, strain cultivation, storage and antibiotics

All bacteria were routinely grown in Luria-Bertani (LB) medium (1% trypton, 0.5% yeast extract, 0.5% NaCl). Liquid media were sterilised by autoclaving (121 °C at 1 bar for 20 min). For solid agar, 1.5% agar were added. If required, media were supplemented with the

respective antibiotic. Bacteria on LB-agar were cultivated over night at 37°C, if not stated otherwise. Bacteria in liquid culture were cultivated over night at 37°C on a shaking platform.

Table 5: Antibiotics used for selection of prokaryotic and eukaryotic cells.

Antibiotic	Abbreviation	Dissolved in	Working concentration
Ampicillin	Amp	H_2O	50 µg/ml
Kanamycin	Kan	H_2O	20 µg/ml
Puromycin	Puro	H_2O	575 ng/ml
Hygromycin	Hyg	H_2O	100 µg/ml

4. Molecular genetic methods

4.1 Plasmid Mini-Preparation

3 ml of a bacterial over night culture were spun down at 15.000 x g for 1 min in a bench top centrifuge. The supernatant was discarded and the bacterial cell pellet was resuspended with 200 µl of resuspension buffer P1 (Qiagen). After addition of 200 µl of lysis buffer P2 (Qiagen), the sample was mixed and incubated at RT for 5 min. After addition of 200 µl of neutralisation buffer P3 (Qiagen), the sample was mixed and centrifuged for 10 min at 15.000 x g. The supernatant was transferred to a new Eppendorf tube, and 250 µl of phenol:chloroform:isoamylalcohol (PCI, 25:24:1) were added under the extractor hood. The sample was centrifuged at 15.000 x g for 2 min and the upper phase was transferred to a new Eppendorf tube. Thirty µl of sodium acetate (3 M NaAc, pH 5) and 800 µl of ice cold 100% ethanol were added. The solution was mixed and the tube was centrifuged at 15.000 x g for 5 min. The supernatant was completely removed and the pellet was resuspended in 100 µl H_2O.

4.2 Determination of the DNA concentration

The concentration of DNA was determined using spectrophotometry. One microlitre (µl) of DNA solution was measured in 49 µl dH_2O using the Ultrospec 3000 spectrophotometer (Pharmacia Biotech). The concentration of the DNA was calculated using Lambert Beers Law which states concentration of double-stranded DNA in mg/ml = A_{260} x 1000 x dilution factor (50), whereby A is the adsorption coefficient of DNA at 260 nm. The solution was also measured to determine the absorption at 280 nm for proteins. Only DNA samples with a A_{260}/A_{280} ratio of 1.7-2.0 were used for experiments.

4.3 Electro-transformation of bacteria

The DNA was added to micro-centrifuge tubes. For re-transformations, 1 µl of the plasmid DNA were used. For newly ligated plasmids, up to 2 µl of the ligation mix were used. DH10B electro-competent cells were thawed on ice. Forty µl of competent cells were transferred to 0.2 cm cuvettes and plasmid DNA was added. Samples were electroporated with a BioRad GenePulser® II electroporator using the following conditions: 2.5 kV, 200 Ω, 25 µF. 1 ml of LB medium was immediately added after transformation and the suspension was transferred to a 1.5 ml Eppi. The transformed bacteria were shaken for 1 h at 37°C and subsequently plated on the appropriate solid agar.

4.4 Manipulation of plasmid DNA

4.4.1 Restriction digestion of DNA and agarose gel electrophoresis

Plasmid DNA or DNA fragments generated by PCR were digested with 4 units of the respective restriction endonuclease in a total volume of 20 µl for analytic digests or 80 µl for preparative digests. Digests were performed for at least 1 h at the respective cleaving temperature. Cleaved DNA samples were loaded onto 1 % agarose gels containing ethidium bromide for visualisation.

4.4.2 Isolation of DNA fragments from agarose gels

After running the 1% agarose gel, the respective band of DNA was cut out. Exposure of the DNA to UV light was reduced to a minimum to avoid mutations. The cut out gel slices were transferred to Eppendorf tubes and the DNA was isolated using a Qiagen gel extraction kit according to the manufacturer's instructions. The DNA fragments were eluted in 30 µl dH_2O.

4.4.3 Dephosphorylation of DNA fragments

Dephosphorylation of the digested vector DNA was performed to prevent self-ligation. DNA was dephorphorylated using 1U of calf intestinal phosphatase (CIP) at 37ºC for 1 h. CIP was inactivated by incubation at 75 ºC for 10 min. The dephosphorylated DNA was extracted with phenol:chloroform:isoamylalcohol and ethanol precipitation. The precipitated DNA was re-suspended in 20 µl of dH_2O.

4.4.4 Ligation of DNA fragments and plasmids

According to the size of the respective fragments/plasmids used in the ligation reaction, an excess of insert DNA was added (plasmid:insert 1:4). 400 units of T4 DNA ligase were used in an as small as possible reaction volume. Ligation was performed for 1 h at room temperature. A control ligation without insert was always included.

4.5 DNA-Sequencing and bioinformatic analysis

To control the correctness of cloned DNA fragments and to obtain DNA-sequences for the design of oligonucleotides, plasmid and viral vector DNA were sent for sequencing to MWG (Martinsried). Obtained sequences were analysed using the basic local alignment search tool for nucleic acids (BLASTN). Furthermore, the obtained sequences were analysed by sequence alignments to known DNA sequences. Both programmes were provided by the National Center for Biotechnology Information (NCBI). The database and the respective algorithms are available at http://blast.ncbi.nlm.nih.gov/Blast.cgi.

4.6 Isolation of DNA from eukaryotic cells

4.6.1 Isolation of genomic DNA from cell pellets

The cell pellet was resuspended in 200 µl DPBS and vortexed for complete resuspension. Two hundred µl lysis buffer (10 mM Tris, 10 mM EDTA, 0.5% SDS) were added, supplemented with 30 µl 10% SDS and 15 µl proteinase K (20 µg/ml). The suspension was mixed thoroughly and incubated over night at 55°C. The next day, 2 µl RNase A were added and the suspension was incubated for 30 min at 37°C. Porteins were removed by adding 350 µl of phenol:chloroform:isoamylalcohol. This suspension was mixed and subsequently centrifuged for 2 min at high speed in a bench top centrifuge. The supernatant was transferred to a fresh tube. Now, the phenol:chloroform:isoamylalcohol extraction was repeated as described above. The supernatant was transferred to a fresh tube and DNA was precipitated by addition of 50 µl NaAc (pH 5.0, 3 M) and 1 ml ice cold 100% ethanol. The DNA was pelleted by centrifugation for 10 min at high speed in a bench top centrifuge. The supernatant was removed and the pellet was washed by adding 500 µl of 70% ethanol and thorough mixing. This solution was centrifuged for 2 min at high speed in a bench top centrifuge. Subsequently, the supernatant was discarded. The pellet was dried and

subsequently dissolved in 120 µl of water. For determination of DNA concentration and purity a test gel was run and the OD_{260} was measured.

4.6.2 Isolation of low molecular weight DNA from eukaryotic cells

For isolation of low molecular weight (LMW) DNA, particularly viral and vector DNA, the DNA was treated according to a modified Hirt-protocol. Tissue culture plates were washed once with cold PBS, removed from the plate, and pelleted. Cells were resuspended by adding 200 µl lysis buffer/Hirt-solution (10 mM Tris; 10 mM EDTA, pH 7.5; 0.6% SDS). One hundred µl of 5 M NaCl were added. The sample was incubated over night at 4°C. After centrifugation at 13.000 rpm at 4°C for 40 min, the pellet was discarded and 25 µg of proteinase K (34 U/mg of protein) were added to the supernatant and incubated at 37°C for 1 h. LMW DNA was extracted twice with phenol/chloroform/isoamylalcohol (25:24:1, Invitrogen) extraction. The viral DNA was precipitated at –80°C for at least 3 h by adding two volumes of 100% ice cold ethanol and 3 M NaAc (pH 5), followed by centrifugation at 13.000 rpm at 4°C for 1 h. The pellet was washed with 70% ethanol and centrifuged for 5 min. The DNA was partially dried and the pellet was resuspended in 100 µl of sterile H_2O.

4.6.3 Isolation of genomic DNA from liver

Two ml lysis buffer (40 mM NaCl, 10 mM Tris pH 7.5, 10 mM EDTA) were filled into 50 ml tubes (Falcon) and 300 µl of 10% SDS were added. Approximately 0.1 g of liver tissue was used. Small pieces of tissue were immediately transferred to the prepared lysis buffer. Ten µl of Proteinase K (20 mg/ml) were added and the samples were incubated at 55°C for 2 h with permanent gentle shaking. Subsequently, 15 µl RNase A (10 mg/ml) were added and the samples were incubated over night at 37°C on a shaking platform.

On the next day the samples were extracted twice with 500 µl phenol:chloroform: isoamylalcohol (25:24:1). After centrifugation at 3000 rpm for 2 min the supernatant was transferred to a 15 ml Falcon tube. For precipitation of the DNA, three volumes of ice cold 100% ethanol were added and the samples were mixed thoroughly. Subsequently, the samples were centrifuged at 2000 rpm for 10 min. Afterwards, the supernatant was discarded and the DNA-pellet was briefly air-dried. To solve the DNA 500 µl freshly prepared TE buffer (10 mM Tris pH 7.5, 1 mM EDTA, 10 µg/ml RNase A) were added and the samples were gently shaken for 2 h at room temperature. For long-time storage the samples were frozen at -20°C.

4.7 Pulsed Field Gel Electrophoresis (PFGE)

Cells were removed from culture plates, pelleted and resuspended in 0.25 ml of phosphate-buffered saline (Gibco) which was mixed with 0.25 ml of liquid 2% low-melting-point Incert Agarose (Biozym). This solution was cast into blocks and, after solidification, incubated at 55°C for 24 h in 1% SDS/125 mM EDTA with proteinase K at 5 mg/ml. The samples can be stored at 4°C in storage buffer (10 mM Tris, 10 mM EDTA). One half of an agarose block was loaded on each line of the gel (Gold Agarose, Biozym) and the pockets of the gel were sealed with Incert Agarose (1%). As molecular size marker the MidRange II PFG Marker (NEB) was used. All gels were electrophoresed in 0.5 x TBE buffer (890 mM Tris, 12.5 mM EDTA, 890 mM boric acid) at a ramped pulse frequency of 1-20 sec, at 6 V/cm^2 for 24 h (CHEF-DR III, Biorad).

4.8 Southern blotting

4.8.1 Southern transfer of agarose gels

After fractionating the DNA by gel electrophoresis, the gel was soaked in 200 ml of 0.2 N HCl for 30 min. The gel was rinsed several times with deionised water. Then, DNA was denatured by soaking in alkaline transfer buffer (1 M NaCl, 0.4 M NaOH). Alkaline transfer buffer was used to transfer DNA to charged nylon membranes (Hybond-XL, Amersham). Transfer of DNA was for 48 h. After transfer, the membrane was neutalised with Neutralization buffer II (1 M NaCl, 0.5 M Tris-HCl (pH 7.2)).

4.8.2 Southern blot hybridisation/Labelling of the probe

The blotted membrane was wetted with 2X SSC and pre-hybridised in 25 ml church buffer (7% SDS, 0.5 M NaPhosphate (pH 7.5), 1 mM EDTA) at 65°C for 30 min. The probe was marked with radioactive αp^{32}CTP according to the manufacturer's instructions (Prime-It II Random Primer Labeling Kit, Stratagene). Subsequently, the probe was purified and non-incorporated nucleotides were removed using the nucleotide removal kit (Qiagen). The purified probe was eluted in 100 µl of elution buffer. One hundred µl of the radioactively labelled probe were added to 25 ml of pre-warmed (65°C) church buffer. The membrane was hybridised at 65°C in a roller oven over night. On the next day, the hybridised membrane was washed with 50 ml washing buffer 1 (2% SSC / 0.1% SDS) for 5 min at 65°C. Subsequently, the first wash was removed and a second wash with another 50 ml of washing buffer 1 was

performed for 15 min. Additionally, the membrane was washed two times with washing buffer 2 (0.1% SSC / 0.1% SDS) for 15 min. Hybridised membranes were detected on photo imager plates (Fujifilm) or normal photo films (Kodak).

4.8.3 Probes used for Southern hybridisation

The adenoviral probe was a PCR product from base pair 1 to 354 of the left arm of the adenoviral Ad5 genome. The hFIX specific probe (1.6 kb) was generated by cutting plasmid pAAV-EF1a-hFIX (Nakai *et al.* 2001) with *Hind*III and *Eco*RI.

4.8.4 Stripping of hybridised Southern membranes

Stripping of hybridised Southern membranes was performed using 0.4 M NaOH for 30 min at 42°C.

4.9 Analysis of RNA

4.9.1 Isolation of RNA

For isolation of total RNA from eukaryotic cells, the RNeasy kit (Qiagen) was employed according to the manufacturer's instructions. The concentration of isolated RNA was determined using a spectrophotometer (Pharmacia). The integrity of the isolated RNA was evaluated by agarose gel elecrophoresis. All work was done on ice. Isolated RNA was stored at -80°C.

4.9.2 Reverse Transcription of mRNA into cDNA/Analysis of cDNA by PCR

Transcription of mRNA into cDNA was done according to the NEB First Strand Synthesis Protocol using 2 µg of purified total RNA. 5 µl of cDNA were directly used for PCR. PCR conditions were:

Denaturation:	94°C 2 min	
Denaturation:	94°C 30 sec	} 20 - 25 cycles
Annealing:	55°C 30 sec	
Elongation:	72°C 30 sec	

4.10 Polymerase chain reactions (PCR)

4.10.1 Standard PCR

Reaction components:

Template DNA	0.1-500 ng
Oligonucleotide 1 (10 µM)	2 µl
Oligonucleotide 2 (10 µM)	2 µl
dNTPs (2 mM)	2 µl
10 x Taq buffer	5 µl
Taq polymerase (5 U/µl)	0.5 µl
H_2O	ad 50 µl

Cycling parameters:

Denaturation:	94°C 5 min	
Denaturation:	94°C 30 sec	30 - 35 cycles
Annealing:	xx°C 30 sec	
Elongation:	72°C y min	
Final extension:	72°C 10 min	

x: Annealing temperature is dependent on the Tm (melting temperature) of oligonucleotides.
y: Elongation is typically 1 min per kb of amplified DNA using Taq polymerase.

4.10.2 PCR for concatemer and circular monomer detection

PCRs for GD vector were run with the oligonucleotides Rev436, For-1700 and Ad5ITRfw1-24, respectively. PCRs for *ΔE4* virus and first generation vectors were run with the oligonucleotides H5dl1004For and Rev436. The GD AdV concatemer PCR was run with 45 cycles at 95°C for 5 min, 95°C for 30 sec, 55°C for 30 sec, 72°C for 2 min. The *ΔE4* and *ΔE1/E3* AdV concatemer PCRs were run under the same conditions, however, elongation was at 72°C for 1 min. PCRs for linear monomer detection were also run under the same conditions, but with an elongation time of 30 sec. Input of Hirt-extracted DNA was 400 ng in a total volume of 50 µl.

4.10.3 Real-time PCR for detection of **Xho***I-cleavage/GD AdV-replication*

DNA was digested with *Xho*I or *Nco*I for 24 h. Two hundred ng of total DNA were used in a final volume of 20 µl. *Xho*I cleavage was detected running a PCR-program with 40 cycles. Amplification and data collection were at 60 °C for 1 min. As probe was used SondeRep (500 nM), forward oligonucleotide was TaqFor (200 nM), reverse oligonucleotide was TaqRev (200 nM). Probes and oligonucleotides were designed with primerExpress3.0

software (Applied Biosystems) and obtained from Operon, Germany. The Taqman Fast Universal PCR Master Mix (Applied Biosystems) was used in corporation with a Taqman 7500 Fast Real Time PCR System (Applied Biosystems).

5. Enzyme activity assays

5.1.1 Luciferase assay **in vitro**

Cells were routinely grown on 24 well plates and were transfected 48 h before luciferase measurement. One hundred µl freshly prepared Passive Lysis Buffer (PBL) were added to each well to detach the cells from the plate and for cell lysis. The cell suspension was transferred into Eppendorf tubes. For complete cell lysis, the cell suspension was once frozen in liquid nitrogen and thawed subsequently at 37°C. The samples were measured in white 96 well plates (Nunc). First, 35 µl of Luciferase Assay Reagent II (LAR) were filled into each well. 5 µl of the samples (cell-PBL-lysate) were added and mixed thoroughly. Firefly luciferase was measured at 492 nm and 25°C for 5 seconds. After the firefly luciferase measurement, 35 µl of Stop-and-Glow reagent were added. Having mixed the reagents thoroughly, renilla luciferase was detected as an internal transfection control in a second measurement. Promoter activity was calculated by dividing the firefly luciferase activity through the renilla activity. All reagents were purchased from Promega.

5.1.2 Luciferase life imaging

D-Luciferin potassium salt (Xenogen) in DPBS was used. Ten µl/g body weight of luciferin were administered intraperitoneally 15 min before imaging. For bioluminescence luciferase imaging the Xenogen IVIS Imaging System 200 Software was used. Object height (1.5 cm) was adjusted as measuring surface. Luciferase expression was detected by an exposure time of 60 seconds. Photographs were taken with a CCD camera at an operating temperature of at least – 80°C.

5.2 Measurement of alanine aminotransferase (ALT)/Analysis of hepatic injury

For the quantitative determination of ALT in murine serum as signal for liver toxicity, the reagents of a kit (Randox) were used according to the manufacturer's instructions. Fifteen µl of murine serum were applied to each reaction.

5.3 Enzyme Linked Immunosorbent Assay (ELISA)

Detection of hFIX in murine serum was performed in a sandwich ELISA. 96 well plates (Nunc MaxiSorp) were coated with an anti-hFIX antibody (Sigma). The anti-hFIX antibody was used in a dilution of 1:2000 in coating buffer (0.1 M $NaHCO_3$, pH 9.4). The plates were incubated using 50 µl per well at 4°C over night. For blocking, plates were washed with 200 µl TBST (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20) per well. After removal of the TBST solution, 200 µl dilution buffer (TBST + 5% FBS) per well were added and the plates were incubated 1 h at room temperature. The plates were washed twice with TBST. Samples were diluted in appropriate relations. For generation of a standard curve, purified hFIX (ProSpec-Tany Technogene) was used. Fifty µl per well were loaded and the plates were incubated for 2 h at 37°C. The plates were washed twice with TBST. For detection an anti-hFIX-HRPO antibody (Biozol) was used. The anti-hFIX-HRPO antibody was applied in a 1:1000 dilution. Fifty µl per well were loaded and the plates were incubated for 2 h at 37°C. The plates were washed 4x with TBST. For development 50 µl substrate solution (Sigma Fast OPD) per well were used and the plates were incubated for 10 min at room temperature. To stop and enhance the reaction, 50 µl of 2 M H_2SO_4 were added to each well. The samples were read at 492 nm in a plate reader (Tecan, Magellan3 software).

6. Cell culture

6.1 Cell culture media

Growth Medium:

Modified eagle medium (MEM, PAA) or Dulbecco's modified eagle medium (DMEM, PAA), supplemented with 10% (v/v) fetal bovine serum (FBS, PAA). For liver cell lines, non-essential amino acids (PAA) were added.

Freezing Medium:

DMEM or MEM supplemented with 10% (v/v) FBS and filter sterilised dimethylsulfoxide (DMSO).

Phosphate Buffered Saline (PBS) (PAA):

137 mM NaCl
2.6 mM KCl
10 mM Na_2HPO_4
1.8 mM KH_2PO_4

Trypsin-EDTA Solution (PAA)

0.53 mM tetra-sodium ethylene diamine tetra-acetic acid (EDTA)

0.05% trypsin

6.1.1 Cultivation of eukaryotic cell lines

All cells were grown in a humidified incubator at 37°C and 5% CO_2.

Human embryonic kidney 293 cells (ATCC CRL-1573) and HeLa cells (ATCC CCL-2) were grown in MEM supplemented with 10% FBS.

Huh7, Sk-Hep-1 (ATCC HTB 52) and murine Hepa 1A cells were kept in DMEM containing 10% FBS and 0.1 mM non-essential amino acids.

116 cells (Palmer and Ng 2003) were used for production of GD vectors. This cell line carries a hygromycin resistance gene, expresses Cre recombinase, and was grown in MEM supplemented with 10% FBS and 100 μg/ml hygromycin B.

W162 cells (ATCC CRL-2783), a Vero cell derivative stably expressing the E4 region of Ad5, supports the growth of adenoviral *E4* deletion mutants. This cell line was maintained in MEM with 2 mM L-glutamine, adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate and 10% FBS.

293M cells were grown in MEM supplemented with 10% FBS and 100 μg/ml hygromycin for selection of *Pae*R7 methylase expressing cells. 293M cells were generated in the present study.

116M cells were routinely grown with DMEM supplemented with 10% FBS, 100 μg/ml hygromycin (PAA) for selection of Cre expression and 575 ng/ml puromycin (PAA) for selection of *Pae*R7 methylase expression. 116M cells were generated in the present study.

6.1.2 Establishment of cell cultures from cryostocks

The thawed cell suspension was transferred to a tube containing growth medium. Cells were collected by centrifugation at 200 g for 3 min at room temperature. The growth medium was removed, the cell pellet was re-suspended with 1 ml of fresh growth medium and transferred to cell culture dishes containing the appropriate growth medium.

6.1.3 Preparation of permanent cell culture stocks in liquid nitrogen

Cells were grown to at least 50% confluency. Cells were trypsinised and collected by centrifugation at 200 g for 3 min at room temperature. The supernatant was removed and the cell pellet was re-suspended in 3 ml of freezing medium. 500 µl of the cell suspension were aliquoted into cryovials and kept in liquid nitrogen for long-term storage.

6.1.4 Passaging of eukaryotic cell lines

At a concluence of more than 50%, cells were split at a 1:10 ratio. Cells were removed from the dish with trypsin-EDTA solution (PAA). Trypsinised cells were immediately transferred to new cell culture plates containing the appropriate growth medium.

6.1.5 Establishment of stable eukaryotic cell lines

Plasmids were transfected into 116 and 293 cells using Superfect transfection reagent according to the manufacturer's instructions. Transfected cells were incubated with the respective antibiotics. After 20 days of incubation under selection pressure, single cell colonies were isolated. Cells were further expanded in the respective growth medium.

6.2 Gene-deleted adenoviral vector hFIX (GD AdV)

GD AdV hFIX was mainly used for the present study. GD AdV AdFTC-hFIX-lucRNAi was based on the parental plasmid pAdFTC (Ehrhardt and Kay 2002) and contains Ad5 5'- and 3'- inverted terminal repeats (ITR) and a packaging signal (Ψ). The stuffer DNA of this vector consists of the matrix attachment region (MAR) of the murine immunoglobulin κ locus and alphoid repeat DNA sequences from human chromosome 17. The alphoid repeat DNA is equivalent with a centromeric region and contains potential mammalian origins of replication. The human blood coagulation factor IX (hFIX) expression cassette was described previously (Miao *et al.* 2000) and contained two liver specific enhancers - apolipoprotein E (ApoE) enhancer and the hepatic control region (HCR) - and a human alpha-1-antitrypsin promoter (hAAT-p) controlling expression of the hFIX minigene. Additionally, this GD AdV contained an expression cassette encoding small hairpin RNA (shRNA) against firefly luciferase driven by human U6 snRNA promoter (hU6-p) [**figure 4**]. The capsid of this vector is identical to that of Ad5 wt.

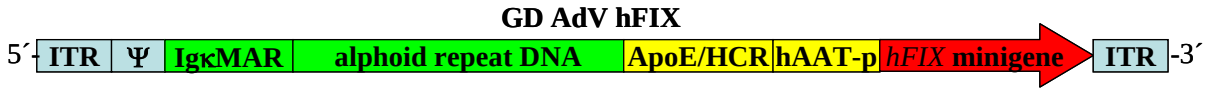


Figure 4: Schematic representation of the genome of gene-deleted adenoviral vector hFIX (GD AdV hFIX). For description of the single elements see text.

6.3 Large-scale production and purification of viral vectors and viruses

6.3.1 Pre-amplification steps

The linearised GD AdV DNA construct was transfected into the producer cell line 116 according to the manufacturer's instructions (Superfect, Qiagen). Sixteen to 18 h post transfection, co-infect with helper virus at an MOI of 5. Cells and supernatant (= passage P0) were harvested 48 h post-infection and frozen at –80°C. Now, 3 serial passaging steps (P1 - P3) on 6 cm, 10 cm and 15 cm dishes are required for pre-amplification of GD AdV. Usually, one third of the freezed-thawed lysate of the previous passage is applied to infect the next plate. Co-infection with helper virus was always at an MOI of 5. Lysate was generally harvested when complete cytopathic effect was observed.

6.3.2 Large-scale production of GD AdV

Large-scale GD AdV production was performed as described earlier (Parks *et al.* 1996; Palmer and Ng 2003; Jager *et al.* 2008 submitted; **figure 5**). In brief, 10 confluent 15 cm dishes of 116 cells were transferred into 1 litre of MEM (Gibco) supplemented with 10% FBS (Sigma) and 100 μg/ml hygromycin B (Invitrogen). For production of methylated GD AdV, 116M cells growing with 575 ng/ml puromycin were used. Over the next days, the same growth medium with all necessary supplementals was added to a final volume of 3 litres (500 ml on days 2 and 3, 1000 ml on day 4). On day five, 3 litres of 116 cells at 3 to $4x10^5$ cells/ml were harvested by centrifugation (8 min at 1500 rpm in a clinical centrifuge), resuspended in 120 ml volume medium and co-infected with 100% of the crude lysate from the 15 cm dish of serial passaging step 3 and AdNG163R-2 helper virus (Palmer and Ng 2003) at an MOI of 1 PFU/cell. In an alternative simplified protocol, purified GD AdV instead of crude lysate was used as inoculum at an MOI of 100 for co-infection of 3 litres of 116 cells.Virus adsorption was performed at 37°C on a magnetic stir plate for 2 h, after which medium (MEM supplemented with 5% FBS, Sigma) was added to a final volume of 2 litres. Co-infected cells were harvested 48 h later for lysis via centrifugation for 10 min at 1500 rpm in a clinical

centrifuge and were re-suspended in a total volume of 30 ml PBS. Subsequently, GD AdV virions were purified.

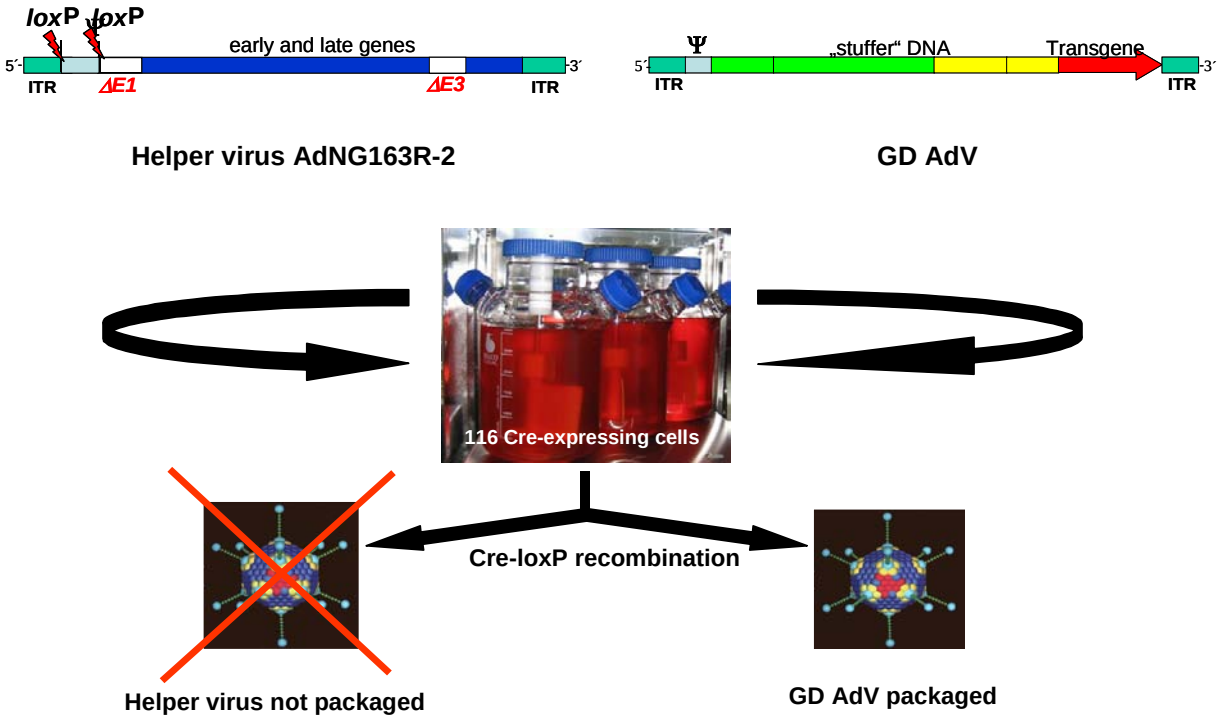


Figure 5: Large-scale production of GD AdV. The helper virus provides all necessary genes for GD AdV production *in trans*. Helper virus genomes are not efficiently packaged due to excision of their packaging signal Ψ in Cre-expressing 116 cells. This method results in high titres of GD AdV. In the upper part of the figure, all relevant characteristics of the helper virus and the GD AdV are indicated.

6.3.3 Large-scale production of Ad5 wt, ΔE4 mutants and 1st generation vector

Ad5 *ΔE4* mutants (H5*dl*1004 lacking nucleotides 2845 to 981 of *E4*) were amplified in W162 trans-complementing cells and Ad5 wild type was amplified in adherent 293 or 293M cells. Amplification of the E1 and E3 deleted recombinant 1st generation adenovirus (*Δ*E1/E3 AdV) was performed in 293 cells. All of these viruses/vectors were produced on 15-30 confluent 15 cm cell culture plates. Viral particles were purified using CsCl density centrifugation (see also 6.3.5).

6.3.4 Purification of adenoviruses and adenoviral vectors

Cells and supernatant of infected dishes were collected in conic 500 ml bottles. Cells containing the virus were spun down for 8 min at 200 x g. Supernatant was removed and the cell pellet was resuspended in 14-28 ml DPBS. This suspension can be stored at -80°C. To

release the virus from the cells, the suspension was four times freezed in liquid nitrogen and thawed in a 37°C waterbath. The lysate was spun down for 8 min at 200 x g and the supernatant containing the virus was collected.

For purification of adenovirus preparations, the suspension obtained above was loaded onto one step and one continuous gradient. The step gradient was established by pipetting CsCl solutions with different densities in the following order:

0.5 ml	1.5 g/cm^3 CsCl
3 ml	1.35 g/cm^3 CsCl
3.5 ml	1.25 g/cm^3 CsCl
5 ml	virus/vector

This step gradient was spun for 1.5 h at 35000 rpm (226000 x g)/ 12°C in an ultracentrifuge. The lower bluish band containing the virus was collected and mixed with 1.35 g/cm^3 CsCl (final volume 12 ml). This continuous gradient was spun for at least 20 h at 35000 rpm (226000 x g)/ 12°C. The virus band was collected in an as little volume as possible. The purified virus was dialysed over night at 4°C in 2 l dialysis solution (10 mM Tris (pH 7.5, 10% Glycerol, 1 mM $MgCl_2$) at very low stir speed and aliquotted.

6.4 Adenovirus DNA analysis

Fifty µl CsCl-purified virus were incubated in 150 µl lysis buffer (10 mM Tris, 10 mM EDTA, 0,5% SDS) supplemented with 10 µl proteinase K (Qiagen, 600 mAU/ml = 20 mg/ml) for 2 h at 55°C. Subsequently, samples were purified with phenol:chloroform:isoamylalcohol. Samples were centrifuged 2 min at full speed in a bench top centrifuge and upper phase was transferred to a new tube. DNA was precipitated with ice cold 100% ethanol for 20 min at -80°C and centrifugation at full speed and 4°C for 10 min. Precipitated DNA was resuspended in 50 µl water. For DNA analysis, 15 µl DNA were digested with restriction endonuclease for 1.5 h.

6.5 Titering of adenoviral vector preparations

6.5.1 Determination of the physical titre

Viral DNA was released from virions obtained from CsCl gradients in TE buffer (10 mM Tris, 10 mM EDTA) with 0.1% SDS. The A_{260} was measured to determine the viral titre. By the well-characterized absorption of pure adenovirus at 260 nm (Maizel *et al*. 1968), an OD_{260} of 0.01 corresponds to a viral particle concentration of 1.1 x 10^9 VP/ml based on a 36

kb size of the wild type particle genome. Thus, total viral particles for GD AdV and other adenoviruses were calculated as follows: VP/ml x (OD_{260} x dilution factor x 36 kb)/(size of the vector [kb] x 9.09 x 10^{-13}); where 9.09 x 10^{-13} is the extinction coefficient for wild-type adenovirus.

475 µl of measurement buffer (0.1 M TE, 10 mM EDTA, 0.1% SDS) were added to a 25 µl aliquot of the CsCl purified virus. Suspensions were vigorously shaken at 1200 rpm for 15 min in a table top shaker. Subsequently, samples were briefly centrifuged and subjected to OD_{260nm} measurement. Fifty µl of each of 5 aliquots were used for determination.

6.5.2 Determination of the infectious titre by Southern blot

To control that similar amounts of *ΔE4* and GD AdV genomes were administered, 293 cells were infected with either virus at OD_{260} MOIs of 1, 11 and 33. Cells were harvested after 4 h of incubation with trypsin. Thirty µg of total DNA were analysed on a Southern blotted gel. The intensity of the respective band indicates the relative infectious titres. We found that *ΔE4* and GD AdV show similar titres at the respective MOI [**Appendix figure 3**].

6.5.3 Determination of the infectious titre by quantitative real-time PCR

Moreover, the number of total genomes and infectious genomes were determined by quantitative real-time PCR (qPCR) employing the oligonucleotides Rev436 and Ad5ITRfw1-24. For determination of total particles, 10 vp/cell were directly administered to pelleted 293 cells before starting Hirt extraction. Subsequently, total particles were determined analyzing 200 fg of Hirt-extracted DNA in a total volume of 20 µl. Infectious particles were determined analyzing 200 fg of Hirt-extracted DNA of infected (10 vp/cell) 293 cells in a total volume of 20 µl. These infected 293 cells were harvested with trypsin after 4 h of incubation to remove unabsorbed virions. The LightCycler FastStart DNAMaster^{Plus} SYBR Green I (Roche) detected the PCR-products. The programme of the LightCycler (Applied Biosystems) was set as follows: Pre-incubation at 95°C for 10 min, amplification in 45 cycles at 95°C for 10 sec, 55°C for 5 sec, 72°C for 30 sec. All MOI indicated in the present study relate to titre determinations by OD_{260} measurements [**Appendix figure 2**].

6.5.4 Determination of the helper virus contamination of GD AdV preparations

The determination of the helper virus conatamination was performed using purified adenoviral DNA. To purify adenoviral DNA from CsCl solutions, three independent adenoviral preparations, each 20 µl, were incubated with 400 µl of proteinase K-SDS solution (proteinase K [0.5 mg/ml], 10 mM Tris-HCl [pH 7.5], 0.5% sodium dodecyl sulfate [SDS], 10 mM EDTA [pH 8]) for 3 h at 37°C. DNA was then precipitated by addition of 1/10 volume of 3 M sodium acetate (pH 5.2) and a 2.5 vol of 95% ethanol, rinsed with 70% ethanol, dried, and resuspended in 25 µl of water. Preparations of GD AdV were evaluated in three different dilutions per assay: 10^{-3} to 10^{-5}. In a total volume of 20 µl per reaction, 5 µl of the template DNA was added.

Quantitative real-time PCR for helper virus contamination was carried out by the Taqman 7500 Fast Real Time PCR System (Applied Biosystems) amplifying a 235 bp area of the adenoviral late gene 3 (L3). Utilizing the oligonucleotides L3 forward (400 nM) and L3 reverse (400 nM) together with an L3 specific probe (300 nm) (Ella Biotech) the PCR was run with the following programme: AmpErase UNG reaction at 50°C for 2 min, pre-incubation/activation at 95°C for 10 min, amplification and data collection in 40 cycles at 95°C for 15 sec and 60°C for 1 min. Universal Fast PCR Master Mix (Applied Biosystems) was used for Taqman qPCR. Oligonucleotides, probe and protocol parameters were chosen according to Puntel *et al.* 2006.

The contamination of GD adenoviral vector with helper virus lays at 0.09% compared to the number of total particles [**Appendix figure 2**]. This amount is well in the range reported by others (Puntel *et al.* 2007).

7. Transduction of cell lines

Cell lines were seeded 24 h before transduction at 70 to 80% confluency. At the day of transduction, CsCl-purified virions were directly applied to the culture medium at an appropriate MOI. Determination of the MOI generally relied on physical (OD_{260}) titres.

8. Animal studies

All mice (C57Bl/6 and Balb/c) were kept and treated according to the regulations of the Government of Upper Bavaria. All mice were injected via the tail with a total volume of 200 µl containing $7.5x10^9$ or $1x10^{10}$ transducing particles diluted in Dulbecco´s phosphate

buffered saline (Gibco), respectively.

Rapid cell cycling of murine liver cells was induced by intraperitoneal administration of 50 µl carbon tetrachloride (CCl_4, Sigma) solution (1:1 dilution in mineral oil, Sigma).

For hydrodynamic plasmid delivery to murine liver, animals were injected via the tail vein with 20 µg of plasmid DNA in a total volume of 2 ml PBS within 5 to 8 seconds (Liu *et al.* 1999).

8.1 Transduction of C57Bl/6 mice with GD AdV ApoE HCR hAAT hFIX

Mouse 1 received GD AdV, was CCl_4-treated and sacrificed in week seven post infection. Mice 2 and 3 received GD AdV and murine livers were isolated in week five. Mice 4 and 5 received GD vector, were CCl_4-treated and were sacrificed in week nine. Mouse number six was infected with GD vector and killed in week nine. Mice 7 and 8 received GD AdV and CCl_4 and were sacrificed in week nine. Mice 9 and 10 represent the negative control for this experiment, were not infected with GD AdV and were killed in week nine. Mouse 10 was CCl_4-treated. For the analysis of the replicative state of GD AdV, the mice were grouped as follows: group 1 (GD AdV and CCl_4-treated) contains mice 1, 4 and 5. Group 2 (GD AdV-transduced) consists of mice 2, 3, 7 and 8. Group 3 consists of the untransduced mice 9 and 10.

9. The model transgene human blood coagulation factor IX

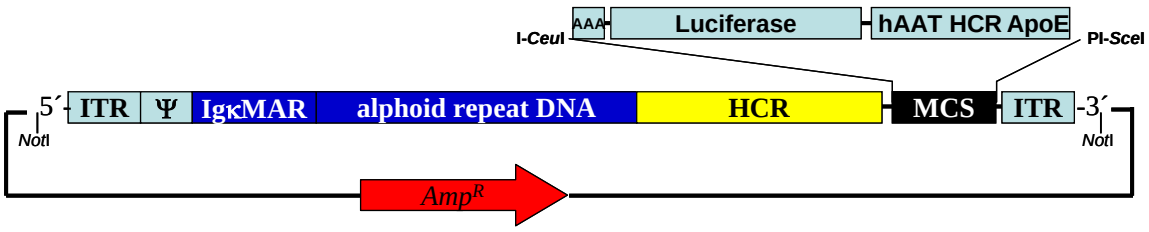
Since Ad5-derived rAdV mainly turn to the liver when injected into animals, the disease model of this survey is haemophila B. In patients affected by haemophilia B, the human blood coagulation factor IX (hFIX) is not expressed or not functional. In healthy individuals, hFIX is produced by hepatocytes. Therefore, rAdV carrying an hFIX transgene can be effectively used to transfer a functional hFIX gene to the natural site of hFIX production. Once expressed in hepatocytes, hFIX can be incorporated in the blood clotting cascade, if necessary.

C Results

1.1 Analysis of tropism and persistence of recombinant adenoviral vectors (rAdV) in vivo

1.1.1 Generation of a luciferase-expressing gene-deleted adenoviral vector (GD AdV)

First, the features of rAdV *in vivo* were analysed. Therefore, a new GD AdV was constructed and produced, to visualise the tropism of and to investigate the duration of transgene expression from GD AdV in mice. This GD AdV contains the same stuffer DNA and promoter sequences as GD AdV hFIX [**figure 4**] but expresses firefly luciferase instead of hFIX. To construct the production plasmid for the GD AdV expressing luciferase, the liver-specific promoter and enhancer region (ApoE HCR hAAT; 1.3 kb) was PCR-amplified using pBS ApoE HCR hAAT hFIX (Miao *et al.* 2000) as template. This fragment was cloned into an upstream position of the luciferase gene of the plasmid pGL3 basic by *Sac*I/*Nhe*I ligation resulting in pGL3 ApoE HCR hAAT luc [**Appendix figure 5**]. The whole enhancer-promoter-luciferase-polyA DNA fragment (3.2 kb) was then cloned by the restiction enzymes *Sac*I/*Sal*I into the pHM5 shuttle plasmid that was named pHM5 ApoE HCR hAAT luc [**Appendix figure 6**]. By PI-*Sce*I and I-*Ceu*I restriction enzyme digest the liver-specific luciferase construct was cloned into pAdFTC (Ehrhardt and Kay 2002), resulting in pAdFTC ApoE HCR hAAT luc [**figure 6**].



pAdFTC ApoE HCR hAAT luc

Figure 6: pAdFTC ApoE HCR hAAT luc. This plasmid was used for the production of GD AdV liver-specifically expressing luciferase. The plasmid carries an ampicillin resistance gene. Relevant recognition sites for endonucleases are indicated.

This plasmid was characterised by DNA-sequencing and restriction analyses and was tested for luciferase functionality *in vitro* (data not shown). Luciferase expression was liver-specific (high expression in Sk-Hep and Huh7 cells), but was inhibited in non-hepatic 293 and HeLa cells (data not shown). Using this plasmid pAdFTC ApoE HCR hAAT luc, GD luciferase expressing vector particles (GD AdV luc) were produced. Using these GD AdV particles,

luciferase functionality in the GD AdV context was confirmed in cell culture by luciferase assays (data not shown). Luciferase expression was liver-specific (high expression in Sk-Hep and Huh7 cells), but was inhibited in non-hepatic 293 and HeLa cells (data not shown).

1.1.2 Investigation of tropism, transduction efficacy, transgene expression and persistence of recombinant AdV employing luciferase life-imaging

Balb/c mice were transduced to test the liver transduction efficacy and the transgene expression of rAdV. GD AdV luc and a 1st generation vector expressing luciferase driven by an SV40 promoter (1st gen. luc) were administered via tail vein injection at a dose of 1x10^{10}. A hydrodynamic plasmid injection with 20 µg of pGL3 SV40 luc was performed in a control mouse.

One week after vector administration, high levels of luciferase (up to 1.2 x 10^7 light units, LU) were detected in the liver of the mouse receiving GD AdV luc and 1st gen. Luc, respectively [**figure 7A**]. Expression levels from pGL3 were relatively low (2 x 10^6 LU). Seven weeks p.i., luc expression from GD AdV luc was high (up to 9.8 x 10^5 LU) throughout the liver indicating persistence of GD AdV genomes [**figure 7B**]. Sixteen weeks p.i, transgene expression from GD AdV luc was hardly detectable suggesting either vector genome loss, transgene promoter silencing or induction of vector specific immune response in Balb/c mice [**figure 7C**]. Luciferase expression from pGL3 SV40 luc and 1st gen. luc dropped to undetectable levels two and three weeks p.i., respectively (data not shown). Taken together, the *in vivo* experiment using rAdV showed that rAdV particles transduce the liver and that the GD AdV genomes persist within the organ for several months. These findings are in concordance with work previously published (Ehrhardt and Kay 2002, McCormack *et al.* 2006). In the following it was investigated how persistence of rAdV might be mediated.

1.2 Do gene-deleted adenoviral vectors (GD AdV) replicate?

GD AdV genome replication could explain vector persistence, since replication of vector genomes would increase the amount of GD AdV DNA in transduced host cells. The GD AdV used in the present study contains putative mammalian origins of replication within its alphoid repeat DNA [**figure 4**]. The question whether GD AdV replicate episomally was addressed by a methylase/restriction endonuclease-based system.

The *Pae*R7 methylase derived from *Pseudomonas aeruginosa* transfers methyl moieties to the N^6-position of the adenine base of its recognition site. Since mammalian cells do not possess

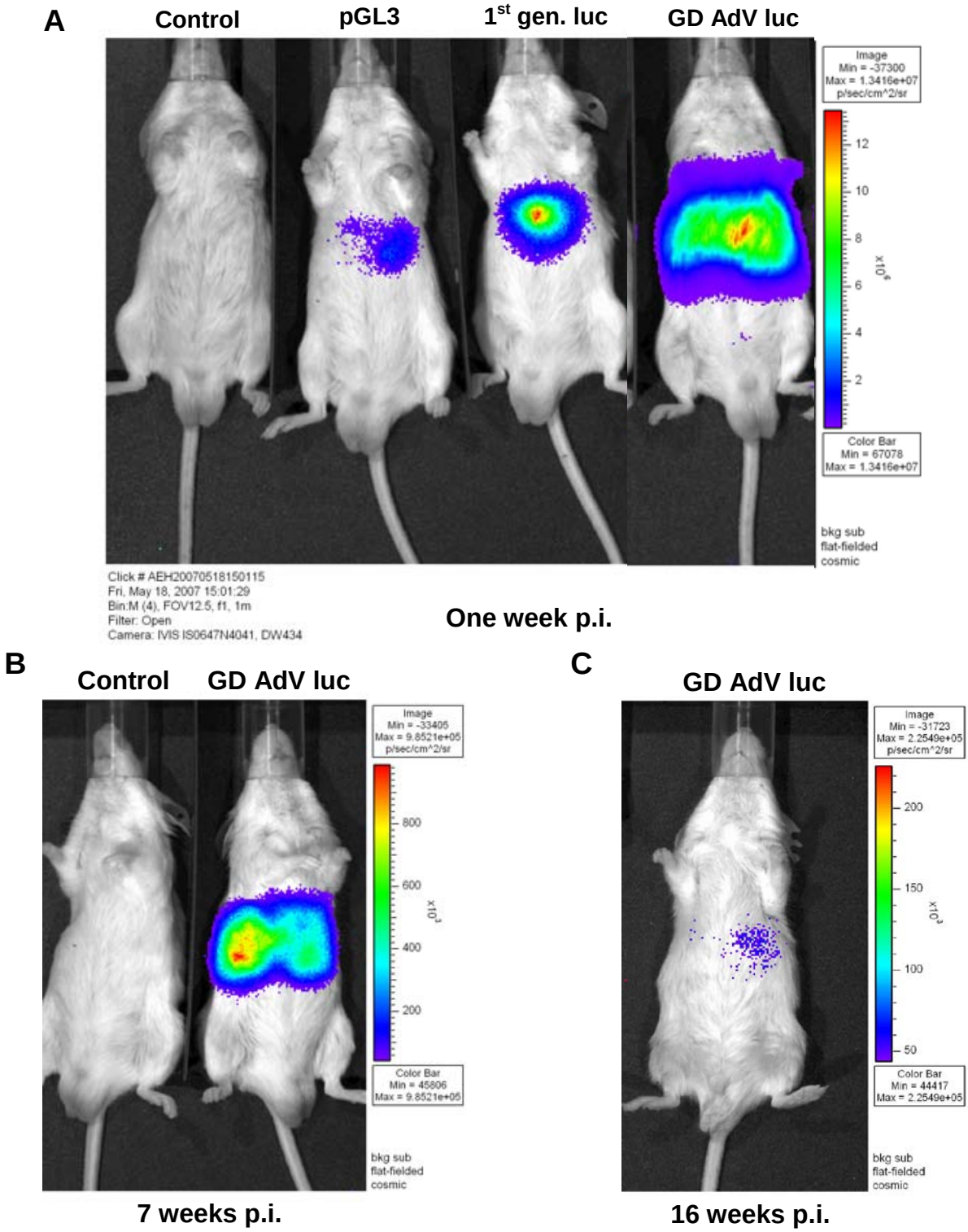


Figure 7: *In vivo* luciferase imaging of Balb/c mice dosed with different adenoviral vectors. A time course is shown [1 - 16 weeks post injection (p.i.)]. **A:** Luciferase expression one week post injection of different vectors: from left to right: control mouse (injection of PBS), pGL3 (hydrodynamic tail vein injection of 2 ml PBS containing 20 µg plasmid pGL3), mouse 3 received $1x10^{10}$ viral particles of a first generation adenoviral vector (1^{st} gen. luc) and mouse 4 was transduced with $1x10^{10}$ GD AdV luc particles. All mice received a total volume of 200 µl, if not stated otherwise. **B:** Seven weeks p.i. only the mouse transduced with GD AdV luc shows luciferase expression within the whole liver. **C:** Sixteen weeks p.i., luciferase expression from the mouse receiving GD AdV luc is hardly detectable.

methylases that target adenine bases, the procaryotic *Pae*R7 methylase uniquely marks the DNA. The *Xho*I endonuclease is an isoschizomer of the corresponding *Pae*R7 endonuclease and was used to test the methylation status of DNA. Only unmethylated DNA is cleaved whereas methylated DNA is not. To analyse the methylation/replication status of GD AdV, vector genomes were specifically methylated. The only way to remove the specific methylation from originally methylated GD AdV is by *de novo* viral genome synthesis. Thus, GD AdV genomes that have replicated, can be cleaved by *Xho*I whereas the originally methylated input GD AdV DNA stays methylated and can not be cleaved [**figure 8**]. To set up this system, cell lines that stably express the *Pae*R7 methylase were generated. Producing GD AdV and Ad5 wt virus in these cell lines, results in specific methylation of *Pae*R7/*Xho*I recognition sites.

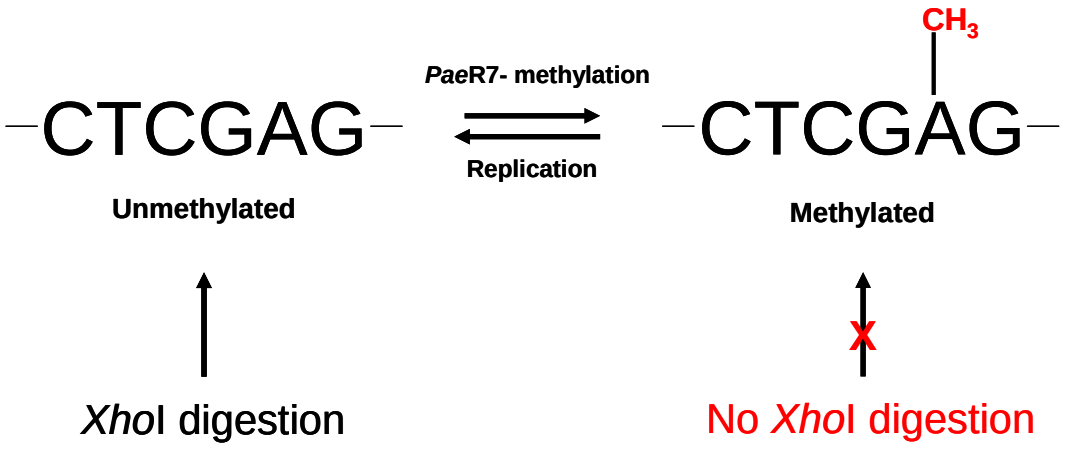


Figure 8: The methylase/restriction endonuclease-based system. The addition of a methyl group to the N^6-position of the adenine base of *Xho*I sites renders the DNA *Xho*I-uncleavable. Replication restores *Xho*I cleavage by loss of methylation. *Xho*I is an isoschizomer of the respective *Pae*R7 methylase.

1.3 Establishment of the methylase/restriction endonuclease-based system

1.3.1 Construction of the **PaeR7** *methyl transferase expression plasmid pIRESpuro2 PMT*

The plasmid pCEP-PMT (Nelson and Kay 1997) was used to excise the *Pae*R7 methyl-transferase (PMT) gene (1.9 kb) with *Bam*HI. This fragment was cloned into the pIRESpuro2 plasmid via *Bam*HI ligation, resulting in pIRESpuro2 PMT. The expression cassette of pIRESpuro2 PMT consists of cytomegalovirus promoter, the PMT gene, a synthetic intron, an internal ribosomal enty site and the puromycin resistence gene followed by a polyA signal [**figure 9**]. The correct orientation of the PMT insert was verified and the

plasmid pIRESpuro2 PMT was further characterised by restriction analyses, PCR and DNA-sequencing (data not shown).

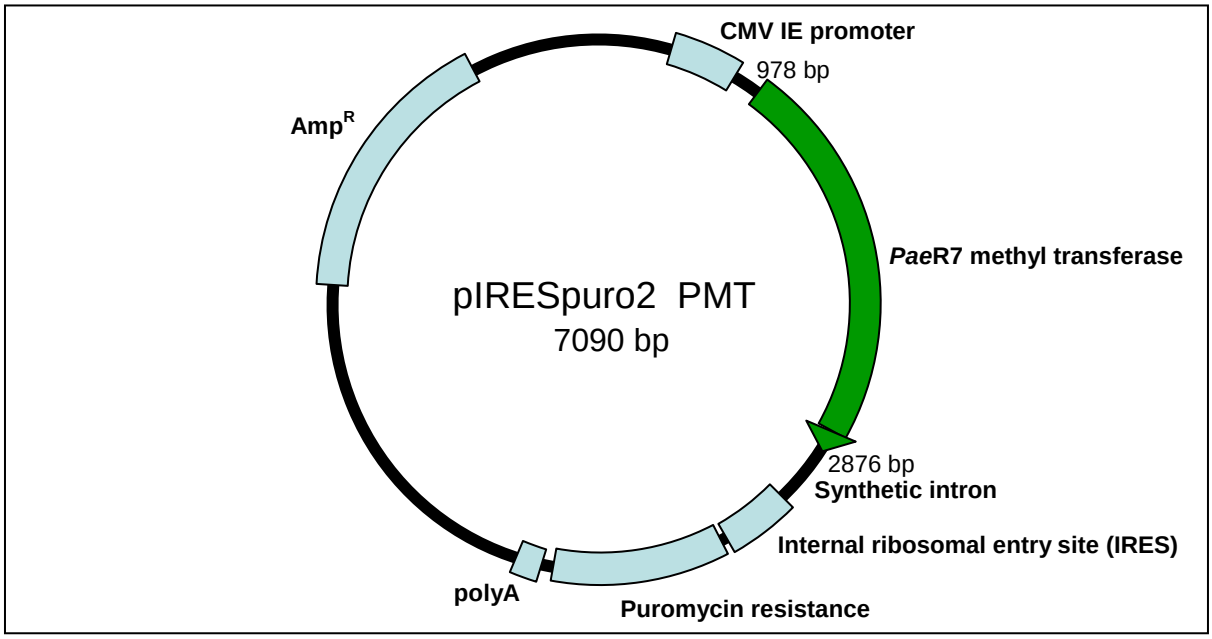


Figure 9: pIRESpuro2 PMT. Expression plasmid used for cloning of *Pae*R7 methyl transferase and generation of stably methylase expressing cells lines. Note the bicistronic expression of the PMT and puromycin resistance gene. CMV IE: Cytomegalovirus immediate early, Amp^R = ampicillin resistance gene, polyA = polyA signal, bp = base pairs.

1.3.2 Generation of stable cell lines for PaeR7 *methyl transferase expression*

To define an appropriate puromycin concentration for the establishment of stable 116 cell lines expressing PMT, a dose escalating study was performed in 116 cells [**figure 10A**]. 116 cells were used because production and packaging of GD AdV is optimised in these cells (Palmer and Ng 2003). A useful puromycin concentration was 575 ng/ml. pIRESpuro2 PMT was then transfected into (hygromycin resistant, Cre recombinase expressing) 116 cells and after 20 days of puromycin selection, cell clones were isolated and further expanded [**figure 10B**]. These new cell lines expressing *Pae*R7 methyl transferase were named 116M. Several 116M cell clones were analysed for PMT expression by isolating total RNA and reverse transcription PCR (RT-PCR) of *Pae*R7 methylase mRNA into cDNA to detect specific transcripts. All stable cell clones analysed expressed PMT [**figure 11**].

Furthermore, to amplify also methylated Ad5 wt, 293 cells were transfected with pCEP-PMT and incubated for 20 days under hygromycin selection. Single colonies were picked and further expanded. These methylase expressing cells were termed 293M cells. PMT expression

in 293M cells was confirmed by RT-PCR [**figure 12**]. Morphology and adherence of 116M and 293M cells were similar to original 293 and 116 cells. However, the doubling time of both methyl transferase expressing cell lines, however, was slightly longer (23 h) (data not shown).

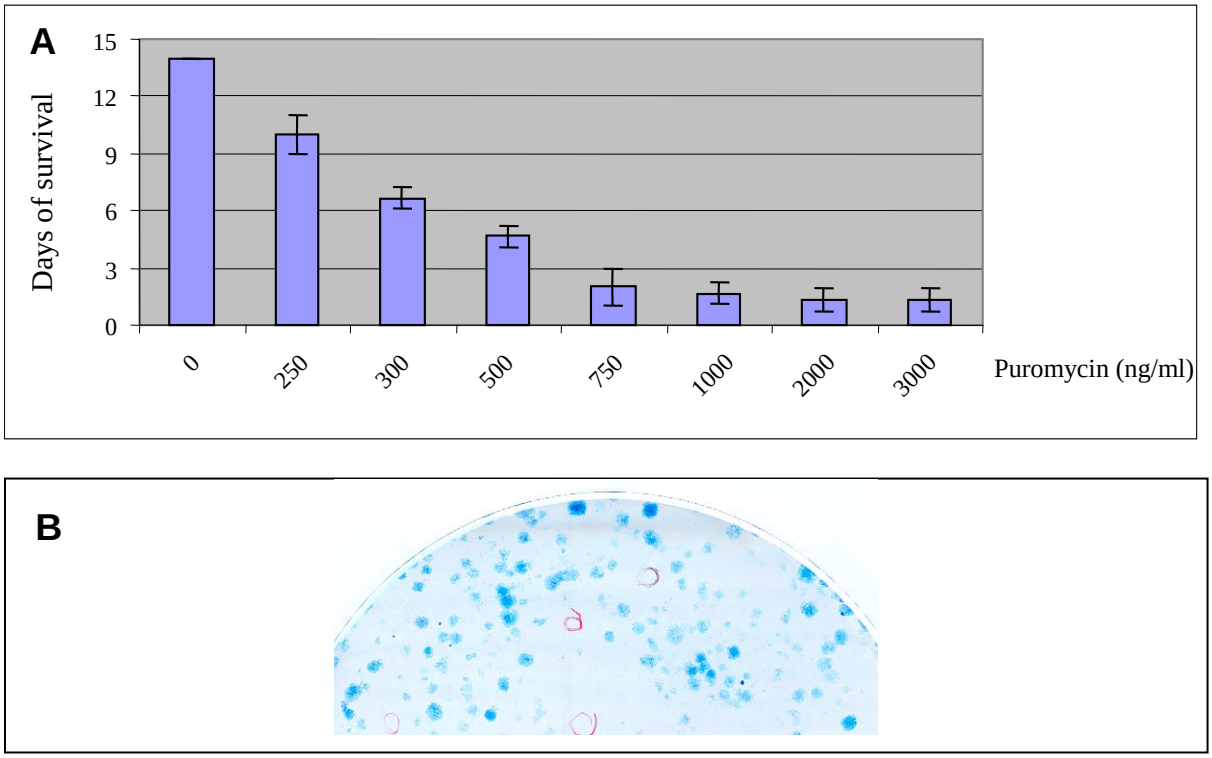


Figure 10: Establishment of methylase expressing cell lines. A: Determination of a useful puromycin concentration for selection of 116 cells. **B:** Selection of single cell colonies of methylase expressing cell lines. Methylene blue stained cell colonies are shown after 20 days of incubation under puromycin selection pressure. Removed colonies selected for further expansion are marked by circles.

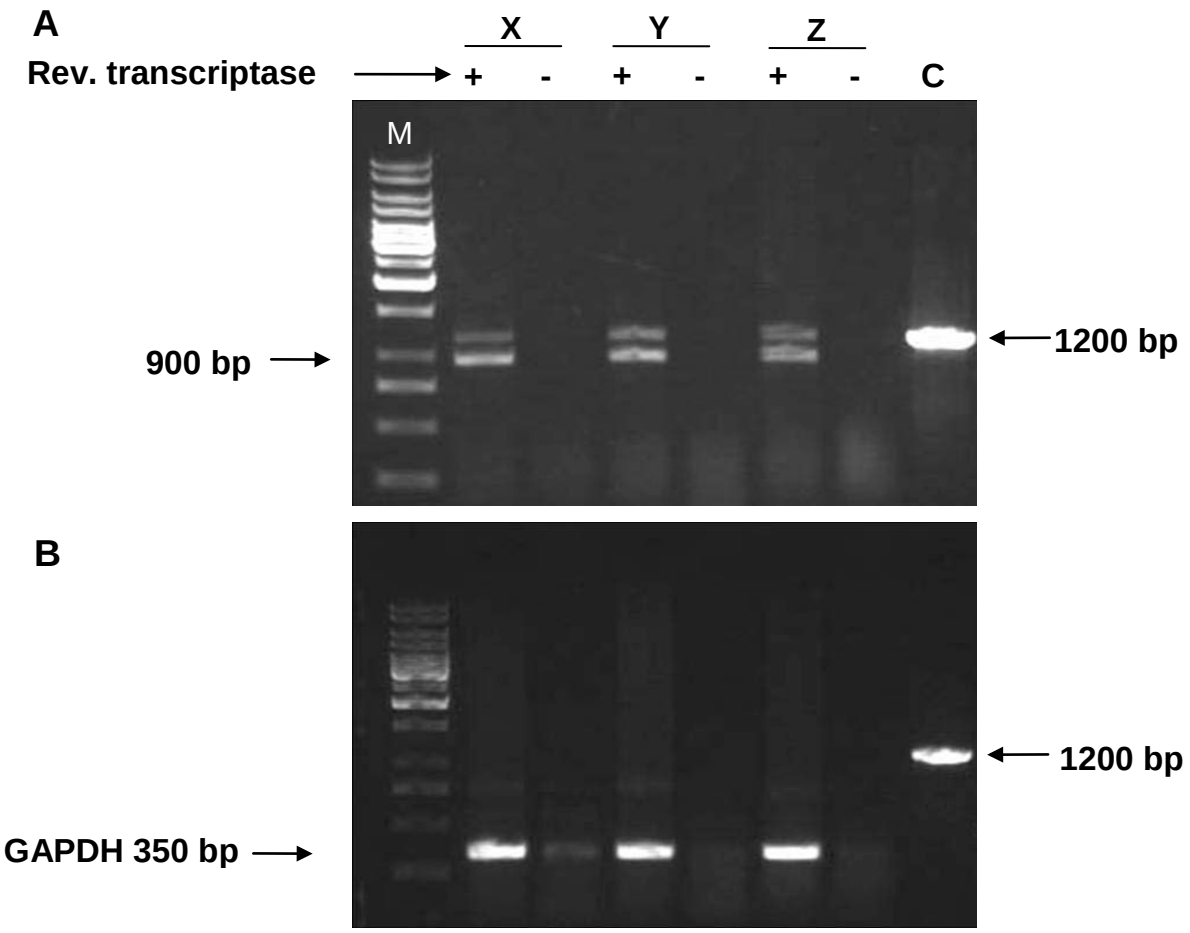


Figure 11: Reverse transcription PCR to evaluate *Pae*R7 methyl transferase expression in 116M cells. Two µg of total RNA were used for transcription into cDNA. Five µl of cDNA of three different cell clones were analysed (X, Y, Z). Samples were run on a 1% agarose gel. **A:** A PCR product of 900 bp indicates spliced PMT transcripts, a PCR product of 1200 bp indicates unspliced (PMT + puromycin mRNA) transcripts. The control (C) is derived from plasmid DNA pIRESpuro2 PMT. **B:** GAPDH control PCR from the same samples as above. A band of 350 bp represents GAPDH transcripts. Control (1200 bp) as above. (+) indicates addition of reverse transcriptase, (-) w/o reverse transcriptase. (M) = molecular size marker [**figure Appendix 1**].

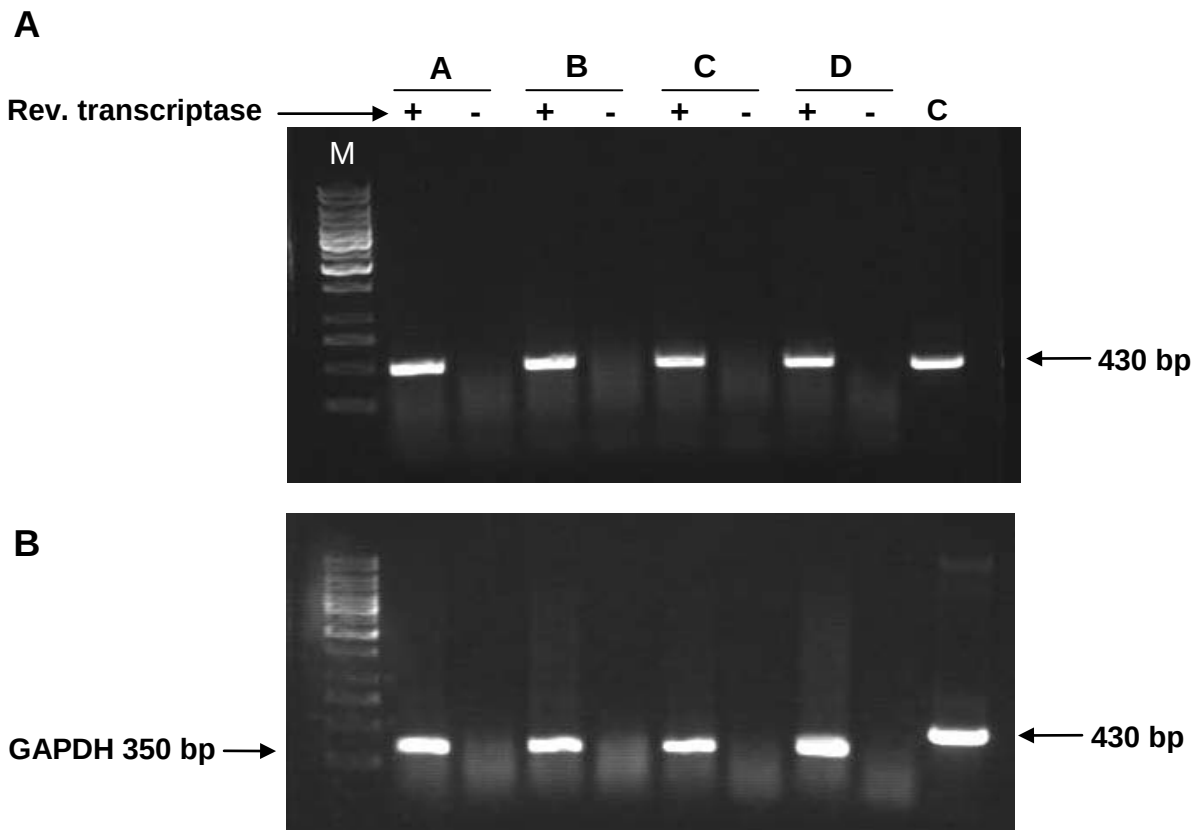
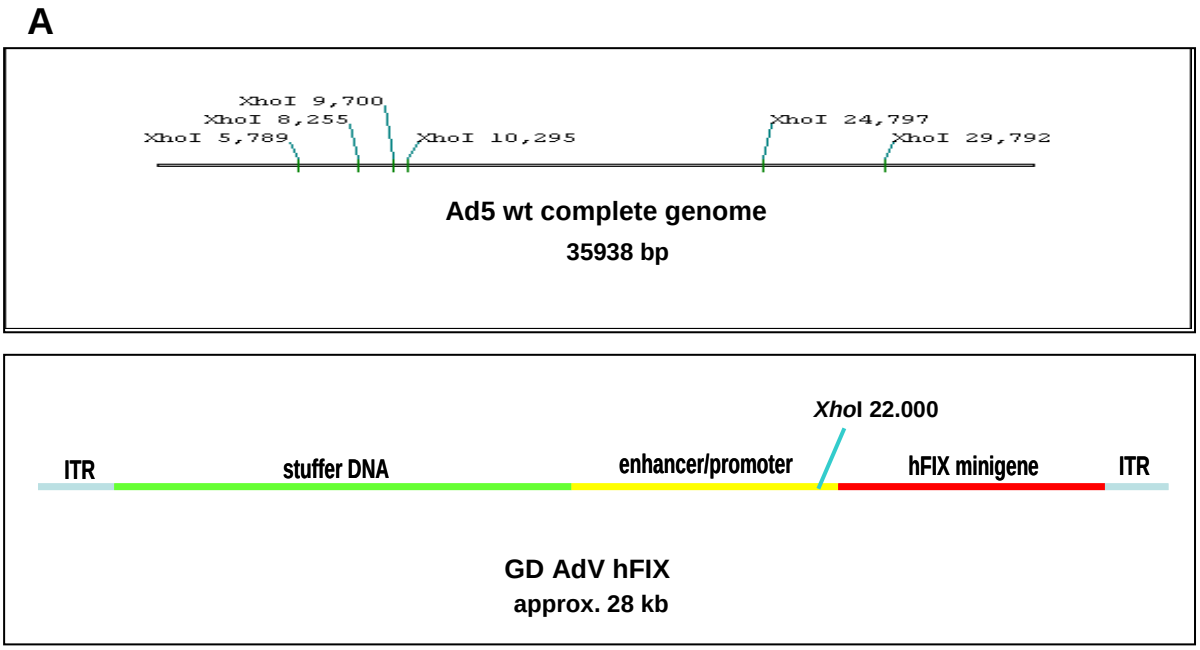


Figure 12: Reverse transcription PCR to evaluate *Pae*R7 methyl transferase expression in 293M cells. Two µg of total RNA were used for transcription into cDNA. Five µl of cDNA of four different cell clones were analysed (A, B, C, D). Samples were run on a 1% agarose gel. **A:** A PCR product of 430 bp indicates PMT transcripts. The control (C) is derived from DNA of pCEP PMT. **B:** GAPDH control reaction from the same samples as above. A band of 350 bp represents GAPDH transcripts. A band at 430 bp is amplified from pCEP PMT as relation. (+) indicates addition of reverse transcriptase, (-) w/o reverse transcriptase. (M) = molecular size marker.

1.3.3 Evaluation of PMT-functionality in 116M and 293M cells

To evaluate methyl transferase function in 116M and 293M cells, Ad5 wt and GD AdV hFIX were produced in these cell lines. As control, the Ad5 wt virus and the GD AdV were produced in original 116 and 293 cells. After CsCl purification, virions were disintegrated and purified adenoviral and GD AdV genomes were analysed by *Xho*I restriction enzyme digest [**figure 13A and 13B**]. Ad5 wt and GD AdV DNA produced in PMT-expressing cells were resistant to *Xho*I cleavage, indicating methylation of the respective *Xho*I recognition sites. However, *Pae*R7 methylation had no effect on digestion of Ad5 wt DNA with *Eco*RI [**figure 13B**]. In conclusion, the 116M and 293M cells lines express significant amounts of functional *Pae*R7 methyl transferase and retain their potential to amplify high titres of wild type virus or GD AdV, respectively (data not shown).



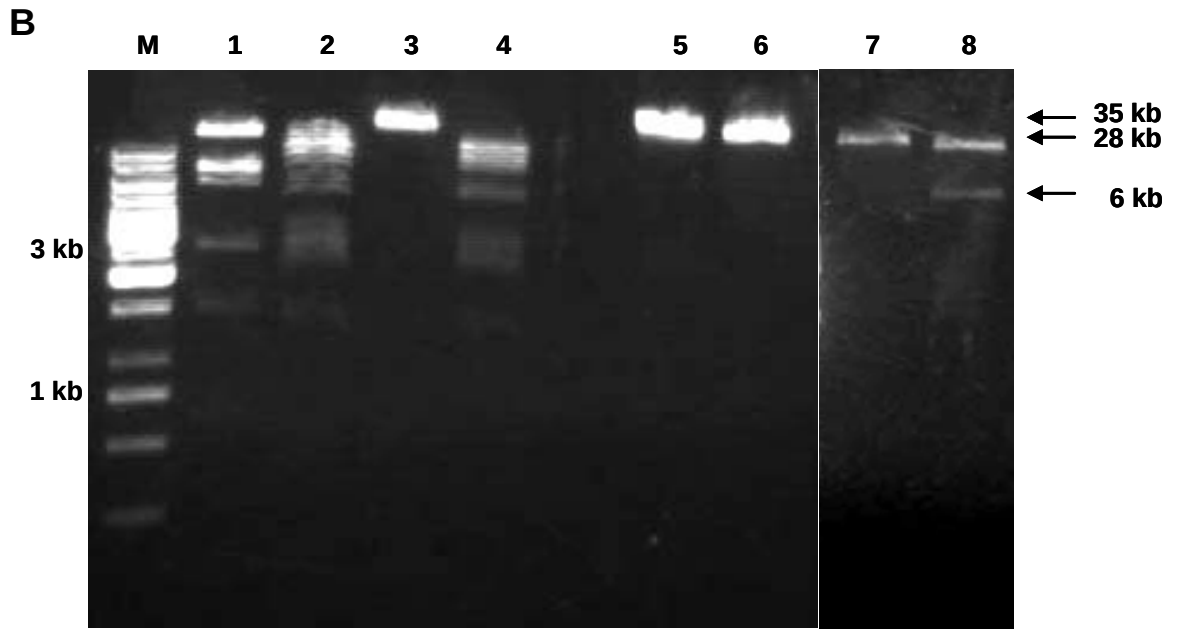


Figure 13: Analysis of *Xho*I sites in Ad5 wt and GD AdV genomes. A: Restriction maps for *Xho*I recognition sites of Ad5 wt (upper panel) and GD AdV hFIX genomes (lower panel). **B:** Methylated adenoviral genomes are insensitive to *Xho*I digestion. To analyse whether the site specific methylation is effective and renders the adenoviral genome insensitive to *Xho*I, un-methylated and methylated CsCl-purified adenoviral genomes were compared on a 1% agarose gel. *Xho*I digest of un-methylated (1) and methylated Ad5 wt (3), *Eco*RI digest of un-methylated (2) and methylated (4) Ad5 wt. Undigested controls of un-methylated (5) and methylated Ad5 wt DNA (6). *Xho*I digest of methylated (7) and un-methylated GD AdV DNA (8). Uncleaved Ad5 wt and GD AdV DNA run at approx. 35 kb or 28 kb, respectively. An additional band at 6 kb indicates *Xho*I cleavage of GD AdV DNA (8). *Xho*I or *Eco*RI cleavage of Ad5 wt DNA results in characteristic banding patterns. (M) = molecular size marker.

1.3.4 PMT-methylation is removed by replication

Next, it was tested whether methylation of adenine residues within *Xho*I recognition sites can be removed by replication. 293 cells and the liver-derived cell lines Huh7, Sk-Hep and Hepa 1A were infected with methylated Ad5 wt at MOIs of 10 to 3000. 293 cells were transduced with methylated GD AdV at an MOI of 1000 and co-infected with helper virus at an MOI of 10 to promote replication of GD AdV genomes. After an incubation time of 48 to 96 h, low molecular weight DNA was extracted from cells and subjected to *Xho*I digestion. It was found that formerly methylated and uncleavable DNA [**cf. figure 13**] can be cleaved after replication [**figure 14**].

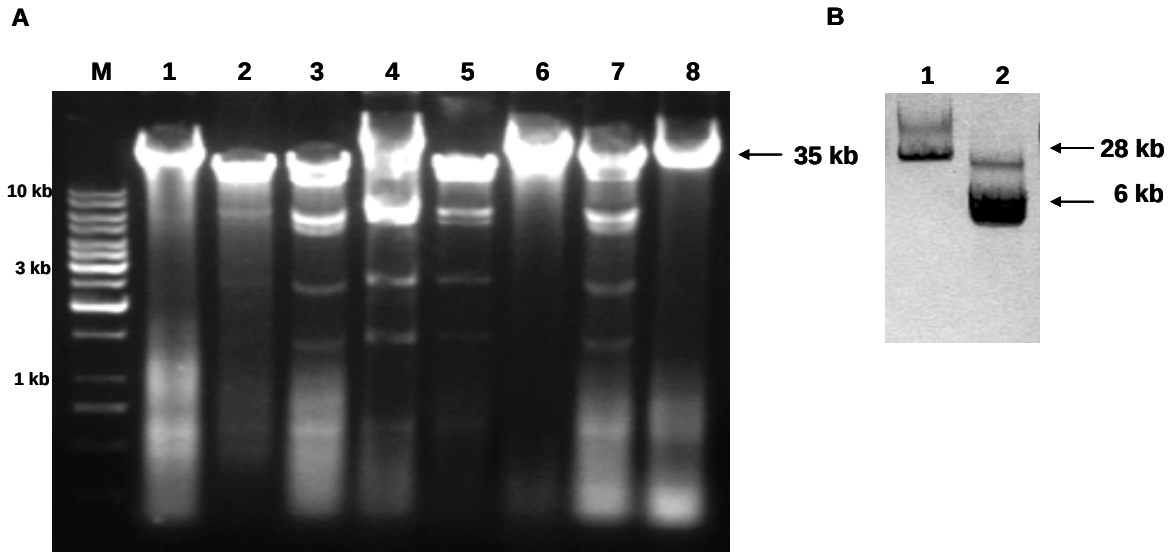


Figure 14: *Xho*I-specific methylation can be removed by replication. A: Replicative status of methylated Ad5 wt after infection of mammalian cells. Various cell lines were infected with methylated Ad5 wt and incubated for 48 h, if not stated otherwise. DNA was isolated via Hirt protocol, digested with *Xho*I and run on a 1% agarose gel. Sk-Hep cells infected with methylated Ad5 wt at an MOI of 100 (1), Sk-Hep cells infected with methylated Ad5 wt at an MOI of 100 for 96 h (2), Sk-Hep cells infected with methylated Ad5 wt at on MOI of 3000 (3), 293 cells infected at MOI 10 (4), Huh7 cells infected at MOI 10 (5), murine Hepa 1A cells at MOI 10 (6). 293 cells infected with un-methylated Ad5 wt at MOI 10 (7), CsCl-purified methylated Ad5 wt (8). Replication and cleavage of Ad5 wt DNA by *Xho*I are indicated by a characteristic banding pattern. (M) = molecular size marker. **B:** *Xho*I methylation can be removed from methylated GD AdVs by replication. CsCl-purified methylated GD AdV genomes digested with *Xho*I (1). 293 cells were transduced with methylated GD AdV at MOI 1000 + helper virus at MOI 10 (2). 72 h post infection DNA was isolated via Hirt protocol, digested with *Xho*I, run on a 1% agarose gel, Southern-transferred and hybridised with hFIX and ITR/Ψ probes. Uncleaved Ad5 wt and GD AdV DNA run at approx. 35 kb or 28 kb, respectively. An additional band at 6 kb indicates *Xho*I cleavage of GD AdV DNA.

In Sk-Hep cells a high MOI (3000) of Ad5 wt had to be transduced to detect replication. At low MOI (100) Ad5 wt does not replicate in Sk-Hep cells. Since murine Hepa 1A cells are not permissive for human Ad5 wt virus, Hepa 1A cells are an additional control and no replication/removal of methylation was seen. Helper-virus-driven replication of GD AdV genomes and loss of methylation were shown by Southern blotting, using hFIX and ITR/Ψ-specific probes [**figure 14B**]. Thus, *de novo* genome synthesis of Ad5 wt and GD AdV leads to removal of methylation. This removal can be detected by *Xho*I digestion.

1.4 The replicative state of GD AdV genomes in vitro

Next, the replicative state of GD AdV genomes was analysed in cell culture. Numerous cell lines (293, Huh7, Sk-Hep and Hepa 1A) were transduced with methylated GD AdV at an MOI of 100 to 3000, respectively. Transduced cells were harvested 48 to 96 h post infection (p.i.) and low molecular weight DNA was extracted by Hirt protocol. Five µg of purified low molecular weight DNA were analysed by *Xho*I digestion and subsequent Southern blotting using hFIX- and ITR/Ψ-specific probes. As shown by detection of full length GD AdV genomes after *Xho*I cleavage, the GD AdV DNA remained methylated and did not replicate. This result was independent of the cell type and the amount of vector genomes per cell used (MOIs up to 3000) [**figure 15**]. Purified DNA of disintegrated virions from CsCl-purified particles was used as a control in this experiment. Only the unmethylated DNA was cleaved by *Xho*I.

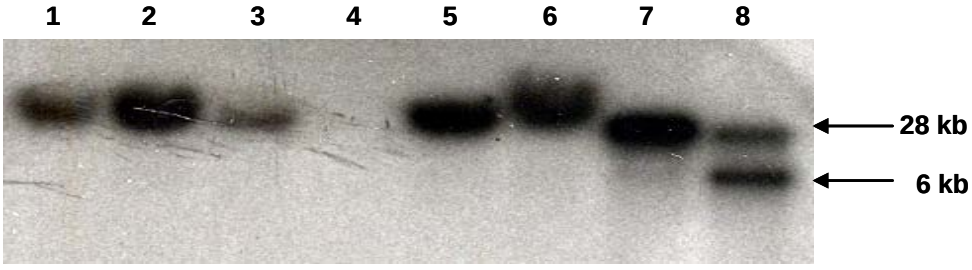


Figure 15: Methylated GD AdV DNA does not replicate after infection of mammalian cells *in vitro*. Cells were transduced with methylated GD AdV and incubated for 48 h, if not stated otherwise. DNA of samples was Hirt-extracted, *Xho*I-digested and subjected to Southern protocol with hybridising probes detecting hFIX and ITR/Ψ. Sk-Hep cells transduced at MOI 2000 for 96 h (1), Sk-Hep cells transduced at MOI 3000 (2), 293 cells transduced at MOI 2000 (3), 293 cells transduced at MOI 100 (4), Huh7 cells transduced at MOI 2000 (5), Hepa 1A cells transduced at MOI 2000 (6). CsCl-purified methylated GD AdV (7), CsCl-purified, un-methylated GD AdV (8). All samples were equally treated with *Xho*I. GD AdV genomes running at 28 kb represent full length DNA. A band at 6 kb indicates cleavage by *Xho*I.

1.5. The replicative state of GD AdV genomes in vivo

To analyse the replicative state of GD AdV in murine liver C57Bl/6 mice were chosen because of known long-term transgene expression and genome persistence in this mouse strain (Ehrhardt and Kay 2002; Belalcazar *et al.* 2003). C57Bl/6 mice (n=7) were injected via the tail vein with $7.5x10^9$ viral particles of methylated GD AdV hFIX. Two control mice were mock-infected and received PBS without viral particles. Moreover, to analyse the replicative state of GD AdV in proliferating liver tissue three mice were treated intraperitoneally with carbon tetrachloride (CCl_4). CCl_4 is a liver-toxic substance and leads to death of affected hepatocytes by necrosis (Weber *et al.* 2003). As a result, the volume of the liver and number of hepatocytes will be reduced. The amount of CCl_4 applied (50 µl) leads to necrosis of at least 70% of total hepatocytes (Das *et al.* 2007). Therefore, CCl_4-treated animals reconstitute the normal liver size by mitosis of (GD AdV-transduced) hepatocytes. With this procedure the question was addressed whether putative mammalian origins of replication within the GD Ad vector genome can be activated by host cell proliferation. An overview over the experimental procedure of the *in vivo* experiment is shown in [**figure 16**].

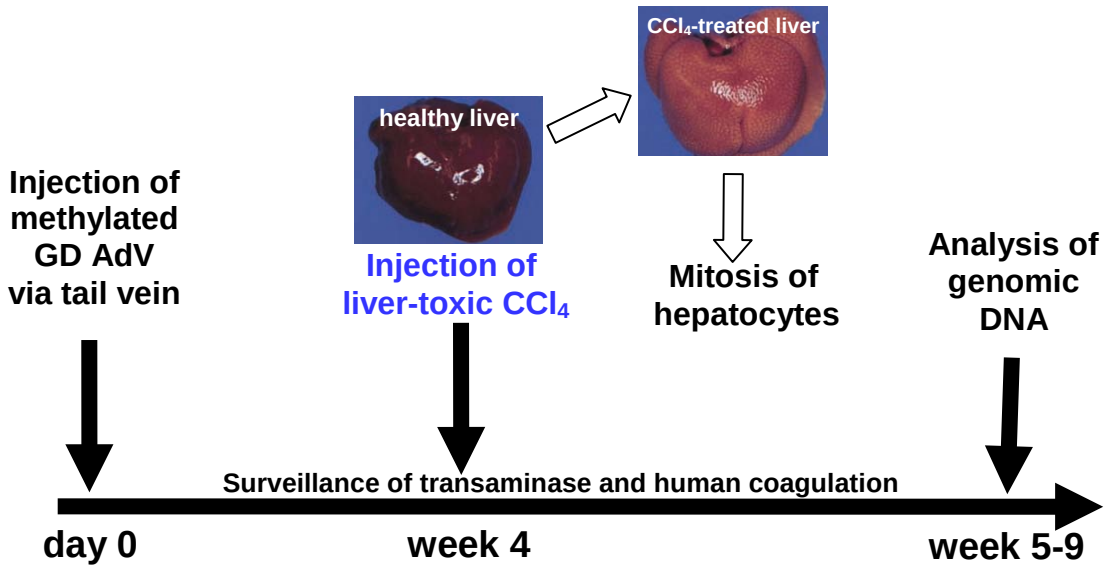


Figure 16: Experimental procedure for the analysis of the replicative state of GD AdV genomes in murine liver. The two photographs were reprinted from Bezerra *et al.* 1999 with modification.

All three groups (GD AdV-, GD AdV + CCl_4-treated and control) were monitored for human blood coagulation factor IX (hFIX) transgene expression and alanine transferase (ALT) levels (Ozer *et al.* 2007) as indicator of liver toxicity for up to 9 weeks p.i. [**figure 17 and figure 18**]. After an initial peak of ALT levels on the first day p.i., these liver enzyme

levels stayed within normal range (9-30 U/l) in all non-CCl_4-treated animals. CCl_4-treated mice showed very high levels of serum transaminase (up to 800 U/l) after drug administration which declined to normal range within one week [**figure 17**]. By 9 weeks p.i. murine livers were collected to extract genomic and viral DNA. At the time of liver resection, the morphology of CCl_4-treated livers was pale and abnormal in residual areas, the size of the liver was normal (data not shown). Livers of CCl_4-untreated mice looked normal.

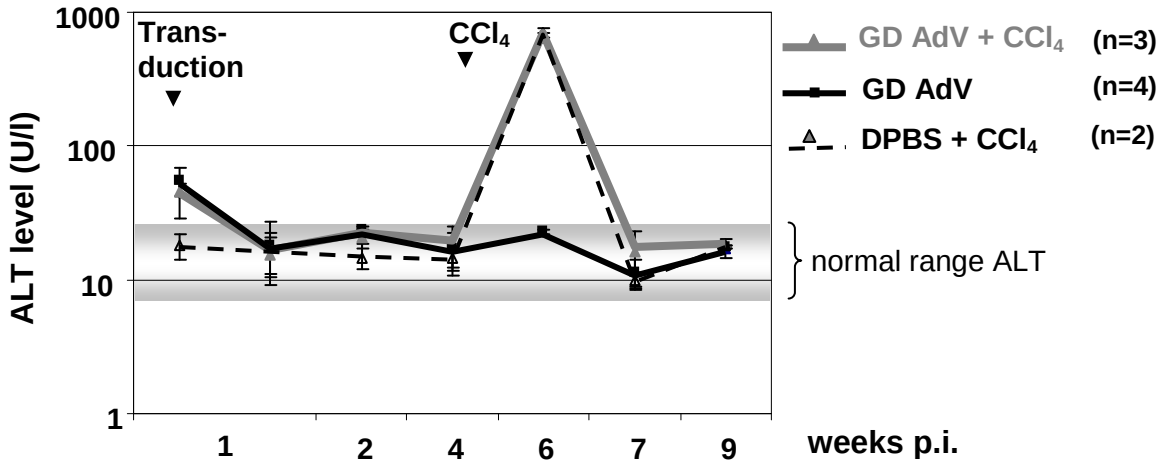


Figure 17: Surveillance of liver alanine transaminase levels for determination of liver toxicity. Alanine transaminase (ALT) levels were determined in murine serum using a commercially available assay (Randox). (U/l) = units per litre; (p.i.) = post infection; (CCl_4) = carbon tetrachloride.

Transgene expression was determined using ELISA (Kay *et al.* 1995). Transgene expression levels before and after CCl_4 administration and at the end of the experiment were of particular interest. Within the first days after GD AdV transduction, high levels (up to 8000 ng/ml) of the human blood coagulation factor IX were detectable [**figure 18**]. These initial levels increased during the next weeks, reaching supra-physiological hFIX levels (>10000 ng/ml). Physiological hFIX levels are ranging from 3000 to 5000 ng/ml. After CCl_4-treatment a drop of 90% in hFIX levels was seen which stabilised at a lower level (about 1000 ng/ml) afterwards. This result is in concordance with earlier experiments (Yant *et al.* 2002). hFIX expression in non-CCl_4-treated mice stayed stable throughout the experiment.

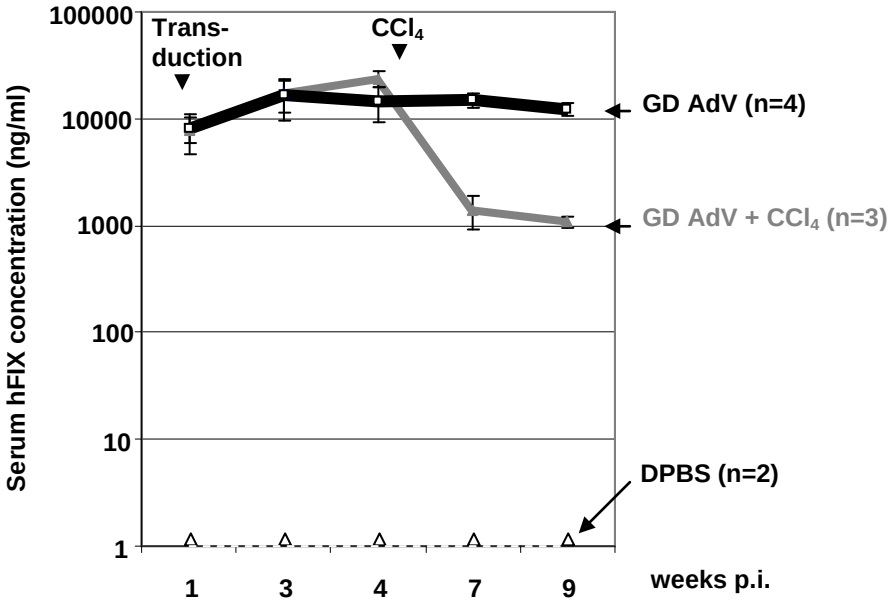


Figure 18: Human blood coagulation factor IX (hFIX) expression profile in C57Bl/6 mice. Serum levels of hFIX were determined analysing murine serum by ELISA. Stable transgene expression was achieved throughout the experiment in mice treated with GD AdV alone (black graph). Note the decrease in hFIX levels after CCl_4 administration (grey graph). As expected, mock injected mice did not produce human factor IX (DPBS, dashed graph). (p.i.) = post infection; (CCl_4) = carbon tetrachloride; (DPBS) = Dulbecco's phosphate buffered saline.

1.5.1 Investigation of the replicative state of GD AdV genomes in vivo *by conventional PCR*

First, a conventional PCR was established to evaluate the presence of a specific *Xho*I recognition site within the GD AdV genome. Purified DNA of CsCl-isolated and disintegrated particles ($1.5x10^7$) was used as templates for PCR. The expected PCR fragment (800 bp) was then isolated from the gel, purified and subjected to *Xho*I digestion. The correctness of the PCR-fragment and the presence of the *Xho*I recognition site were shown by bands of 300 bp and 500 bp after digestion [**figure 19**].

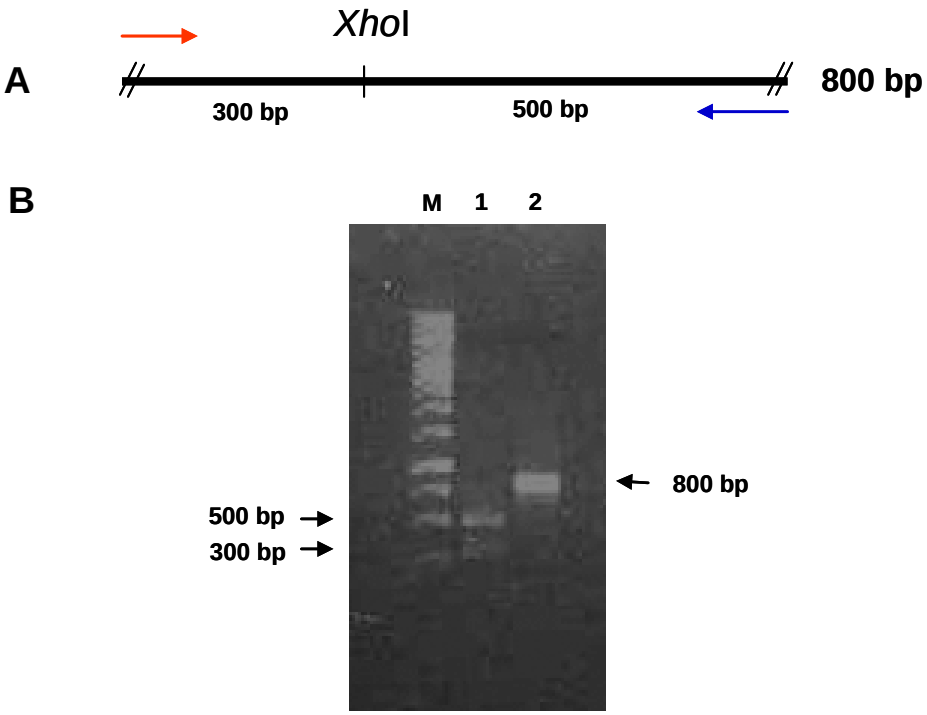


Figure 19: Establishment of a conventional PCR for detection of methylated and unmethylated *Xho*I recognition sites. A: Schematic overview over the GD AdV DNA fragment amplified. The oligonucleotides (red arrow: For-1700 and blue arrow: U6Rev775) and the *Xho*I recognition site are depicted. **B:** Agarose gel after PCR and digestion of PCR products. (1): The PCR product after digestion with *Xho*I. The full length fragment (800 bp) was cleaved into fragments of 500 and 300 bp. (2): Full length PCR fragment (800 bp). (M) = molecular size marker.

Next, this PCR was performed with isolated and purified liver DNA of untransduced and transduced mice. Five hundred pg of total liver DNA were used for PCR before and after *Xho*I digest. If there was replication of GD AdV genomes, the PCR after *Xho*I cleavage should be negative. However, in all transduced mice (n=8), a specific PCR product was seen before and after *Xho*I treatment [**figure 20**]. Comparing the intensity of PCR bands before and after *Xho*I digestion, equal amounts of GD AdV DNA were used as starting material for the PCR The unmethylated control, the plasmid pAdFTC GD hFIX (500 pg) was cleaved by *Xho*I, showing reduced PCR efficacy. The uninfected control mice (no. 9 and 10) were negative for vector DNA. This finding suggests that GD AdV DNA was not replicating in quiescent and proliferating murine liver tissue.

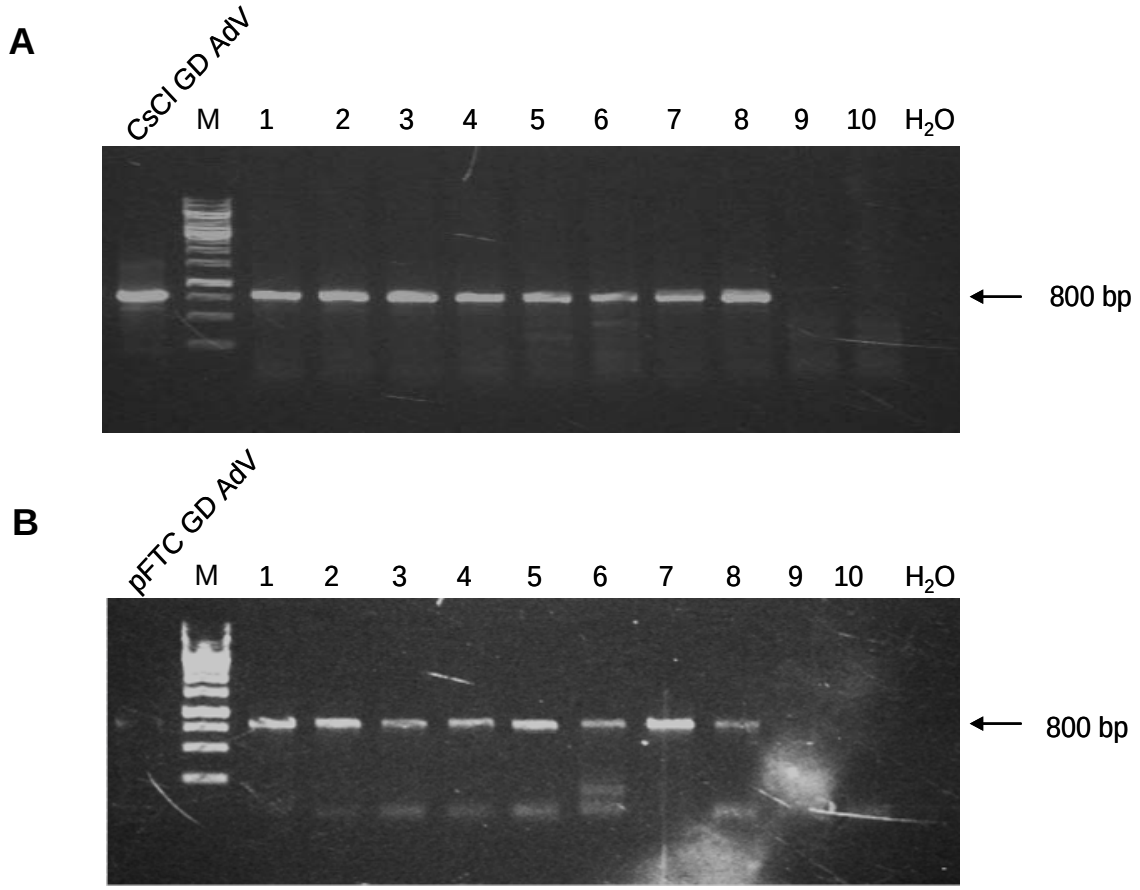


Figure 20: Conventional PCR of murine hepatic DNA before and after *Xho*I digest. A: PCR before *Xho*I digest. All transduced mice (1 to 8) show a positive signal for GD AdV DNA (800 bp), as expected. DNA of un-transduced control mice generated no signal. CsCl purified GD AdV genomes (CsCl GD AdV) serve as positive control in this experiment. **B:** PCR after *Xho*I digest. All transduced mice (1 to 8) show - still - a positive signal for GD AdV DNA (800 bp), demonstrating ineffective *Xho*I cleavage resulting from maintained methylation. The un-methylated plasmid pAdFTC GD AdV, however, was cleaved by *Xho*I resulting in a strongly diminished PCR signal. The weak signals below the 800 bp fragments result probably from unused or dimeric oligonucleotides. (M) = molecular size marker.

1.5.2 Analysis of the replicative state of GD AdV genomes in murine liver by real-time PCR

To detect low amounts of viral DNA that might have lost methylation due to replication, a real-time PCR assay with oligonucleotides that anneal around the specific *Xho*I site within the GD AdV genome was established [**figure 21**]. Genomic liver DNA was treated with *Xho*I and, as a control, with equal amounts of *Nco*I. *Nco*I cleaves GD AdV DNA at arbitrary positions but not between the RT-PCR oligo annealing sites detecting *Xho*I-cleavage.

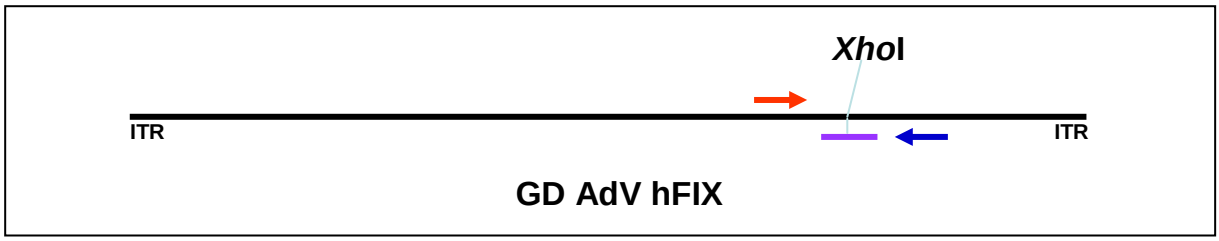


Figure 21: Schematic outline of the real-time PCR experiment for detection of replicated and unreplicated GD AdV genomes. The GD AdV hFIX genome is shown in black. The oligonucleotides TaqFor (red) and TaqRev (blue) as well as the specific FAM-TAMRA-labelled probe (pink) are indicated. The *Xho*I recognition site is located between the annealing sites of the oligonucleotides. The amplified PCR fragment is 102 bp in size. *Nco*I sites are situated at arbitrary positions but of course not between the RT-PCR annealing sites detecting *Xho*I-cleavage (not shown).

To confirm that the methylase/endonuclease-based system is applicable to real-time PCR, replication kinetics were performed. 293 cells were transduced with methylated GD AdV at MOI 100 and helper virus at an MOI of 10. Helper virus co-infection promotes replication of GD AdVs. The double-transduced cells were harvested at the time points indicated, DNA was isolated via Hirt protocol, the samples were digested with *Xho*I or *Nco*I and the described real-time PCR was applied [**figure 22**]. At 1 h and 6 h post transduction the methylated adenoviral input DNA was re-isolated. No difference between *Xho*I and *Nco*I digest was detectable at the early time points. At 12 h p.i. replication and de-methylation of GD AdV had partly begun, indicated by the shift of the crossing point of the *Xho*I-digested sample to higher PCR-cycle numbers [**figure 22**]. A certain level of methylated input GD AdV DNA was still present. In contrast 24 and 48 h p.i., methylated input GD AdV DNA was no longer detectable after *Xho*I-digest, demonstrating that replication promoted by helper virus proteins had taken place. Also, the total amount of GD AdV DNA within these samples increased, indicated by a shift of the crossing points of the *Nco*I-digested samples to lower PCR cycle numbers. As additional controls, digested unmethylated plasmid DNA (pAdFTC) and CsCl-purified methylated GD AdV DNA were analysed. Plasmid DNA was completely cleaved whereas methylated GD AdV DNA was not. In conclusion, RT-PCR can be used to detect unreplicated, partially replicated and completely replicated de-methylated adenoviral DNA.

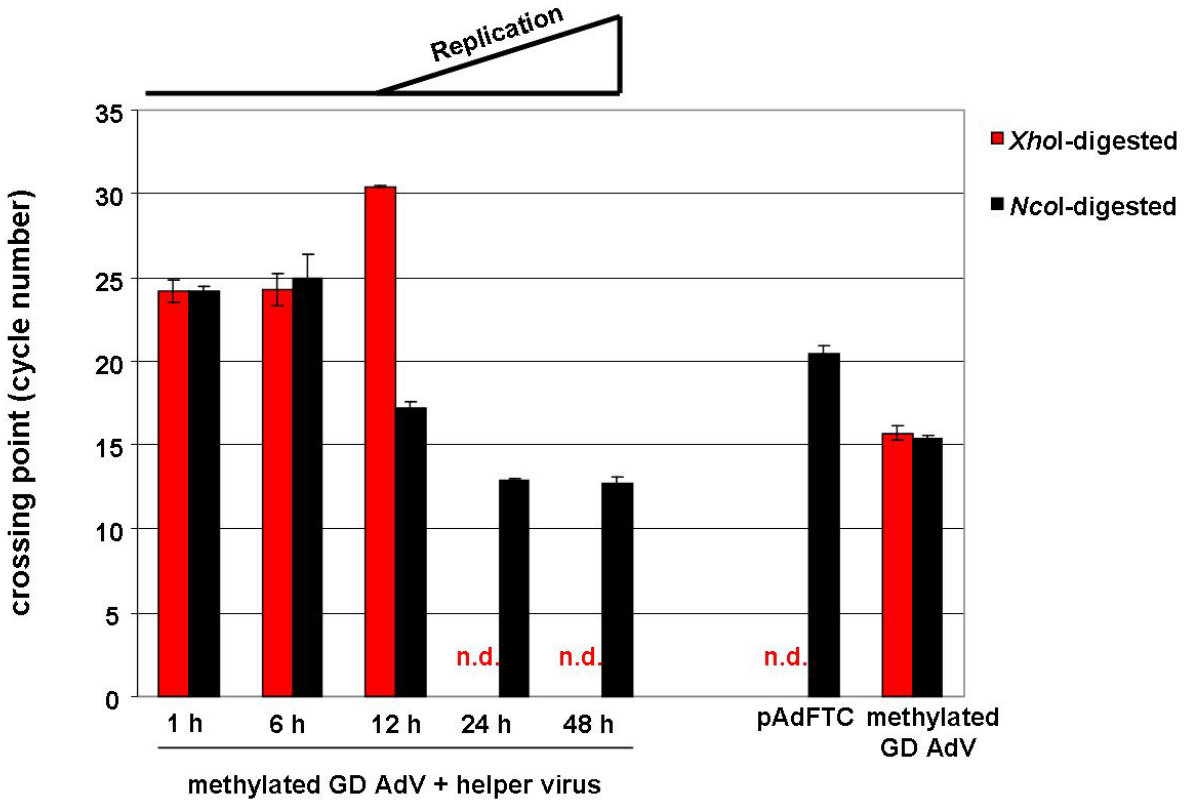


Figure 22: Removal of methylation by replication detected by real-time PCR. Investigated were samples at time points 1 to 48 h after transduction of 293 cells with GD AdV (MOI 100) + helper virus (MOI 10). Samples were harvested at the time points indicated (1 to 48 h), Hirt-extracted and *Xho*I or *Nco*I digested, respectively. 200 ng total DNA were used for real-time PCR analysis. Controls: pAdFTC, an un-methylated plasmid containing the whole GD AdV sequence, CsCl-purified methylated GD AdV DNA. n.d.: not detectable.

Next, the replicative state of GD AdV genomes in murine liver was analysed. In both animal groups, treated with GD AdV + CCl_4 or with GD AdV alone, no difference in *Xho*I-digested and *Nco*I-digested samples was detectable [**figure 23**]. Thus, the methylated input GD AdV genomes did not replicate in quiescent and mitotic hepatocytes. These results indicate that persistence of GD AdV genomes is independent of vector DNA replication.

2.1 Do rAdV form concatemers and/or circles?

After infection of cells with *E4 ORF3/6* double mutants (H5*dl*1004, *ΔE4*) [**figure 24A**], viral genomes are linked to one another at its termini, leading to head-to-head, tail-to-tail and head-to-tail junctions (Weiden and Ginsberg 1994; Sandler and Ketner 1989; Bridge and Ketner 1989; Jayaram and Bridge 2005). To test concatenation of rAdV genomes, pulsed field gel electrophoresis (PFGE) was used to resolve the high molecular viral DNA.

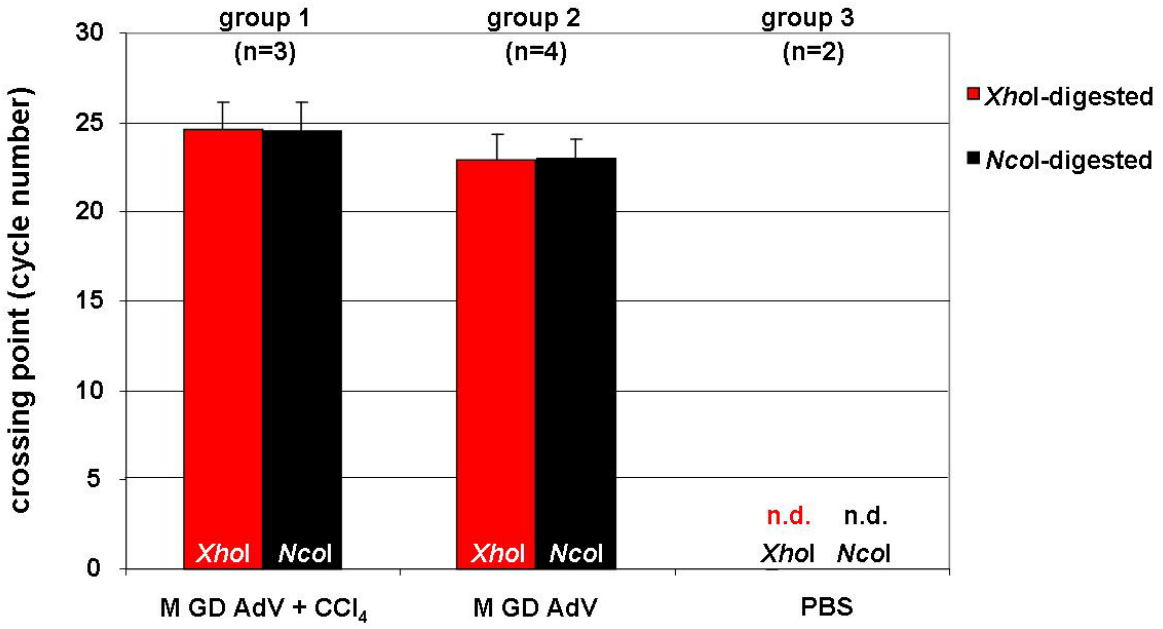


Figure 23: Real-time PCR analysis of the replicative state of GD AdV in murine liver. Total murine liver DNA was isolated and digested with *Xho*I or *Nco*I, respectively. Two hundred ng of total DNA were used for real-time PCR analysis. Group 1: mice receiving methylated (M) GD AdV + CCl_4 (n=3), group 2: mice receiving methylated (M) GD AdV alone (n=4), group 3: mock-infected mice (PBS, n=2). Depicted is the PCR cycle in that the exponential amplification phase begins (crossing point). Mean values +/- standard deviations are shown. n.d.: not detectable. PBS = phosphate buffered saline; CCl_4 = carbon tetrachloride.

2.1.1 Detection of ΔE4 *concatemers using PFGE*

To define the detection limits of this system, dose-dependent studies with the *ΔE4* virus as a positive control for concatemer formation were performed. 293 cells were infected with the *ΔE4* virus at MOIs ranging from 200 to 1. Forty-eight hours p.i. cells were harvested and viral genomes were analysed by PFGE. Southern blot analysis of the pulsed field gel with a radioactively labelled probe specific for the ITR/Ψ sequence of the *ΔE4* virus revealed concatemer formation at high MOIs (100 to 25) and at least dimeric concatemers were detected at an MOI of 6 (**figure 24B**). At the lowest MOIs (3 and 1) only monomeric genomes were detectable. The monomeric genomes as well as the dimeric, trimeric and multimeric concatemers are detectable at 35 kb, 70 kb, 105 kb and as higher molecular bands (x-times 35 kb), respectively (**figure 24B**).

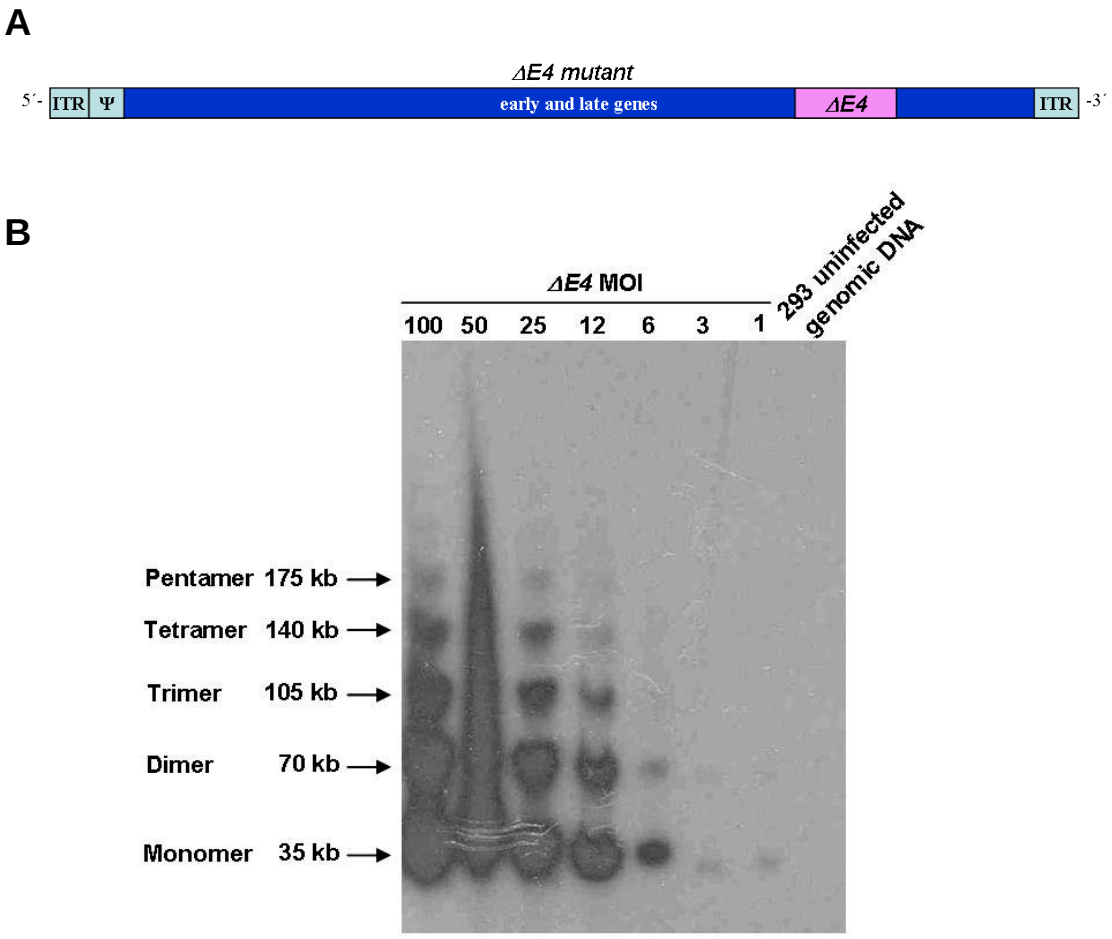


Figure 24: Pulsed field gel electrophoresis (PFGE) to analyse the molecular status of *ΔE4* mutant virus. A: Schematic representation of the genome of *ΔE4* mutant virus. The deletion in the E4 region is indicated. **B:** Sensitivity study for PFGE. Southern-blotted pulsed field gel of 293 cells infected with *ΔE4* virus at MOIs 100 to 1. Forty-eight h p.i. cells were harvested and subjected to the PFGE protocol. Concatemers are detectable down to an MOI of 6. At an MOI of 3 and 1 no concatemers are detectable. Negative control: total genomic DNA of uninfected 293 cells.

2.1.2 Analysis of concatemer formation of GD AdV in PFGE

To test whether GD AdV form concatemers several cell lines were infected with GD AdV hFIX [**figure 25A**]. HEK 293 cells, HeLa cells and hepatic cell lines (Sk-Hep, Huh7, murine Hepa 1A) were infected at different MOIs (MOI 100 to 15000) and harvested at varying time points (16 h to 72 h). PFGE was used to resolve the high molecular viral DNA of GD AdV genomes. After Southern transfer and hybridisation of the blotted membranes with a ITR/Ψ- and hFIX-specific probe, only monomers (ca. 30 kb) and no concatemeric formation of GD AdV genomes were detectable [**figure 25B**]. Even at high MOIs (3600 and 15000) no concatemer formation was observed. This result was independent of the multiplicity of infection (MOI), the cell line and the time period of incubation (only

experiments with high MOIs and an incubation period longer than 20 h are shown). As a positive control DNA of *ΔE4* mutant virus (H5*dl*1004) [**figure 24**] that shows concatenation was used. In contrast to *ΔE4* virus but similar to GD AdV, a single, monomeric band running at 36 kb for wild type adenovirus was detected [**figure 25B**].

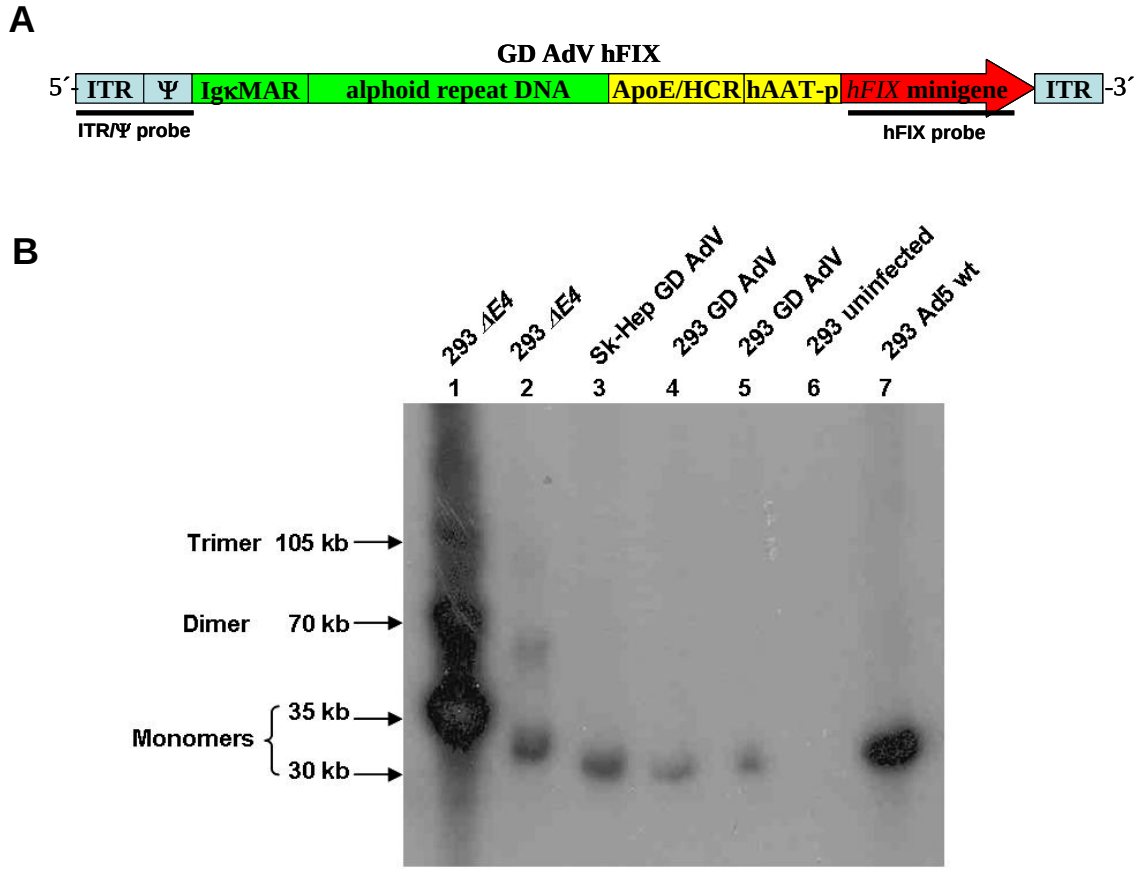


Figure 25: Pulsed field gel electrophoresis to analyse the molecular status of GD AdV hFIX and Ad5 wild type. A: Schematic representation of the GD AdV hFIX genome. The liver-specific hFIX expression cassette and stuffer DNA elements are shown. Additionally, the ITR/Ψ and hFIX probes used for Southern detection are indicated. **B:** GD adenoviral vector genomes do not form concatemers. Cell lines were infected with GD adenoviral vectors for up to 72 h. 293 cells infected with *ΔE4* virus at an MOI of 400 for 48 h (1), 293 cells infected with *ΔE4* virus at an MOI of 200 for 24 h (2), Sk-Hep cells transduced with GD AdV at an MOI of 10000 for 48 h (3), 293 cells transduced with GD AdV at an MOI of 10000 for 20 h (4), 293 cells transduced with GD AdV at an MOI of 15000 for 20 h (5), genomic DNA of uninfected 293 cells (6), 293 cells infected with Ad5 wt at an MOI of 100 for 40 h (7). After pulsed field gel electrophoresis the GD AdV genomes were detected in a monomeric conformation as bands of 28 kb. The monomeric *wt* genome is observable as a prominent band of 35 kb (7). The positive control was provided by the *ΔE4* mutant (H5*dl*1004) that showed multimeric genome concatenation at 35, 70 and 105 kb (1 and 2) in HEK 293 cells. As an additional control uninfected 293 cells were used (6).

2.1.3 Discrimination between GD AdV genomes and helper virus genomes

Usually preparations of GD AdV contain approximately 0.02 to 0.1% helper virus contamination (Palmer and Ng 2003; Puntel *et al.* 2007). Since the results from the hybridisation with the terminal probe do not distinguish between helper virus genomes and GD AdV genomes and the MOIs used are high, GD AdV genomes were specifically detected, using an hFIX-specific probe that detects only GD hFIX vector DNA [**figure 26A, cf. figure 25A**]. After stripping of the membrane, hybridisation was performed with the ITR probe, visualising *ΔE4* concatemers, Ad5 wt genomes and GD AdV hFIX (+ helper virus) genomes [**figure 26B**]. It was found that the amount of isolated DNA is similar comparing *ΔE4*, Ad5 wt and GD AdV genomes. Furthermore, the bands of the hFIX and ITR probes show similar intensity, suggesting that contamination with helper virus is low.

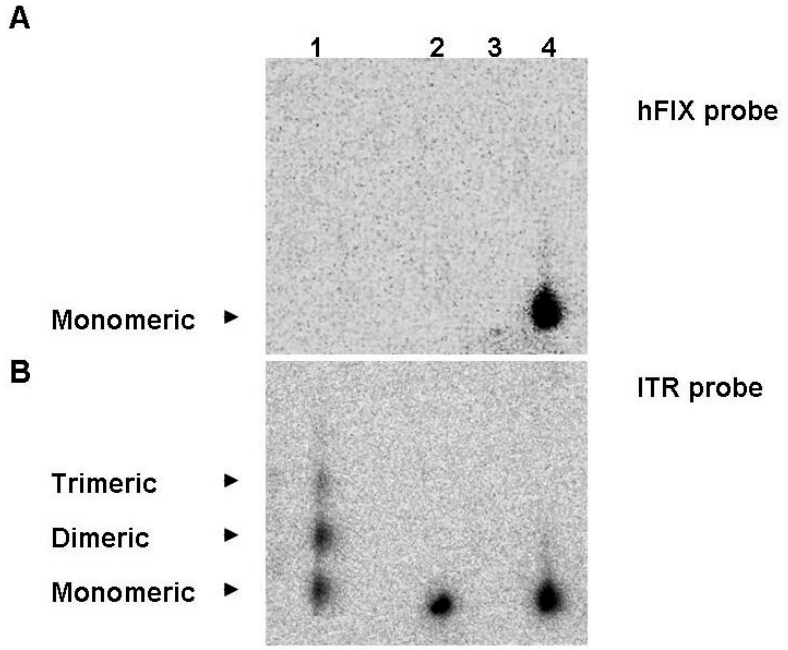


Figure 26: Discrimination between GD AdV, Ad5 wt and *ΔE4* genomes. The same Southern-blotted gel was hybridised with probes against hFIX (GD hFIX-specific, **A**) and ITR (**B**), respectively. 293 cells infected with *ΔE4* at MOI 100 for 24 h (1). 293 cells infected with Ad5 wt at MOI 100 for 24 h (2). Genomic DNA of 293 cells (3). 293 cells infected with GD AdV at MOI 10000 for 24 h (4). Note that the total amount of isolated DNA is similar comparing *ΔE4*, Ad5 wt and GD AdV genomes. Furthermore, the bands of the hFIX and ITR probes show similar intensity, suggesting that contamination with helper virus is low.

2.2 PCR assay for detection of linked adenoviral vector genomes.

To investigate the potential formation of concatemers in more sensitive way and to analyse the possibility of circularisation of recombinant adenoviral vector genomes, a PCR

specific for concatenated and circular genome conformations was developed. Using this approach, head-to-tail concatemers, head-to-tail circular monomers, tail-to-tail or head-to-head concatemers can be detected. If neither concatemers nor circular monomers are present, no PCR-product is generated [**figure 27**].

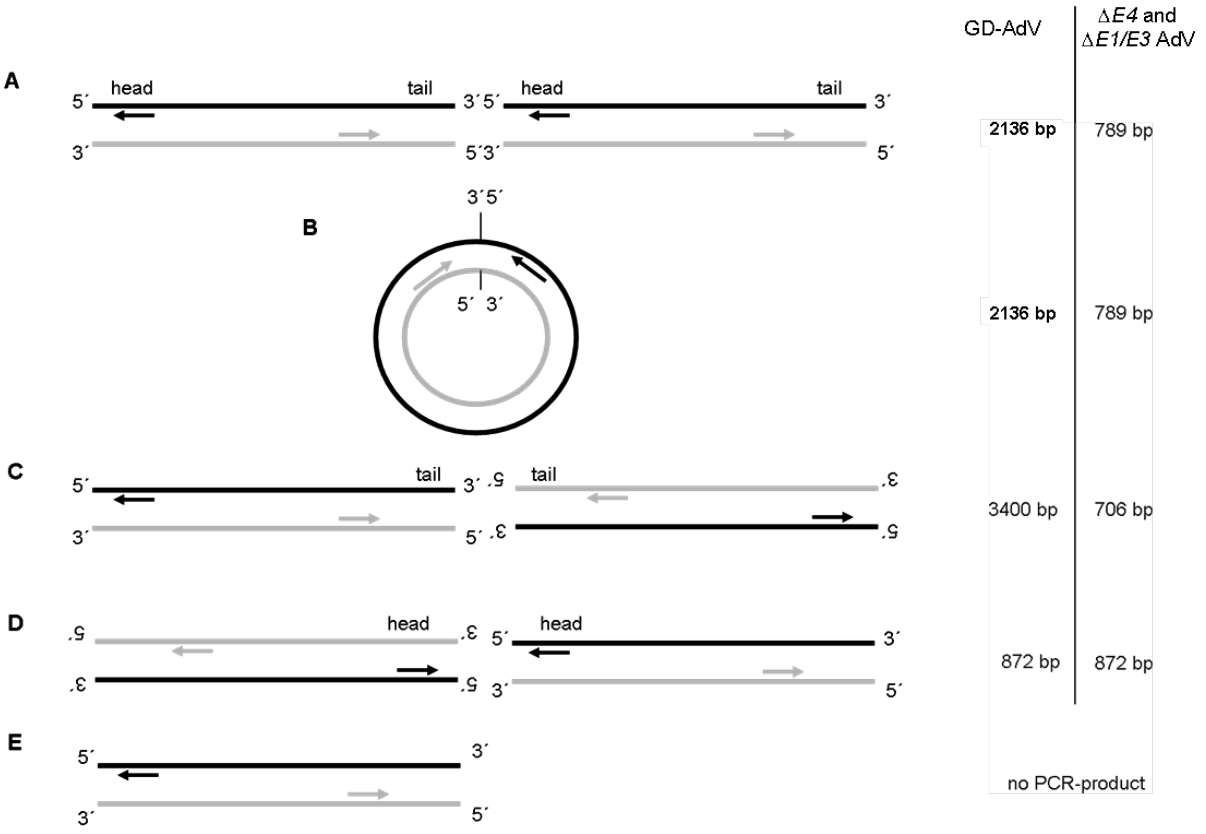


Figure 27: PCR design for detection of concatemers and circular monomers of gene-deleted adenoviral vector genomes, *ΔE4* as well as 1st generation vector *(ΔE1/E3)* genomes. Two oligonucleotides that are specific for the 5´-end of adenoviral DNA (Rev436, black arrow) and the 3´-end of the GD AdV genome (For-1700 or H5dl1004For35585Circ, grey arrow) result in products of indicated sizes and detect head-to-tail concatemers or head-to-tail circular monomers, respectively (**A** and **B**). Tail-to-tail or head-to-head concatemers can be detected with a PCR using forward primer (grey) or reverse primer (black) alone. The respective product sizes are individually indicated (**C** and **D**). If neither concatemers nor circular monomers are present, no PCR-product is generated, as drafted in (**E**).

2.2.1 Determination of the detection limits of this PCR system

To define the detection limits of this PCR, dose-dependent studies with *ΔE4* virus were performed. 293 cells were infected with this virus at MOIs ranging from 200 to 1 [**cf. figure 24B**]. For the PCR, DNA was isolated by Hirt extraction. After PCR a smear of differently joined concatemers was detected starting at an MOI of 3 [**figure 28A**]. Therefore, the PCR assay was more sensitive than the PFGE [**cf. figure 24B**]. At high MOIs (best visible at MOI

200) in addition to the smear two bands were visible indicating that predominant molecular forms of linkage of recombinant viral genome ends may exist.

2.2.2 Analysis of **ΔE4** *concatemers obtained by PCR*

To prove that the smear obtained in the PCR above [**figure 28A**] consists of differently joined *ΔE4* concatemers, the resulting DNA fragments were cloned into the pCRBluntIITopo plasmid (Invitrogen). Subsequently, 15 clones were selected, plasmids were isolated and the inserted DNA fragment was analysed by DNA-sequencing. It was found that each sub-clone was *ΔE4*-derived and showed a different sequence at its concatemeric junctions with large (up to 750 bp) or small deletions (down to 70 bp) in the ITR and/or Ψ regions [**figure 28B]**. Thus, it was concluded that the smear on the gel consists of a variety of differently joined *ΔE4* concatemers.

2.2.3 Sensitivity assay for concatemer and control-of-infection PCRs

To determine the minimal amount of concatemeric DNA necessary for detection, the Hirt-extracted sample of 293 cells infected with *ΔE4* at MOI of 3 [**cf. figure 28A**] was serially diluted. Subsequently, the concatemer and control-of-infection-PCRs were performed. The detection of linear genomes was possible to a 10^{-5} dilution (4.2 pg). The concatemer PCR was 10 fold less sensitive and a signal was detectable to a 10^{-4} dilution (42 pg) [**figure 29A**]. Furthermore, the number of *ΔE4* genomes present in the samples was determined by qPCR. It was found that 237 copies (in the 10^{-5} dilution) generate a signal in the linear control-of-infection PCR. 2730 copies (in the 10^{-4} dilution) are needed to detect concatemers [**figure 29B**].

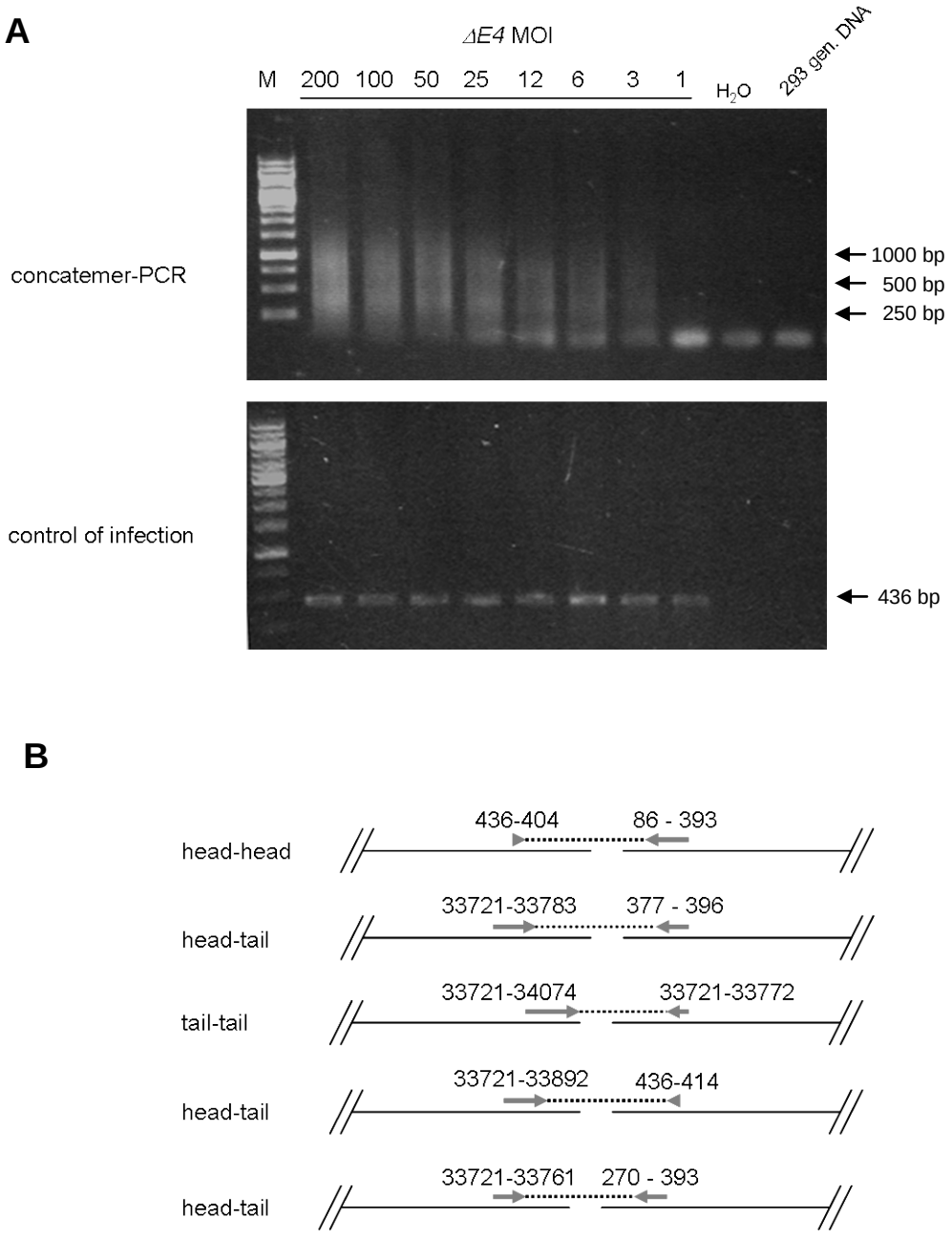


Figure 28: Concatemer detection and molecular characterisation in *ΔE4* mutant virus. A, upper panel: PCR-sensitivity study in *ΔE4* mutant virus. 293 cells were infected with *ΔE4* virus at MOI 200 to 1. 48 h p.i. cells were harvested and subjected to Hirt extraction. PCR with oligonucleotides Rev436 and H5dl1004For reveals concatemers down to MOI 3 on an agarose gel. Controls: H_2O - no template, 293 gen. DNA - genomic DNA of uninfected 293 cells, M = molecular size marker. Bands that are below the smallest marker band (250 bp) represent no PCR product but unused oligonucleotides and oligonucleotide dimers, respectively. **A, lower panel:** Control of viral infection of the samples shown in the upper panel. PCR was performed with oligonucleotides Rev436 and Ad5ITRfw1-24. All virus-infected cells show a specific band at 436 bp. Negative controls: H_2O - no template and genomic DNA of uninfected 293 cells. **B:** Molecular characterisation of the DNA junctions at the ITRs after infection with the *ΔE4* virus. Representative examples are shown. The *ΔE4* genome is schematically drawn as black bar. Concatemeric junctions are shown as grey arrows with its respective position in base pairs. The type of each concatemeric junction is indicated. Deleted regions are represented as dotted line.

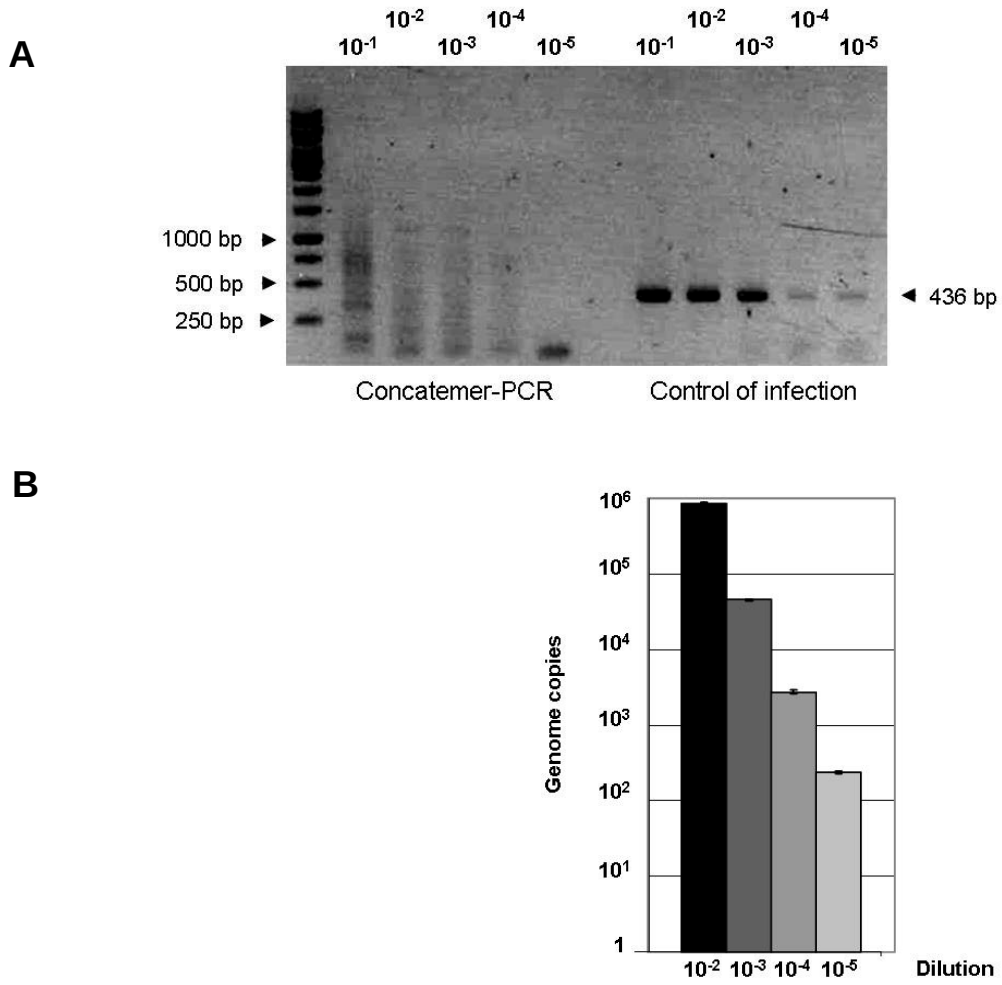


Figure 29: PCR-sensitivity for concatemer detection in *ΔE4* mutants. Serial dilutions (10^{-1} to 10^{-5}) of Hirt-extracted genomic DNA of 293 cells infected with *ΔE4* mutant virus at an MOI of 3 were subjected to PCR. **A, left side:** Concatemer PCR with oligonucleotides Hdl1004circleFor and Rev436 resulting in the typical concatemeric smear. **A, right side:** Control-of-infection-PCR using oligonucleotides Ad5ITRfw1-24 and Rev436 generates a product of 436 bp. **B:** Quantification of the *ΔE4* genome copy number by qPCR. The same samples as in A were used for qPCR with oligonucleotides Ad5ITRfw1-24 and Rev436.

2.3 Investigation of concatemer and circle formation in GD AdV and ΔE1/E3 *AdV genomes in cell culture*

To analyse the molecular status of GD AdV and ΔE1/E3 AdV genomes, HEK 293 cells were infected at an MOI of 1000 and 50, respectively. DNA was isolated and the circle/concatemer-specific PCR was performed. In contrast to the infection control using the *ΔE4* virus, no PCR product was obtained for both, GD AdV and *ΔE1/E3* AdV DNA molecules [**figure 30 and figure 31**].

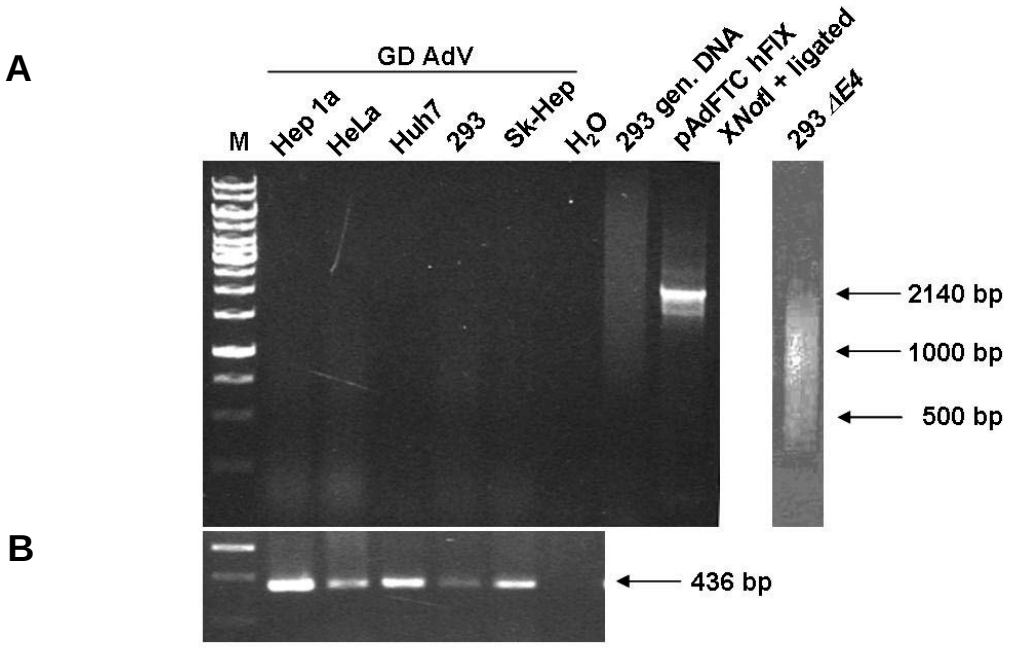


Figure 30: No concatemer formation of GD AdV genomes *in vitro*. A: numerous host cell lines were transduced with GD AdV. Purified DNA was analysed by PCR. Controls: H_2O - no template, 293 gen. DNA – genomic DNA of uninfected 293 cells. Positive circle control: pAdFTC hFIX X*Not*I + ligated - plasmid pAdFTC GD hFIX cut with *Not*I and relegated (see also Appendix 3). Positive control for concatemer formation: 293 ΔE4 - 293 cells infected with ΔE4 mutant virus. **B:** control-of-infection-PCR. Monomeric GD AdV genomes are detectable in all transduced cell lines. (M) = molecular size marker.

To analyse whether concatemer formation is dependent on the infectious dose, HEK 293 cells, HeLa cells, and other hepatic cell lines (Sk-Hep, Huh7, murine Hepa 1A) were infected at an MOI of up to 15000. Low molecular weight DNA was isolated by Hirt extraction and the concatemer/circularisation assay was performed. It was found that, in contrast to the positive controls, no concatenated or circular genome conformations were seen after 72 h of viral incubation *in vitro* (data not shown). In concordance with the PFGE analysis this finding was independent of the cell type used. This finding suggested that linear monomers are the predominant molecular form of GD adenoviral vectors *in vitro*. Altoghether, these results indicate that the incoming recombinant adenoviral DNA molecule was not subject to the double strand break repair (DSBR) machinery of the host cell *in vitro*.

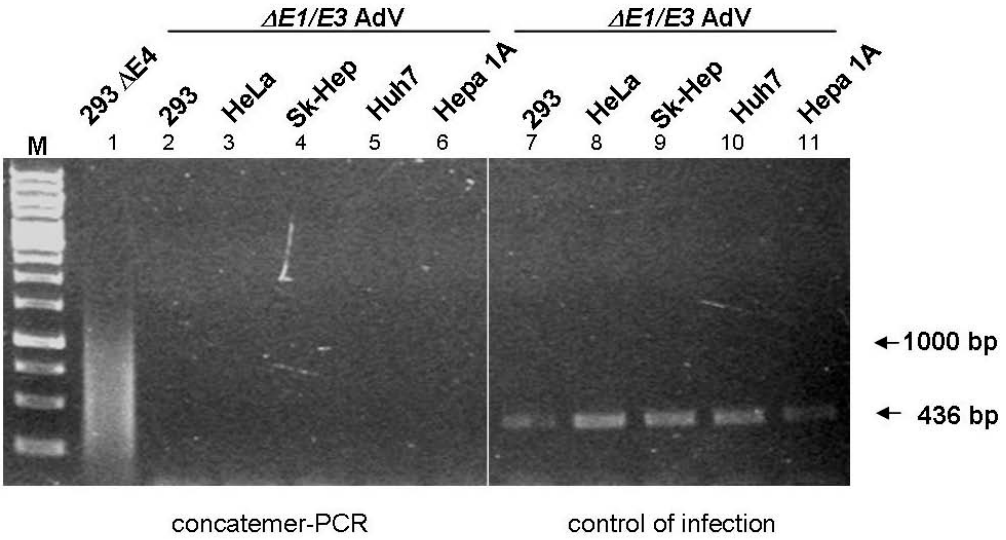


Figure 31: *ΔE1/E3* AdV genomes do not form circles or concatemers *in vitro*. HEK 293 and HeLa cells were infected with 1st generation vector (*ΔE1/E3*) at an MOI of 50, Sk-Hep, Huh7 and Hepa 1A cells were infected with 1st generation vector at an MOI of 1000 for 72 h (lanes 2 - 6). 293 cells infected with *ΔE4* virus at an MOI of 50 serves as positive control for concatemerisation (lane 1). PCR was carried out with oligonucleotides H5dl1004For and Rev436. Samples running on lanes 7 to 11 represent the infection controls for the samples shown on lanes 2 to 6. This control-of-infection-PCR was carried out employing oligos Rev436 and Ad5ITRfw1-24 to detect 1st generation vector DNA as a band of 436 bp. (M) = molecular size marker.

2.4 Analysis of the molecular status of GD AdV genomes in quiescent and cycling cells in vivo.

To analyse whether concatemers and/or circular monomers are formed in murine liver, $7.5x10^9$ transducing units of the GD AdV hFIX were injected into the tail vein of C57Bl/6 mice (n=8). In addition to the examination of the molecular status of viral genomes in quiescent/slowly proliferating hepatic tissue, the question was addressed whether the induction of liver cell proliferation influences the molecular forms of GD AdV DNA. In previous studies it was demonstrated that the expression profile of hepatocytes significantly changes during induction of mitosis (Fukuhara *et al.* 2003). This may lead indirectly to conformational changes of adenoviral DNA molecules. Therefore, carbon tetrachloride (CCl_4) was intraperitoneally administered six weeks after viral infection (n = 3). Mice treated with GD AdV + CCl_4 were killed seven weeks (mouse no. 1) or nine weeks post injection (mouse no. 4 and 5). Another group of mice received the GD AdV (n=5) alone and murine livers were isolated five weeks (no. 2 and 3) and nine weeks (no. 6, 7 and 8) post injection. As a negative control group two mice (no. 9 and 10) were mock-infected with Dulbecco´s phosphate buffered saline (DPBS). Animals were killed at the given time points and total genomic liver

DNA including GD AdV DNA was isolated. This purified DNA was subjected to a PCR protocol as described above to detect concatenation or circularisation of GD AdVs [**figure 27**]. In concordance with the *in vitro* results, no concatenated or circular vector genomes were found in any of the animals transduced with GD AdV [**figure 32A**]. **Figure 32B** shows the infection control PCR. No PCR product was obtained in the negative control group (mouse no. 9 and 10). Thus, the results indicate that linear monomers are the predominant molecular form of GD adenoviral vector genomes also *in vivo*.

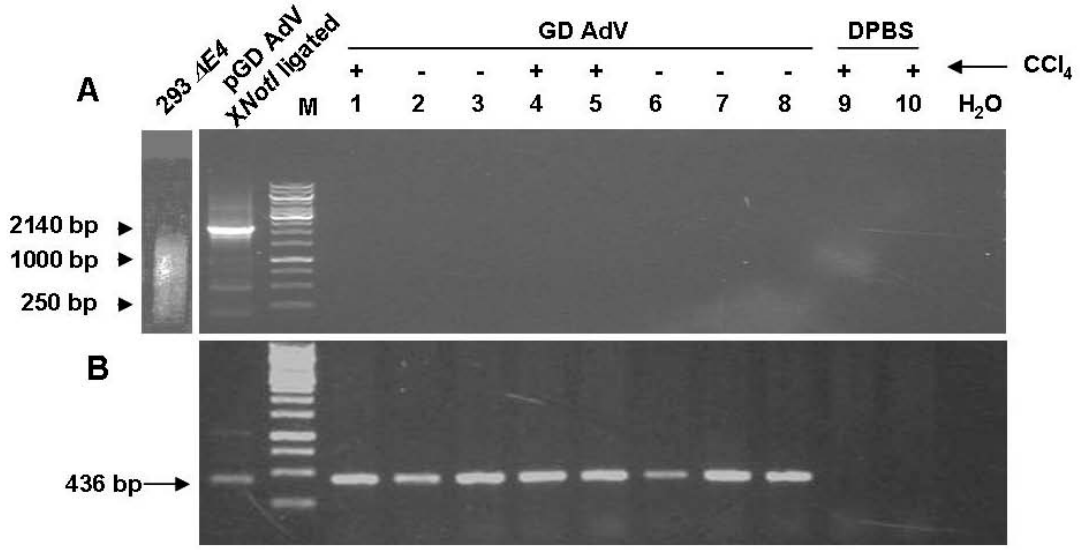


Figure 32: Neither concatemers nor circular monomers of gene-deleted adenoviral vector genomes are detectable by PCR in murine liver. A: C57Bl/6 mice were infected with $7.5x10^9$ transducing units of the GD adenoviral vector (n = 8, lanes 1 to 8). The negative and uninfected control group received PBS alone (lane 9 and 10). After isolation of murine genomic liver DNA, PCR for GD AdV was performed with oligonucleotides Rev436 and For-1700. Re-ligated plasmid pAdFTC and 293 cells infected with *ΔE4* mutant represent the positive controls. M = molecular size marker, H_2O = water control. Treatment with CCl_4 to induce hepatic cell cycling is indicated with (+). **B:** Infection controls for the samples shown in figure 37A. All PCR-reactions were carried out with the oligonucleotide Rev436 (black in figure 26) and an additional oligonucleotide that anneals at the very end of the viral ITR (bp 1-24 Ad5ITRfw1-24). This PCR reveals the presence of viral vector DNA in all infected mice (numbers 1 to 8) resulting in a band of 436 bp. DPBS = Dulbecco's phosphate buffered saline, CCl_4 = carbon tetrachloride.

D Discussion

Recombinant adenoviral vectors (rAdV) are a promising gene therapeutic tool for efficient transduction and long-term transgene expression with limited side effects. We and others (Kreppel *et al.* 2002; Ehrhardt and Kay 2002; Brunetti-Pierri *et al.* 2007; Jozkowicz and Dulak 2005) showed that after injection of a single dose of gene-deleted adenoviral vector (GD AdV) particles the viral genome is maintained. Even after induction of cell cycling of the host cell in murine liver the GD AdV DNA molecule is more stable than non-viral DNA (Ehrhardt *et al.* 2003). Therefore, one part of this study was to reveal the mechanism of persistence of rAdVs. Several mechanisms that potentially could lead to this persistence have been discussed (Jager and Ehrhardt 2007; Kreppel *et al.* 2002; Ehrhardt *et al.* 2003). The rAdV genome may (i) replicate episomally, (ii) form concatemers that were shown to be predominantly responsible for persistent transgene expression in the context of rAAV. Furthermore, (iii) circularisation of rAdV DNA might improve vector DNA maintenance, which was shown for example for members of the *Herpesviridae.* Herpes simplex virus 1 persists in circular conformation in non-dividing host cells without replication (Roizman and Sears 1996). The Epstein-Barr-Virus genome on the other hand, is maintained in a circular conformation in dividing lymphocytes with simultaneous replication (Kieff 1996).

Concatemeric rAdV DNA could be more persistent during host cell mitosis due to a multiplication of stabilising effects of one monomer (centromeric region, matrix attachment region, nuclear retention signals) within one large multimeric molecule. Moreover, if the vector genome replicates as a linear DNA molecule without telomeric regions, concatemers could be more persistent because this large molecule only gradually becomes shorter at its ends after each replication cycle, and therefore, concatemers would have an extended half-life compared to single genomes. Second, circularisation of vector genomes might play an important role for episomally replicating vectors because the difficulties associated with replication of linear DNA would be circumvented.

In the present study these questions were - at least in part – addressed by showing that the formation of concatemers is not responsible for vector genome persistence and transgene expression over a long period of time [**figures 25, 30 and 32**]. Also circularisation of recombinant AdV genomes was excluded [**figure 30 and figure 32**]. Moreover, it was found that GD AdV genomes are defective in replication *in vitro* and in murine liver. This suggests that the listed mechanisms can not explain rAdV genome persistence.

1.1 GD AdV genomes are not replicated in vitro *and* in vivo

The GD AdV contains a putative mammalian origin of replication (ori) within its centromeric region (Krysan *et al.* 1993; Ehrhardt and Kay 2002). We proposed that this mammalian ori could be activated by the host cell replication machinery to prolong the vector's half-life. However, in cell culture studies as well as in replication-activated and quiescent liver tissue no replication of GD AdV genomes was seen.

Cell proliferation of murine hepatocytes was induced by injection of the liver toxic halogenated hydrocarbon CCl_4. It is not possible to monitor the number of removed and subsequently restored hepatocytes. Therefore, the surveillance of alanine transferase (ALT) and human blood coagulation factor IX (hFIX) transgene expression levels was performed as control mechanisms for the animal experiments. The CCl_4 toxicity profile that was compiled in the present study showing transient elevation of ALT levels upon CCl_4 application [**figure 17**], is confirmed by data already published (Das *et al.* 2007; Bezerra *et al.* 1999). At the given dose, at least 70% of affected hepatocytes are removed from the liver and strong cell cycling of the remaining hepatocytes was induced (Das *et al.* 2007). This hepatocellular proliferation should have activated potential origins of replication in the GD AdV genome.
Furthermore, it was reported that the hFIX expression levels drop because of CCl_4-induced hepatic injuries and loss of episomal GD AdV genomes (Yant *et al.* 2002). This finding was in concordance with the present study in which a similar decrease of hFIX expression levels post CCl_4 treatment was detected. This finding further suggests that a significant number of hepatocytes was destroyed. The removed hepatocytes, however, were subsequently replaced by liver regeneration, such that at the ending of the experiment the size of the organ looked normal (data not shown).

GD AdV genome replication would be more effective with circular or concatemeric vector DNA than with a linear monomeric genome. Our studies on circularisation and concatenation, however, showed that GD AdV genomes stay in linear monomeric conformation and argue against episomal replication (Jager and Ehrhardt 2008, submitted).

The real-time PCR assay is sensitive in detecting unmethylated replicated genomes. Yet, one cannot completely exclude that some low level of replication takes place. Another possibility may be that a threshold level is required for replication of GD AdV. This is indicated by the dose-dependent replication of Ad5 wt in Sk-Hep-1 cells was detected [**figure 14**]. At low multiplicity of infection (MOI 100) no replication was detected at high MOI (MOI 3000), however, there was removal of methylation from Ad5 wt genomes. Accordingly, Jayaram and Bridge (2005) demonstrated a severe replication defect of *ΔE4* mutants relative

to Ad5 wt at low MOI (MOI 3) that could partly be rescued by administration of higher MOI (MOI 30).

To control tropism and transduction efficacy of GD AdV hFIX, luciferase life-imaging experiments were performed. GD AdV luc transduced the murine liver [**figure 7**]. Although this is not a direct proof that GD AdV hFIX also transduces the liver with the same efficiency, we suppose that both GD AdV possess similar transduction rates, since both vectors possess identical capsids derived from the same Ad5 helper virus. An efficient transduction rate of hepatocytes using Ad5-derived vectors has previously been reported (Toietta *et al.* 2005; Morral *et al.* 1998; Brunetti-Pierri *et al.* 2006). Furthermore, the administered vector dosages were similar for both vectors (GD AdV hFIX $7.5x10^9$ and GD AdV luc $1x10^{10}$, respectively). Moreover, at the time point of liver resection (5 to 9 weeks p.i.), hFIX expression was high [**figure 18**]. In comparison, the expression of luciferase - driven by the same liver-specific enhancer/promoter construct as hFIX - in week seven was high as well [**figure 7B**]. However, one cannot state that the course of transgene expression and the vector's tropism are identical in the different murine strains (Balb/c or C57Bl/6, respectively) used (Mills *et al.* 2000).

To avoid adverse events *in vivo*, it is useful to apply the minimal vector dose that achieves maximum therapeutic effect. In the *in vivo* experiments using GD AdV hFIX, approximately 75 vector genomes per hepatocyte were applied ($1x10^8$ hepatocytes per murine liver, administered dose $7.5x10^9$ vector particles). This dose, sufficient to transduce the whole liver and to obtain high levels of transgene expression, was well tolerated (Yant *et al.* 2002; **figures 17 and 18**). At this dose GD AdV is replication-inactive [**figures 20 and 23**]. The small percentage of contaminating *ΔE1/E3* helper virus that is copurified during vector preparation (Palmer and Ng 2003, **Appendix figure 2**), does not *in trans* influence replication of the GD AdV DNA molecule in murine liver. Replication of GD AdV genomes driven by helper virus proteins might have been possible because the helper virus provides most genes (besides *E1*) essential for genome replication.

Using a 1st generation vector lacking E1, Nelson and Kay (1997) showed that there are differences in the replication profile of this type of vector *in vitro* and *in vivo*. Notably, their 1st generation vector was able to replicate in human huH7 cells and primary murine and macaque hepatocytes. In the liver of C57Bl/6 mice, the authors did not detect replication. Lieber *et al.* (1997b) speculated that low amounts of E2 proteins produced by 1st generation vectors are necessary for stabilising adenoviral mini-vectors that are only 9 kb in size and lack all viral coding sequences. In contrast, Hodges *et al.* (2000) reported that lack of E2 proteins which renders the virus replication deficient, did not affect DNA maintenance of a 2nd

generation vector. Since this 2nd generation adenoviral vector was found to persist for several months despite the lack of its replication machinery, these findings support the data of the present study implying that replication is not essential for vector genome persistence. Therefore, other features like centromeric regions, nuclear retention signals and/or MARs have to account for episomal GD AdV genome maintenance.

In summary, it was demonstrated that input GD AdV genomes persist without replication *in vitro* and *in vivo*. This finding adds to the already improved safety profile of GD AdV compared to earlier vector generations.

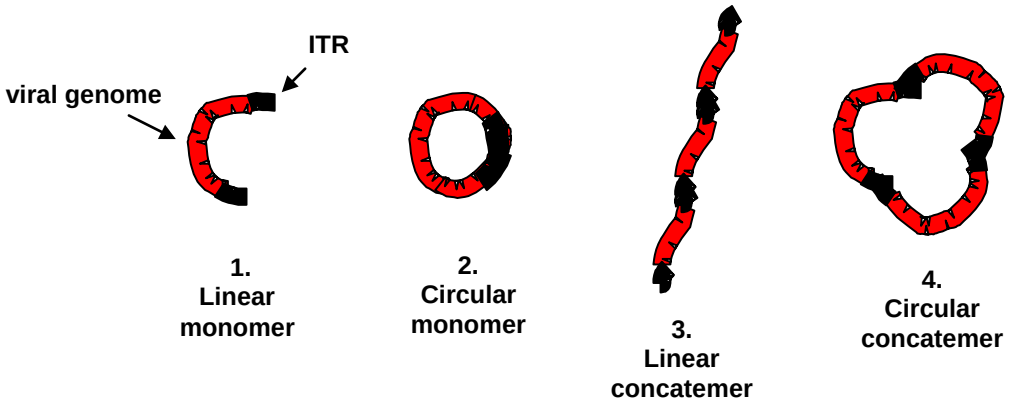
1.2 rAdV genomes do not form concatemers or circles

The infection with Ad exposes the host cell to exogenous linear dsDNA and leads to the activation of cellular double strand break repair (DSBR) proteins that normally respond to damaged chromosomal DNA (Boyer *et al.* 1999). The proteins E4 34 kDa and E4 11 kDa prevent the DSBR system from "repairing" the viral genome by marking the DSBR proteins of the Mre11/Rad50/Nbs1 complex for degradation (Stracker *et al.* 2002). Therefore, the genomes of *ΔE4* mutants are connected to one another during the viral replication process leading to multimeric chains of adenoviral DNA.

It was found in the present study that first generation *ΔE1/E3* AdV recombinant adenovirus lacking the early genes *E1* and *E3* end up as linear monomers in the host cell. This observation might be due to sufficient levels of E4 proteins expressed from the *ΔE1/E3* AdV virus genome inhibiting the DSBR machinery in transduced cells. Because concatemers were not detected, the hypothesis that GD AdV genomes are affected by the DSBR system like *ΔE4* mutant virus could not be confirmed. This finding raises the question, if other proteins involved in the concatemer formation are provided by *ΔE4* mutants but not by GD AdV. Such proteins – both viral (pTP, Ad-Pol, DBP) and cellular (Oct1, NFI) (de Jong and van der Vliet 1999) – that interact at the ITRs of the adenoviral genome could enhance the recruitment of DSBR proteins to the viral termini. Another possibility might be that an amplification of the viral genome is necessary for activation of DSBR proteins. *ΔE4* mutants can amplify their genome by E1 and E2 proteins and cellular factors. This leads to a very high amount of viral DNA inside the host cell that is subsequently concatenated [**figures 24, 28 and 29**]. Until the present study, it was not known if GD AdV show any replication activity independent of the viral E1 and E2 proteins. Replication might have been possible because the GD AdV used in the present study contains DNA sequences that code for potential mammalian origins of

replication (Krysan *et al.* 1993; Ehrhardt and Kay 2002). However, our results indicate that GD AdV are not able to replicate [**figures 30 and 32**]. The idea that viral replication is a prerequisite for activation of the DSBR system, is also supported by the finding that the induction of the host cell DNA damage response is dependent on Herpes virus replication. Replication defective *Herpes simplex* virus or non-permissive host cells like neurons fail to elicit the cellular DNA repair system (Lilley *et al.* 2005; Shirata *et al.* 2005). This suggests that the formation of concatemers is a more complex process.

A comparative overview over the proposed molecular forms of GD AdV genomes, *ΔE4* adenovirus, *ΔE1/E3* AdV, and wild type adenovirus is provided in **figure 33**. Thus, results presented in this study differ from other gene transfer systems based on linear DNA or recombinant AAV. For these vector systems, linear DNA introduced into the cell provides a substrate for the DSBR system. In this regard, GD AdV that also enters the cell as a linear monomer, seems to be unique because it persists as a linear monomer. Perhaps the TP which is covalently bound to the ITRs or cellular proteins interacting with the ITRs prevent concatemer formation by interactions with the DSBR system.



Adenovirus wild type	**+**	**?/n.d**	**-**	**-**
***ΔE4* mutant**	**+**	**?/n.d**	**+**	**?/n.d.**
***ΔE1/E3* AdV**	**+**	**-**	**-**	**-**
Gene-deleted vector	**+**	**-**	**-**	**-**

Figure 33: Proposed molecular forms of gene-deleted and other adenoviruses. (+) = detectable, (-) = not detectable, (?/n.d.) = unknown/not determined. GD AdV are exclusively present as replication-inactive linear monomers.

Interestingly, the junctions between individual *ΔE4* virus genomes are very diverse [**figure 28**]. This phenomenon can not be explained by homologous recombination (HR) or simple

ligation of vector genome termini. As it is known that non-homologous end joining (NHEJ) leads to random deletions at the site of the DNA junctions (Allen *et al.* 2003), it is likely that NHEJ may represent the predominant mechanism of *ΔE4* concatemer formation. Since *ΔE4* mutants show a severe replication defect compared to wild type Ads at low MOIs (MOI 3) (Jayaram and Bridge 2005), one could speculate that hardly any replication takes place at MOIs lower than 3. Thus, it may have been impossible to detect concatemers in the PCR assay at an MOI of 1. However, the PCR assay is sensitive enough to trace low amounts of concatemers. Therefore, it was concluded that the predominant molecular form of GD AdV is the linear monomer.

ΔE4 mutants show a reduced stability of late mRNA (Halbert *et al.* 1985, Sandler and Ketner 1989). This phenomenon could be explained by a disorganisation of the normal coordination of transcription and export of viral mRNA by genome concatenation. The DNA replication centers in *ΔE4* mutants are unusually large and contain aggregations of the 72 kDa DNA binding protein (Bridge *et al.* 2003). Additionally, this disorganisation could be caused by the loss of a stabilising or transport function of E4 proteins in *ΔE4* mutants. Since GD AdV genomes do not form concatemers and are probably not organised in replication centers, the expression of transgenes from the GD AdV is not negatively affected by aggregate formation. This is important because GD AdV was designed to deliver therapeutic transgenes to correct or attenuate a genetic disease in a long-term manner. Ehrhardt and Kay (2002) demonstrated that the same vector used in the present study is capable to reach supraphysiological transgene expression levels driven by a liver-specific promoter for several months. This finding could be confirmed in the present study [**figure 18**].

One might have speculated that concatemer formation or circularisation of GD AdV genomes is even higher than in *ΔE4* mutants, since in contrast to *ΔE4* mutant-infected cells the normal cell cycle control is not disturbed by GD AdV infection. *ΔE4* mutants express the E1B protein that interferes with p53, a crucial tumour suppressor protein regulating cell cycle progression (Russell 2000). In the GD AdV-infected cell, the p53 protein could arrest the cell cycle between G_1 and S phase. By this arrest the cell gains the opportunity to repair chromosomal double strand breaks and to join GD adenoviral linear DNA. This, however, is not the case.

The results of the present study are not in line with those of Kreppel and Kochanek (2004) who found circular molecules of gene-deleted high-capacity (HC) adenoviral vector. They discovered spontaneous circularisation of the HC vectors without Flp-mediated circularisation. Kreppel and Kochanek (2004) used three different HC vectors which were

found to form circular molecules to a varying degree. The vector encoding EBNA1/*oriP* showed the best results in this respect. Thus, the vector's backbone might have yet unknown significant features that support its circularisation. Our GD AdV apparently does not have such elements.

The positive control for circular GD AdV genomes that was used in the present study was generated by ligation. This resulted in a DNA-fragment of defined size (2136 bp) after PCR [**figure 30**]. The variety of differently joined concatemers of *ΔE4* mutant genomes, however, was detected as a smear consisting of an undefined variety of PCR products, suggesting that NHEJ occured. Perhaps circular conformations of DNA are also generated by NHEJ, leading to a variety of differently connected DNA circles. The circular forms that were reported by Kreppel and Kochanek (2004), however, were apparently generated by ligation or HR, since the authors detected defined PCR fragments of a constant size.

Taken together, it was demonstrated that GD adenoviral vectors are present as linear monomers within the host cell. Thus, the reason for vector genome persistence requires further investigation.

In the future it will be of interest to analyse other potential mechanisms of GD AdV persistence inside the host cell. A centomeric function of the GD AdV genome or a nuclear retention signal might play important roles. We expect that further understanding of GD AdV DNA maintenance will not only contribute to our understanding of the molecular persistence of adenoviral vectors as an important gene therapeutic agent but will also help to improve other viral and non-viral vector systems.

E References

Aiyar A, Tyree C, Sugden B. (1998). The plasmid replicon of EBV consists of multiple cis-acting elements that facilitate DNA synthesis by the cell and a viral maintenance element. *EMBO J.* 17(21):6394-403.

Akusjärvi G. (1993). Proteins with transcription regulatory properties encoded by human adenoviruses. *Trends Microbiol.* 1(5):163-70.

Alba R, Bosch A, Chillon M. (2005). Gutless adenovirus: last-generation adenovirus for gene therapy. *Gene Ther.* 12 Suppl 1:S18-27.

Allen C, Halbrook J, Nickoloff JA. (2003). Interactive competition between homologous recombination and non-homologous end joining. *Mol. Cancer Res.* 1(12):913-20.

Barr D, Tubb J, Ferguson D, Scaria A, Lieber A, Wilson C, Perkins J, Kay MA. (1995). Strain related variations in adenovirally mediated transgene expression from mouse hepatocytes in vivo: comparisons between immunocompetent and immunodeficient inbred strains. *Gene Ther.* 2(2):151-5.

Belalcazar LM, Merched A, Carr B, Oka K, Chen KH, Pastore L, Beaudet A, Chan L. (2003). Long-term stable expression of human apolipoprotein A-I mediated by helper-dependent adenovirus gene transfer inhibits atherosclerosis progression and remodels atherosclerotic plaques in a mouse model of familial hypercholesterolemia. *Circulation* 107(21):2726-32.

Benihoud K, Yeh P, Perricaudet M. (1999). Adenovirus vectors for gene delivery. *Curr. Opin. Biotechnol.* 10(5):440-7.

Bezerra JA, Bugge TH, Melin-Aldana H, Sabla G, Kombrinck KW, Witte DP, Degen JL. (1999). Plasminogen deficiency leads to impaired remodeling after a toxic injury to the liver. *Proc. Natl. Acad. Sci. U. S. A.* 96(26):15143-8.

Boyer J, Rohleder K, Ketner G. (1999). Adenovirus E4 34k and E4 11k inhibit double strand break repair and are physically associated with the cellular DNA-dependent protein kinase. *Virology* 263(2):307-12.

Bridge E, Ketner G. (1989). Redundant control of adenovirus late gene expression by early region 4. *J. Virol.* 63(2):631-8.

Bridge E, Mattsson K, Aspegren A, Sengupta A. (2003). Adenovirus early region 4 promotes the localization of splicing factors and viral RNA in late-phase interchromatin granule clusters. *Virology* 311(1):40-50.

Brooks AR, Harkins RN, Wang P, Qian HS, Liu P, Rubanyi GM. (2004). Transcriptional silencing is associated with extensive methylation of the CMV promoter following adenoviral gene delivery to muscle. *J. Gene Med.* 6(4):395-404.

Brunetti-Pierri N, Palmer DJ, Beaudet AL, Carey KD, Finegold M, Ng P. (2004). Acute toxicity after high-dose systemic injection of helper-dependent adenoviral vectors into nonhuman primates. *Hum. Gene Ther.* 15(1):35-46.

Brunetti-Pierri N, Nichols TC, McCorquodale S, Merricks E, Palmer DJ, Beaudet AL, Ng P. (2005). Sustained phenotypic correction of canine hemophilia B after systemic administration of helper-dependent adenoviral vector. *Hum. Gene Ther.* 16(7):811-20.

Brunetti-Pierri N, Ng T, Iannitti DA, Palmer DJ, Beaudet AL, Finegold MJ, Carey KD, Cioffi WG, Ng P. (2006). Improved hepatic transduction, reduced systemic vector dissemination, and long-term transgene expression by delivering helper-dependent adenoviral vectors into the surgically isolated liver of nonhuman primates. *Hum. Gene Ther.* 17(4):391-404.

Brunetti-Pierri N, Stapleton GE, Palmer DJ, Zuo Y, Mane VP, Finegold MJ, Beaudet AL, Leland MM, Mullins CE, Ng P. (2007). Pseudo-hydrodynamic delivery of helper-dependent adenoviral vectors into non-human primates for liver-directed gene therapy. *Mol. Ther.* 15(4):732-40.

Carpenter DE, Stevens JG. (1996). Long-term expression of a foreign gene from a unique position in the latent herpes simplex virus genome. *Hum. Gene Ther.* 7(12):1447-54.

Chen ZY, He CY, Ehrhardt A, Kay MA. (2003). Minicircle DNA vectors devoid of bacterial DNA result in persistent and high-level transgene expression in vivo. *Mol. Ther.* 8(3):495-500.

Chinnadurai G. (1998). Control of apoptosis by human adenovirus genes. *Semin. Virol.* 8: 399-408.

Chuah MK, Schiedner G, Thorrez L, Brown B, Johnston M, Gillijns V, Hertel S, Van Rooijen N, Lillicrap D, Collen D, VandenDriessche T, Kochanek S. (2003). Therapeutic factor VIII levels and negligible toxicity in mouse and dog models of hemophilia A following gene therapy with high-capacity adenoviral vectors. *Blood* 101(5):1734-43.

Cichocki M, Singer G, Beyerlein S, Zeder SL, Schober P, Höllwarth M. (2008). A case of necrotizing enterocolitis associated with adenovirus infection in a term infant with 22q11 deletion syndrome. *J. Pediatr. Surg.* 43(4):e5-8.

Crespo A, Peydró A, Dasí F, Benet M, Calvete JJ, Revert F, Aliño SF. (2005). Hydrodynamic liver gene transfer mechanism involves transient sinusoidal blood stasis and massive hepatocyte endocytic vesicles. *Gene Ther.* (11):927-35.

Cristiano RJ, Smith LC, Kay MA, Brinkley BR, Woo SL. (1993). Hepatic gene therapy: efficient gene delivery and expression in primary hepatocytes utilizing a conjugated adenovirus-DNA complex. *Proc. Natl. Acad. Sci. U. S. A.* 90(24):11548-52.

Das RK, Hossain SU, Bhattacharya S. (2007). Protective effect of diphenylmethyl selenocyanate against CCl4-induced hepatic injury. *J. Appl. Toxicol.* 27(6):527-37.

de Jong JC, Wermenbol AG, Verweij-Uijterwaal MW, Slaterus KW, Wertheim-Van Dillen P, Van Doornum GJ, Khoo SH, Hierholzer JC. (1999). Adenoviruses from human

immunodeficiency virus-infected individuals, including two strains that represent new candidate serotypes Ad50 and Ad51 of species B1 and D, respectively. *J. Clin. Microbiol.* 37(12):3940-5.

de Jong RN, van der Vliet PC. (1999). Mechanism of DNA replication in eukaryotic cells: cellular host factors stimulating adenovirus DNA replication. *Gene* 236(1):1-12. Review.

Dobbelstein M, Roth J, Kimberly WT, Levine AJ, Shenk T. (1997). Nuclear export of the E1B 55-kDa and E4 34-kDa adenoviral oncoproteins mediated by a rev-like signal sequence. *EMBO J.* 16(14):4276-84.

Dudley RW, Lu Y, Gilbert R, Matecki S, Nalbantoglu J, Petrof BJ, Karpati G. (2004). Sustained improvement of muscle function one year after full-length dystrophin gene transfer into mdx mice by a gutted helper-dependent adenoviral vector. *Hum. Gene Ther.* 15(2):145-56.

Ehrhardt A, Kay MA. (2002). A new adenoviral helper-dependent vector results in long-term therapeutic levels of human coagulation factor IX at low doses in vivo. *Blood* 99(11):3923-30.

Ehrhardt A, Xu H, Kay MA. (2003). Episomal persistence of recombinant adenoviral vector genomes during the cell cycle in vivo. *J. Virol.* 77(13):7689-95.

Ehrhardt A, Haase R, Schepers A, Deutsch MJ, Lipps HJ, Baiker A. (2008). Episomal vectors for gene therapy. *Cur.r Gene Ther.* 8(3):147-61.

Fisher KJ, Choi H, Burda J, Chen SJ, Wilson JM. (1996). Recombinant adenovirus deleted of all viral genes for gene therapy of cystic fibrosis. *Virology* 217(1):11-22.

Fukuhara Y, Hirasawa A, Li XK, Kawasaki M, Fujino M, Funeshima N, Katsuma S, Shiojima S, Yamada M, Okuyama T, Suzuki S, Tsujimoto G. (2003). Gene expression profile in the regenerating rat liver after partial hepatectomy. .*J Hepatol.* 38(6):784-92.

Gabler S, Schütt H, Groitl P, Wolf H, Shenk T, Dobner T. (1998). E1B 55-kilodalton-associated protein: a cellular protein with RNA-binding activity implicated in nucleocytoplasmic transport of adenovirus and cellular mRNAs. *J. Virol.* 72(10):7960-71.

Ganem D. (1996). Hepadnaviridae and their replication. In: *Fields Virology*. Third Edition. Raven publishers, Philadelphia. Chapter 85.

Garnett CT, Erdman D, Xu W, Gooding LR. (2002). Prevalence and quantitation of species C adenovirus DNA in human mucosal lymphocytes. *J. Virol.* 76(21):10608-16.

Gräble M, Hearing P. (1990). Adenovirus type 5 packaging domain is composed of a repeated element that is functionally redundant. *J. Virol.* 64(5):2047-56.

Gräble M, Hearing P. (1992). cis and trans requirements for the selective packaging of adenovirus type 5 DNA. *J. Virol.* 66(2):723-31.

Grave L, Dreyer D, Dieterle A, Leroy P, Michou AI, Doderer C, Pavirani A, Lusky M, Mehtali M. (2000). Differential influence of the E4 adenoviral genes on viral and cellular promoters. *J. Gene Med.* 2(6):433-43.

Gussander E, Adams A. (1984). Electron microscopic evidence for replication of circular Epstein-Barr virus genomes in latently infected Raji cells. *J. Virol.* 52(2):549-56.

Halbert DN, Cutt JR, Shenk T. (1985). Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff. *J. Virol.* 56(1):250-7.

Hammerschmidt W, Sugden B. (2004). Epstein-Barr virus sustains Burkitt's lymphomas and Hodgkin's disease. *Trends Mol. Med.* 10(7):331-6. Review.

Hanahan D. (1983). Studies on transformation of Escherichia coli with plasmids. *J. Mol. Biol.* 166(4):557-80.

Harui A, Suzuki S, Kochanek S, Mitani K. (1999). Frequency and stability of chromosomal integration of adenovirus vectors. *J. Virol.* 73(7):6141-6.

Hay RT, Freeman A, Leith I, Monaghan A, Webster A. (1995). Molecular interactions during adenovirus DNA replication. *Curr. Top. Microbiol. Immunol.* 199 (Pt 2):31-48. Review.

Hearing P, Samulski RJ, Wishart WL, Shenk T. (1987). Identification of a repeated sequence element required for efficient encapsidation of the adenovirus type 5 chromosome. *J. Virol.* 61(8):2555-8.

Hierholzer JC. (1992). Adenoviruses in the immunocompromised host. *Clin. Microbiol. Rev.* 5(3):262-74. Review.

Hodges BL, Serra D, Hu H, Begy CA, Chamberlain JS, Amalfitano A. (2000). Multiply deleted [E1, polymerase-, and pTP-] adenovirus vector persists despite deletion of the preterminal protein. *J. Gene Med.* 2(4):250-9.

Horridge JJ, Leppard KN. (1998). RNA-binding activity of the E1B 55-kilodalton protein from human adenovirus type 5. *J. Virol.* 72(11):9374-9.

Horwitz M. (1996). The adenoviridae and their replication. In: *Fields Virology*. Third Edition. Raven publishers, Philadelphia.

Humar A, Doucette K, Kumar D, Pang XL, Lien D, Jackson K, Preiksaitis J. (2006). Assessment of adenovirus infection in adult lung transplant recipients using molecular surveillance. *J. Heart Lung Transplant.* 25(12):1441-6.

Jager L, Ehrhardt A. (2007). Emerging adenoviral vectors for stable correction of genetic disorders. *Curr. Gene Ther.* 7(4):272-83. Review.

Jager L, Ehrhardt A. (2008). Extrachromosomal linear monomers are responsible for persistence of recombinant adenovirus *in vitro* and *in vivo.* Nucleic Acids Research. Submitted.

Jayaram S, Bridge E. (2005). Genome concatenation contributes to the late gene expression defect of an adenovirus E4 mutant. *Virology* 342(2):286-96.

Jozkowicz A, Dulak J. (2005). Helper-dependent adenoviral vectors in experimental gene therapy. *Acta Biochim. Pol.* 52(3):589-99.

Kay MA, Graham F, Leland F, Woo SL. (1995). Therapeutic serum concentrations of human alpha-1-antitrypsin after adenoviral-mediated gene transfer into mouse hepatocytes. *Hepatology* 21: 815-819.

Kieff E. (1996). Epstein-Barr virus and its replication. In: *Fields Virology*. Third Edition. Raven publishers, Philadelphia. Chapter 74.

Kim IH, Józkowicz A, Piedra PA, Oka K, Chan L. (2001). Lifetime correction of genetic deficiency in mice with a single injection of helper-dependent adenoviral vector.*Proc. Natl. Acad. Sci. U. S. A.* 98(23):13282-7.

Kochanek S, Clemens PR, Mitani K, Chen HH, Chan S, Caskey CT. (1996). A new adenoviral vector: Replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length dystrophin and beta-galactosidase. *Proc. Natl. Acad. Sci. U. S. A.* 93(12):5731-6.

Krätzer F, Rosorius O, Heger P, Hirschmann N, Dobner T, Hauber J, Stauber RH. (2000). The adenovirus type 5 E1B-55K oncoprotein is a highly active shuttle protein and shuttling is independent of E4orf6, p53 and Mdm2. *Oncogene* 19(7):850-7.

Kreppel F, Luther TT, Semkova I, Schraermeyer U, Kochanek S. (2002). Long-term transgene expression in the RPE after gene transfer with a high-capacity adenoviral vector. *Invest. Ophthalmol. Vis. Sci.* 43(6):1965-70.

Kreppel F, Kochanek S. (2004). Long-term transgene expression in proliferating cells mediated by episomally maintained high-capacity adenovirus vectors. *J. Virol.* 78(1):9-22.

Krysan PJ, Smith JG, Calos MP. (1993). Autonomous replication in human cells of multimers of specific human and bacterial DNA sequences. *Mol. Cell Biol.* 13(5):2688-96.

Lee BE, Pang XL, Robinson JL, Bigam D, Monroe SS, Preiksaitis JK. (2008). Chronic norovirus and adenovirus infection in a solid organ transplant recipient. *Pediatr. Infect. Dis. J.* 27(4):360-2.

Lewin B. (1991). Gene, Lehrbuch der molekularen Genetik, Zweite Auflage, VCH, Weinheim, pp. 600-604.

Li JJ, Kelly TJ. (1984). Simian virus 40 DNA replication in vitro. *Proc. Natl. Acad. Sci. U. S. A.* 81(22):6973-7.

Lieber A, He CY, Meuse L, Schowalter D, Kirillova I, Winther B, Kay MA. (1997a). The role of Kupffer cell activation and viral gene expression in early liver toxicity after infusion of recombinant adenovirus vectors. *J. Virol.* 71(11):8798-807.

Lieber A, He CY, Kay MA. (1997b). Adenoviral preterminal protein stabilizes mini-adenoviral genomes in vitro and in vivo. *Nat. Biotechnol.* 15(13):1383-7.

Lilley CE, Carson CT, Muotri AR, Gage FH, Weitzman MD. (2005). DNA repair proteins affect the lifecycle of herpes simplex virus 1. *Proc. Natl. Acad. Sci. U. S. A.* 102(16):5844-9.

Liu F, Song Y, Liu D. (1999). Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther.* 6(7):1258-66.

Lusky M, Christ M, Rittner K, Dieterle A, Dreyer D, Mourot B, Schultz H, Stoeckel F, Pavirani A, Mehtali M. (1998). In vitro and in vivo biology of recombinant adenovirus vectors with E1, E1/E2A, or E1/E4 deleted. *J. Virol.* 72(3):2022-32.

Maizel JV Jr, White DO, Scharff MD. (1968). The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. *Virology* 36(1):115-25.

Matecki S, Dudley RW, Divangahi M, Gilbert R, Nalbantoglu J, Karpati G, Petrof BJ. (2004). Therapeutic gene transfer to dystrophic diaphragm by an adenoviral vector deleted of all viral genes. *Am. J. Physiol. Lung Cell Mol. Physiol.* 287(3):L569-76.

McCormack WM Jr, Seiler MP, Bertin TK, Ubhayakar K, Palmer DJ, Ng P, Nichols TC, Lee B. (2006). Helper-dependent adenoviral gene therapy mediates long-term correction of the clotting defect in the canine hemophilia A model. *J. Thromb. Haemost.* 4(6):1218-25.

McGrath D, Falagas ME, Freeman R, Rohrer R, Fairchild R, Colbach C, Snydman DR. (1998). Adenovirus infection in adult orthotopic liver transplant recipients: incidence and clinical significance. *J. Infect. Dis.* 177(2):459-62.

Miao CH, Snyder RO, Schowalter DB, Patijn GA, Donahue B, Winther B, Kay MA (1998). The kinetics of rAAV integration in the liver. *Nature Genetics* 19, 13 - 15.

Miao CH, Ohashi K, Patijn GA, Meuse L, Ye X, Thompson AR, Kay MA. (2000). Inclusion of the hepatic locus control region, an intron, and untranslated region increases and stabilizes hepatic factor IX gene expression in vivo but not in vitro. *Mol. Ther.* 1(6):522-32.

Michaels MG, Green M,Wald ER, Starzl TE. (1992). Adenovirus infection in pediatric liver transplant recipients. *J. Infect. Dis.* 165: 170–4.

Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM. (2000). M-1/M-2 macrophages and the Th1/Th2 paradigm. *J. Immunol.* 164(12):6166-73.

Mizuguchi H, Kay MA. (1999). A simple method for constructing E1- and E1/E4-deleted recombinant adenoviral vectors. *Hum. Gene Ther.* 10(12):2013-7.

Morral N, Parks RJ, Zhou H, Langston C, Schiedner G, Quinones J, Graham FL, Kochanek S, Beaudet AL. (1998). High doses of a helper-dependent adenoviral vector yield supraphysiological levels of alpha1-antitrypsin with negligible toxicity. *Hum. Gene Ther.* 9(18):2709-16.

Morral N, O'Neal W, Rice K, Leland M, Kaplan J, Piedra PA, Zhou H, Parks RJ, Velji R, Aguilar-Córdova E, Wadsworth S, Graham FL, Kochanek S, Carey KD, Beaudet AL. (1999). Administration of helper-dependent adenoviral vectors and sequential delivery of different vector serotype for long-term liver-directed gene transfer in baboons. *Proc. Natl. Acad. Sci. U. S .A.* 96(22):12816-21.

Nakai H, Yant SR, Storm TA, Fuess S, Meuse L, Kay MA. (2001). Extrachromosomal recombinant adeno-associated virus vector genomes are primarily responsible for stable liver transduction in vivo. *J. Virol.* 75(15):6969-76.

Nelson JE, Kay MA. (1997). Persistence of recombinant adenovirus in vivo is not dependent on vector DNA replication. *J. Virol.* 71(11):8902-7.

Ozer J, Ratner M, Shaw M, Bailey W, Schomaker S. (2008). The current state of serum biomarkers of hepatotoxicity. *Toxicology* 245(3):194-205. Review.

Palmer D, Ng P. (2003). Improved system for helper-dependent adenoviral vector production. *Mol. Ther.* 8(5):846-52.

Parks RJ, Chen L, Anton M, Sankar U, Rudnicki MA, Graham FL. (1996). A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. *Proc. Natl. Acad. Sci. U.S.A.* 93: 13565–13570.

Parks RJ, Bramson JL, Wan Y, Addison CL, Graham FL. (1999). Effects of stuffer DNA on transgene expression from helper-dependent adenovirus vectors. *J. Virol.* 73(10):8027-34.

Perez D and White E. (1998). E1B 19K inhibits Fas-mediated apoptosis through FADD-dependent sequestration of FLICE. *J. Cell Biol.* 141(5):1255-66.

Pfüller R, Hammerschmidt W. (1996). Plasmid-like replicative intermediates of the Epstein-Barr virus lytic origin of DNA replication. *J. Virol.* 70(6):3423-31.

Puntel M, Curtin JF, Zirger JM, Muhammad AK, Xiong W, Liu C, Hu J, Kroeger KM, Czer P, Sciascia S, Mondkar S, Lowenstein PR, Castro MG. (2006). Quantification of high-capacity helper-dependent adenoviral vector genomes in vitro and in vivo, using quantitative TaqMan real-time polymerase chain reaction. *Hum. Gene Ther.* 17(5):531-44.

Ramachandra M, Padmanabhan R. (1995). Expression, nuclear transport, and phosphorylation of adenovirus DNA replication proteins. *Curr. Top. Microbiol. Immunol.* 199 (Pt 2):50-88. Review.

Raper SE, Chirmule N, Lee FS, Wivel NA, Bagg A, Gao GP, Wilson JM, Batshaw ML. (2003). Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol. Genet. Metab.* 80(1-2):148-58.

Rekosh DM, Russell WC, Bellet AJ, Robinson AJ. (1977). Identification of a protein linked to the ends of adenovirus DNA. *Cell* 11: 283±295.

Ritzi M, Tillack K, Gerhardt J, Ott E, Humme S, Kremmer E, Hammerschmidt W, Schepers A. (2003). Complex protein-DNA dynamics at the latent origin of DNA replication of Epstein-Barr virus. *J. Cell Sci.* 116(Pt 19):3971-84.

Roizman B, Sears AE. (1996). Herpes simplex viruses and their replication. In: *Fields Virology*. Third Edition. Raven publishers, Philadelphia. Chapter 72.

Rowe WP, Huebner RJ, Gilmore LK, Parrott RH, Ward TG. (1953). Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proc. So.c Exp. Biol. Med.* 84(3):570-3.

Russell WC. (2000). Update on adenovirus and its vectors. *J. Gen. Virol.* 81(Pt 11):2573-604. Review.

Sambrook J. and Russell DW. (2001). Editors. *Molecular Cloning*, Cold Spring Harbor Laboratory Press, New York, Third Edition.

Sandler AB, Ketner G. (1989). Adenovirus early region 4 is essential for normal stability of late nuclear RNAs. *J. Virol.* 63(2):624-30.

Schiedner G, Morral N, Parks RJ, Wu Y, Koopmans SC, Langston C, Graham FL, Beaudet AL, Kochanek S. (1998). Genomic DNA transfer with a high-capacity adenovirus vector results in improved in vivo gene expression and decreased toxicity. *Nat. Genet.* 18(2):180-3.

Schowalter DB, Himeda CL, Winther BL, Wilson CB, Kay MA. (1999). Implication of interfering antibody formation and apoptosis as two different mechanisms leading to variable duration of adenovirus-mediated transgene expression in immune-competent mice. *J. Virol.* 73(6):4755-66.

Shenk TE. (1996). Adenoviridae: The viruses and their replication. In: *Fields Virology*. Third Edition. Chapter 67. Raven publishers, Philadelphia.

Shirata N, Kudoh A, Daikoku T, Tatsumi Y, Fujita M, Kiyono T, Sugaya Y, Isomura H, Ishizaki K, Tsurumi T. (2005). Activation of ataxia telangiectasia-mutated DNA damage checkpoint signal transduction elicited by herpes simplex virus infection. *J. Biol. Chem.* 280(34):30336-41.

Steegenga WT, Riteco N, Jochemsen AG, Fallaux FJ, Bos JL. (1998). The large E1B protein together with the E4orf6 protein target p53 for active degradation in adenovirus infected cells. *Oncogene* 16(3):349-57.

Stehle IM, Scinteie MF, Baiker A, Jenke AC, Lipps HJ. (2003). Exploiting a minimal system to study the epigenetic control of DNA replication: the interplay between transcription and replication. *Chromosome Res.* 11(5):413-21.

Steven SL, Kochanek, S. (2007). Chromosomal integration of adenovirus vector DNA in vitro and in vivo. European congress of virology, Nuremberg, Abstract book p. 27. *unpublished.*

Stewart PL, Fuller SD, Burnett RM. (1993). Difference imaging of adenovirus: bridging the resolution gap between X-ray crystallography and electron microscopy. *EMBO Journal* 12: 2589±2599.

Stracker TH, Carson CT, Weitzman MD. (2002). Adenovirus oncoproteins inactivate the Mre11-Rad50-NBS1 DNA repair complex. *Nature* 418(6895):348-52.

Swaminathan S, Thimmapaya B. (1995). Regulation of adenovirus E2 transcription unit. *Curr. Top. Microbiol. Immunol.* 199 (Pt 3):177-94. Review.

Toietta G, Mane VP, Norona WS, Finegold MJ, Ng P, McDonagh AF, Beaudet AL, Lee B. (2005). Lifelong elimination of hyperbilirubinemia in the Gunn rat with a single injection of helper-dependent adenoviral vector. *Proc. Natl. Acad. Sci. U. S .A.* 102(11):3930-5.

Tollefson AE, Scaria A, Hermiston TW, Ryerse JS, Wold LJ, Wold WS. (1996). The adenovirus death protein (E3-11.6K) is required at very late stages of infection for efficient cell lysis and release of adenovirus from infected cells. *J. Virol.* 70(4):2296-306.

Van der Vliet PC. (1995). Adenovirus DNA replication. *Curr. Top. Microbiol. Immunol.*199 (Pt 2):1-30. Review.

Van Linthout S, Collen D, De Geest B. (2002). Effect of promoters and enhancers on expression, transgene DNA persistence, and hepatotoxicity after adenoviral gene transfer of human apolipoprotein A-I. *Hum. Gene Ther.* 13(7):829-40.

Wagner EK, Bloom DC. (1997). Experimental investigation of herpes simplex virus latency. *Clin. Microbiol. Rev.* 10(3):419-43. Review.

Weber LW, Boll M, Stampfl A. (2003). Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. *Crit. Rev. Toxicol.* 33(2):105-36. Review.

Wadsworth SC, Zhou H, Smith AE, Kaplan JM. (1997). Adenovirus vector-infected cells can escape adenovirus antigen-specific cytotoxic T-lymphocyte killing in vivo. *J. Virol.* 71(7):5189-96.

Weiden MD, Ginsberg HS. (1994). Deletion of the E4 region of the genome produces adenovirus DNA concatemers. *Proc Natl Acad Sci U S A.* 91(1):153-7.

Weterings E, van Gent DC. (2004). The mechanism of non-homologous end-joining: a synopsis of synapsis. *DNA Repair* 3(11):1425-35. Review.

Wold WS, Tollefson AE, Hermiston TW. (1995). E3 transcription unit of adenovirus. *Curr. Top. Microbiol. Immunol.* 199 (Pt 1):237-74. Review.

Wold WS, Doronin K, Toth K, Kuppuswamy M, Lichtenstein DL, Tollefson AE. (1999). Immune responses to adenoviruses: viral evasion mechanisms and their implications for the clinic. *Curr. Opin. Immunol.* 11(4):380-6. Review.

Yant SR, Ehrhardt A, Mikkelsen JG, Meuse L, Pham T, Kay MA. (2002). Transposition from a gutless adeno-transposon vector stabilizes transgene expression in vivo. *Nat. Biotechnol.* 20(10):999-1005.

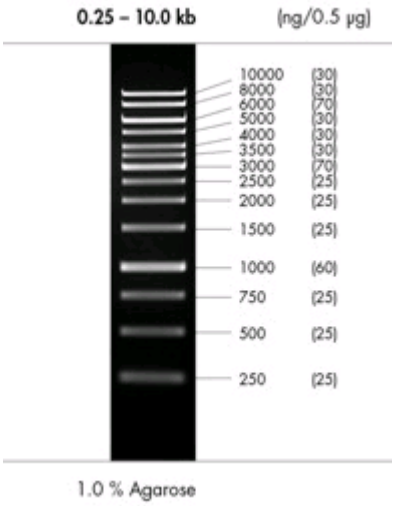
Zhang WW. (1999). Development and application of adenoviral vectors for gene therapy of cancer. *Cancer Gene Ther.* 6(2):113-38. Review.

Zhang Y, Chirmule N, Gao GP, Qian R, Croyle M, Joshi B, Tazelaar J, Wilson JM. (2001). Acute cytokine response to systemic adenoviral vectors in mice is mediated by dendritic cells and macrophages. *Mol. Ther.* 3(5 Pt 1):697-707.

Zhang G, Gao X, Song YK, Vollmer R, Stolz DB, Gasiorowski JZ, Dean DA, Liu D. (2004). Hydroporation as the mechanism of hydrodynamic delivery. *Gene Ther.* (8):675-82.

F Appendix

1. Molecular size marker



Appendix figure 1: peqGOLD 1 kb DNA marker. This molecular size marker was used for approximate determination of DNA fragments on 1% agarose gels. The size of the single bands is indicated. Size range: 250 bp to 10000 bp. Three enhanced bands (1000, 3000 and 6000 bp) help orientation. Reprinted from peqlab Biotechnologie GmbH.

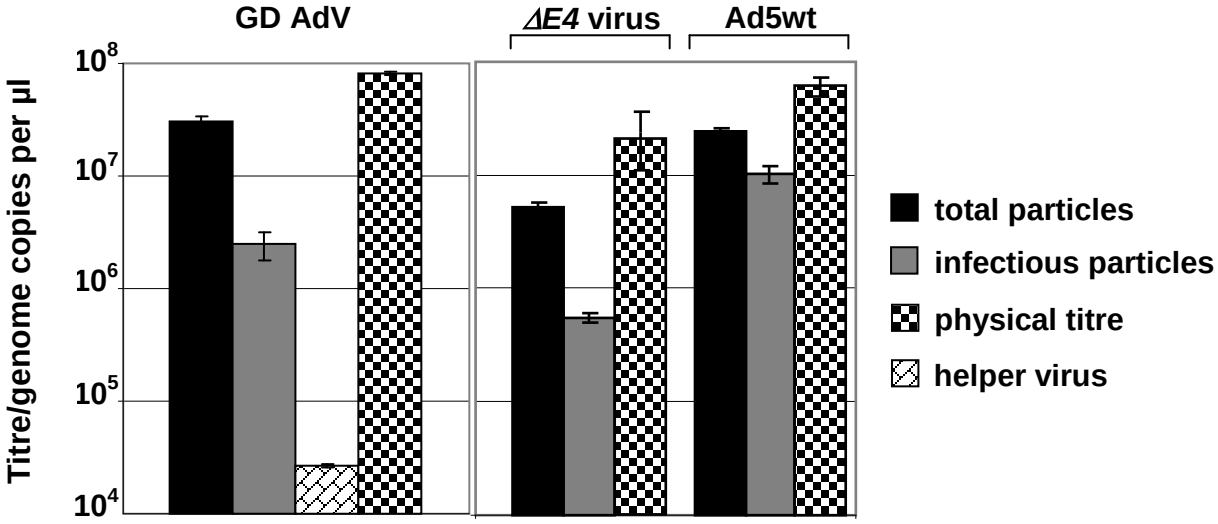
2. Characterisation of virus and vector preparations

2.1 Determination of infectious titres and total genomes by real-time PCR

Infectious genomes and total genomes were determined by quantitative real-time PCR (qPCR). It was found that the number of total genomes detected by qPCR is generally lower than the number of total genomes calculated by OD_{260} measurement. This is true for all three analysed virus and vector preparations (GD AdV, *ΔE4* and Ad5 wt). As expected, the number of infectious genomes is again only a fraction of total genomes [**Appendix figure 2**]. Importantly, the relations between these fractions (total titre:infectious titre, OD_{260} titre:total titre and OD_{260} titre:infectious titre) show similar tendency for each virus and vector preparation, respectively. Therefore, using OD_{260} titres for calculation of the MOI for transducing host cells instead of infectious titres, as done in the present study, does not influence the relative amounts of delivered genomes. The total amount of readily transducing particles, however, has to be corrected to a lower level.

2.2 Determination of the helper virus contamination in GD AdV preparations by quantitative PCR

Since it is of useful to know, whether GD AdV could be influenced by the helper virus that is inevitably co-purified during vector preparation and the MOIs used in some of the experiments are high, the level of helper virus contamination was determined by qPCR. 5 µl of CsCl purified the GD vector preparation were used for helper virus titre determination. Helper virus was specifically detected by a probe against the late protein 3 (L3). The contamination of GD vector with helper virus lays at 0.09% compared to the number of total particles [**Appendix figure 2**]. This amount is well in the range reported by others (Puntel *et al.* 2007).

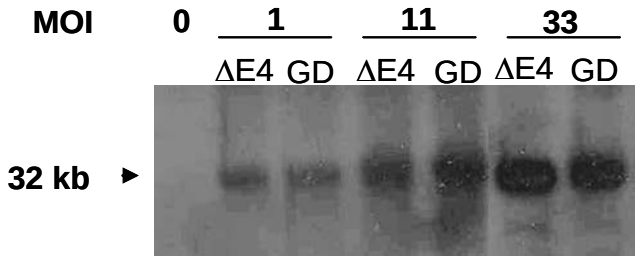


Appendix figure 2: Determination and comparison of GD AdV hFIX, *ΔE4* and Ad5 wt titres by qPCR. Physical titres were determined by OD_{260} measurements. Molecular titration using qPCR was carried out to measure helper virus contamination (L3 probe), infectious and total particles. 5 µl of CsCl-purified GD vector were used for helper virus titre determination. Total particles and infectious particles were determined analysing 200 fg of Hirt-extracted DNA of infected (MOI 10) 293 cells. Titres indicated represent the particle/genome number per µl. All MOI indicated in the present study, however, relate to titre determinations by OD_{260} measurements.]

2.3 Comparison of the infectious titre of ΔE4 *mutants and GD AdV by Southern blot*

To control that similar amounts of *ΔE4* and GD AdV genomes were administered, 293 cells were infected with either virus at OD_{260} MOIs of 1, 11 and 33. Cells were harvested after

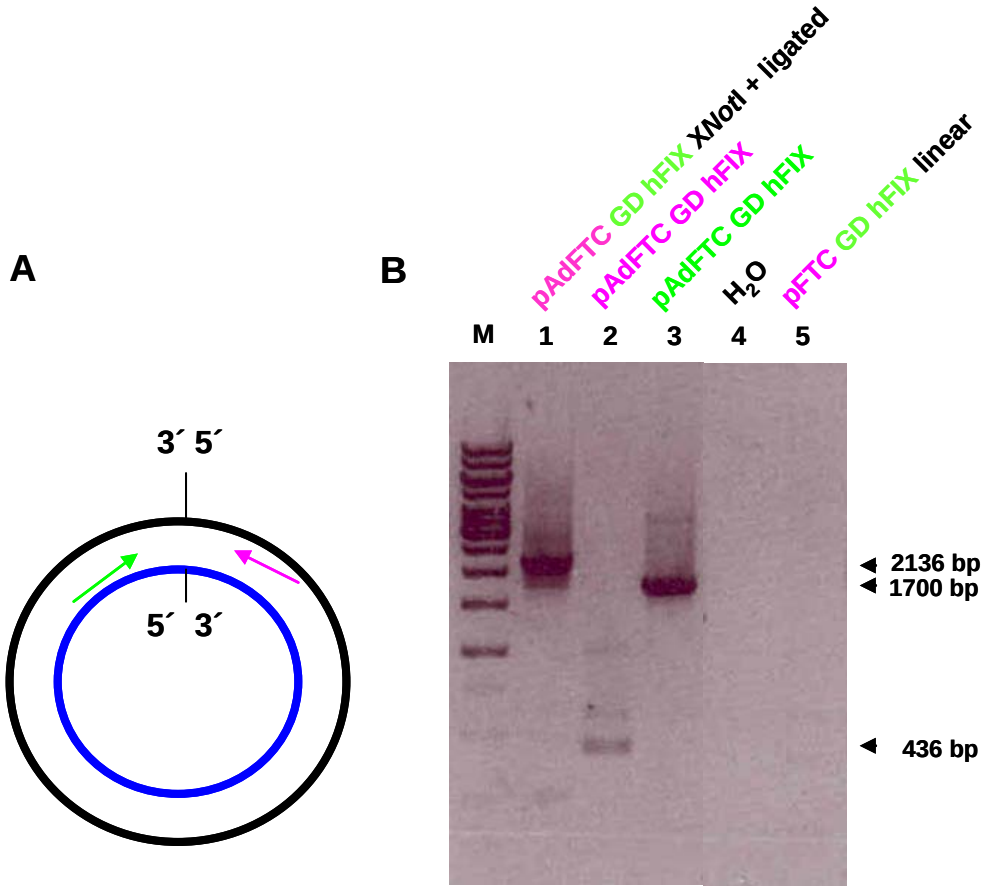
4 h of incubation with trypsin. Thirty µg of total DNA were analysed on a Southern blotted gel. The intensity of the respective band indicates the relative infectious titres. We found that *ΔE4* and GD AdV show similar titres at the respective MOI [**Appendix figure 3**].



Appendix figure 3: Comparison of infectious *ΔE4* and GD AdV titres by Southern blotting. 293 cells were infected with virus or vector at OD_{260} MOIs of 1, 11 and 33. The cells were harvested after 4 h of incubation with trypsin and genomic DNA was isolated. Twenty µg of total genomic DNA were analysed on a Southern blotted gel.

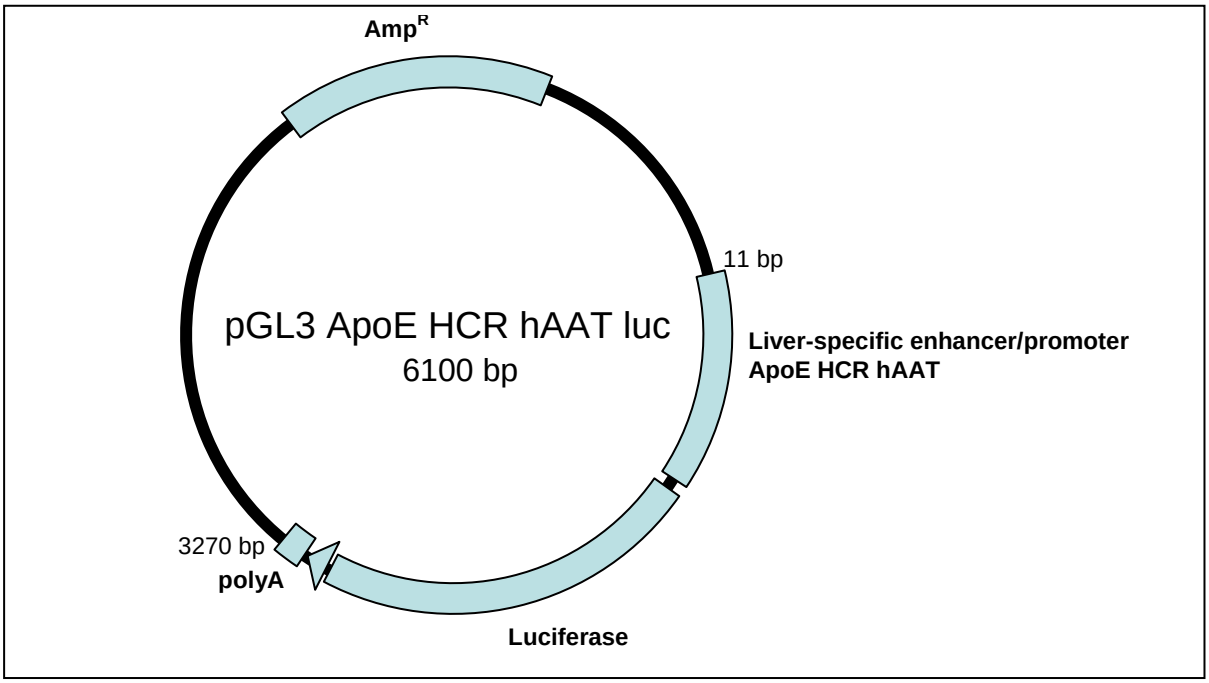
3. Establishment of a PCR assay for detection of circular GD AdV genomes

To provide a positive control for vector circularisation, CsCl-purified GD AdV DNA was extracted with phenol:chloroform:isoamylalcohol. This leads to removal of capsid proteins and the terminal protein (TP). After precipitation of GD AdV DNA, the genome was ligated with T4 ligase. The ligase was heat-inactivated. As an alternative, the GD AdV genome was released from pAdFTC GD hFIX with *Not*I and ligated subsequently. The ligase was heat-inactivated. These ligated DNA were used as template for PCR. Using the oligonucleotides For-1700 and Rev436, a PCR product of 2136 bp indicates the presence of circular GD hFIX genomes [**Appendix figure 4A**]. As controls, different combinations of oligonucleotides were used to verify the specificity of the PCR [**Appendix figure 4B**]. In conclusion, the circular conformation of GD AdV genomes can be specifically detected as a 2136 bp amplification product.

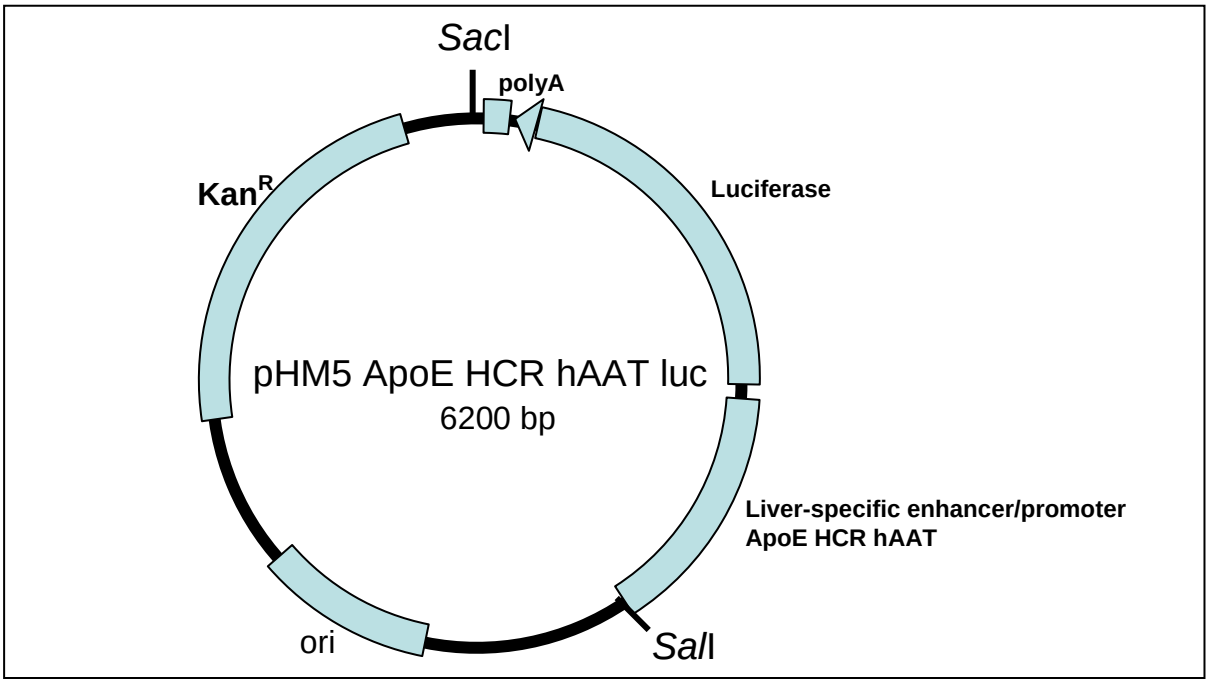


Appendix figure 4: Establishment of a PCR assay for detection of circular GD AdV genomes. A: Schematic overview over the circular PCR. Oligonucleotides are indicated as arrows (For-1700 and Rev436). **B:** Agarose gel after PCR. pAdFTC GD hFIX DNA was cleaved with NotI to release the GD AdV DNA. This released GD AdV DNA was subsequently ligated. Using oligonucleotides For-1700 and Rev436, the circular pAdFTC GD hFIX DNA can be detected as a band of 2136 bp (1). Using oligonucleotides Ad5ITRfw1-24 and Rev436, the left terminus of the construct is amplified (436 bp) (2). Utilising Ad5ITRfw1-24 and For-1700, the right terminus is amplified (1700 bp) (3). No template control (4). Applying the same oligonucleotides For-1700 and Rev436 on un-ligated, linear GD AdV DNA, no PCR band is visible, further confirming the correctness of the generated circular PCR products (5). M = molecular size marker.

4. Plasmid maps



Appendix figure 5: pGL3 ApoE HCR hAAT luc. This plasmid was used for the construction of GD AdV expressing firefly luciferase. pGL3 ApoE HCR hAAT luc was constructed by cloning of the liver-specific promoter/enhancer region ApoE HCR hAAT in front of the luciferase gene. ApoE HCR = Apolipoprotein E, hepatic control region (enhancer), hAAT = human alpha-1-antitrypsin promoter, luc = luciferase, Amp^R = ampicillin resistance gene.



Appendix figure 6: pHM5 ApoE HCR hAAT luc. This plasmid was used for construction of GD AdV luc. Luciferase is under control of the liver-specific promoter/enhancer region ApoE HCR hAAT. This plasmid mediates resistence against kanamycin. Relevant recognition sites for endonucleases are indicated.

Parts of this study were presented at the following conferences and published or prepared for publication in the following journals:

Conferences

May/June 2008	11th Annual Meeting of the American Society of Gene Therapy Boston, MA, USA Oral Presentation
March 2008	Annual Meeting of the Gesellschaft für Virologie, Heidelberg Oral Presentation
September 2007	3rd European Congress of Virology, Nuremberg Poster Presentation
July 2007	14th Annual Meeting of the Deutsche Gesellschaft für Gentherapy, Heidelberg Poster Presentation

Awards

May 2008	Travel Award of the American Society of Gene Therapy 2008

Publications

Jager L, Ehrhardt A. (2007). Emerging adenoviral vectors for stable correction of genetic disorders. *Curr. Gene Ther.* 7(4):272-83. Review.

Jager L, Häusl M, Rauschhuber C, Wolf NM, Ehrhardt A. A rapid protocol for construction and production of gene-deleted adenoviral vectors. *Nature Protocols* (in press).

Jager L, Ehrhardt A. Persistence of high-capacity adenoviral vectors as replication-defective monomeric genomesin virtro and in murine liver. *Human Gene Therapy* (in press).

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Erklärungen

Die vorliegende Dissertation habe ich selbständig und ohne unerlaubte Hilfe angefertigt. Ich habe zuvor noch nicht versucht, die Dissertation bei einer anderen Fakultät oder Universität einzureichen oder mich anderweitig einer Doktorprüfung zu unterziehen. Zudem wurde die Dissertation weder als Ganzes noch in Teilen einer anderen Prüfungskommission vorgelegt.