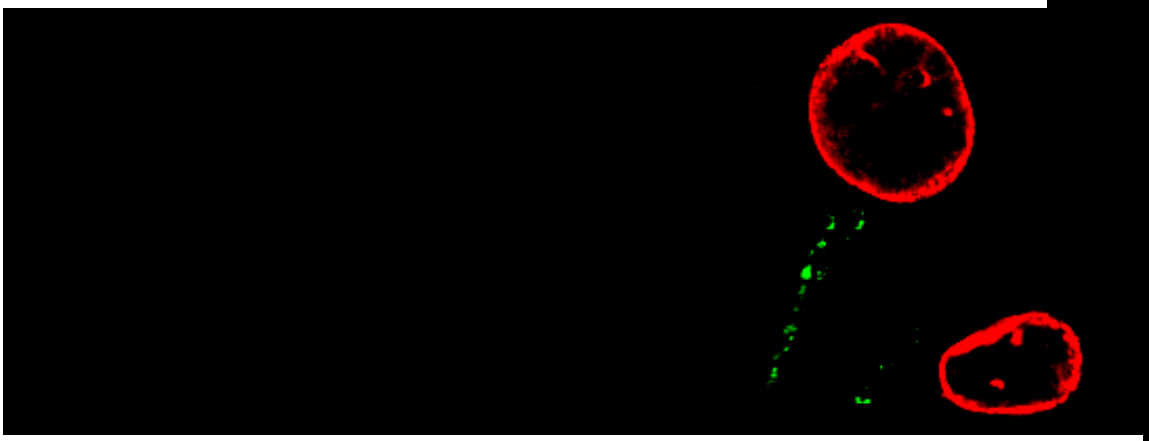
DISSERTATIONS IN
HEALTH SCIENCES

Anssi J. Mähönen

The Challenge of Enhanced and Long-term Baculovirus-mediated Gene Expression in Vertebrate Cells



Publications of the University of Eastern Finland
Dissertations in Health Sciences

UNIVERSITY OF EASTERN FINLAND

A.I.VIRTANEN INSTITUTE

ANSSI J. MÄHÖNEN

The Challenge of Enhanced and Long-term Baculovirus-mediated Gene Expression in Vertebrate Cells

To be presented by permission of the Faculty of Health Sciences, University of Eastern Finland,
for public examination in Tietoteknia Auditorium, Tietoteknia building,
University of Eastern Finland
on Saturday 27th February 2010, at 12 noon

Publications of the University of Eastern Finland
Dissertations in Health Sciences
5

University of Eastern Finland
Faculty of Health Sciences
A.I. Virtanen Institute for Molecular Sciences
Department of Biotechnology and Molecular Medicine
Kuopio
2010

Kopijyvä Oy
Kuopio, 2010
Editors: Prof. Veli-Matti Kosma
Prof. Hannele Turunen
Distribution:
Eastern Finland University Library / Sales of publications
P.O. Box 1627, FI-70211 Kuopio, Finland
http://www.uef.fi/kirjasto

ISBN: 978-952-61-0021-0 (print)
ISBN: 978-952-61-0022-7 (pdf)
ISSN: 1798-5706 (print)
ISSN: 1798-5714 (pdf)
ISSNL: 1798-5706

Author's address:

Department of Biotechnology and Molecular Medicine
A.I. Virtanen Institute for Molecular Sciences
Faculty of Health Sciences
University of Eastern Finland
P.O. Box 1627
FI-70211 KUOPIO
FINLAND
E-mail: anssi.mahonen@uef.fi

Supervisors:

Professor Seppo Ylä-Herttuala, M.D., Ph.D.
Department of Biotechnology and Molecular Medicine
A.I. Virtanen Institute for Molecular Sciences
Faculty of Health Sciences
University of Eastern Finland
E-mail: seppo.ylaherttuala@uef.fi

Professor Kari Airenne, Ph.D.
Department of Biotechnology and Molecular Medicine
A.I. Virtanen Institute for Molecular Sciences
Faculty of Health Sciences
University of Eastern Finland
E-mail: kari.airenne@uef.fi

Olli Laitinen, Ph.D.
Vactech Oy
Biokatu 8
FI-33520 TAMPERE
FINLAND
E-mail: olli.laitinen@vactech.fi

Reviewers:

Docent Varpu Marjomäki, Ph.D.
Department of Biological and Environmental Science
Nanoscience Center
University of Jyväskylä
Survontie 9
FI-40500 JYVÄSKYLÄ
FINLAND
E-mail: varpu.s.marjomaki@jyu.fi

Docent Vesa Hytönen, Ph.D.
Institute of Medical Technology
University of Tampere
FM1, 4-108
FI-33014 TAMPERE
FINLAND
E-mail: vesa.hytonen@uta.fi

Opponent:

Professor Arto Urtti, Ph.D.
Centre for Drug Research
Faculty of Pharmacy
University of Helsinki
P.O. Box 56 (Viikinkaari 5 E)
FI-00014 UNIVERSITY OF HELSINKI
FINLAND
E-mail: arto.urtti@helsinki.fi

Front cover picture: Detection of baculoviruses displaying EGFP on their capsids (green). The nuclei of DMEM cultured Ea.hy926 cells were recognized by anti-lamin A/C antibody (red).

Back cover picture: Detection of intermediate filaments (vimentin) (red). The nuclei of DMEM cultured Ea.hy926 cells were stained with DAPI (blue).

Mähönen, Anssi J. The Challenge of Enhanced and Long-term Baculovirus-mediated Gene Expression in Vertebrate Cells. Publications of the University of Eastern Finland. Dissertations in Health Sciences, 5. 2010. 111 p.

ISBN: 978-952-61-0021-0 (print)
ISBN: 978-952-61-0022-7 (pdf)
ISSN: 1798-5706 (print)
ISSN: 1798-5714 (pdf)
ISSNL: 1798-5706

ABSTRACT

Gene therapy involves the transfer of genetic material into a cell, tissue or whole organ in order to cure a disease or to improve the clinical status of the patient. The aim of this thesis was to enhance and extend the transient gene expression properties of baculovirus. In addition, the effect of cytoskeleton on the baculoviral transduction of mammalian cells was studied.

We found that the high and long-term production of cre-recombinase is toxic to mammalian cells. In addition, the cre-recombinase gene cloned under a mammalian promoter was expressed also in *E.coli* causing serious problems in the cloning procedures. In order to prevent the side effects, an optimized expression cassette was successfully constructed for the cre-recombinase to be used in all applications of Cre-loxP technology.

The transgene expression varies often in baculovirus-mediated transduction and is generally lower in cell types other than those of hepatic origin. We found that the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) boosted transgene expression. Culture conditions were also found to have a strong effect on the transduction efficacy. The highest gene expression was achieved with the combination of optimized cell culture medium together with a WPRE-bearing virus.

The cytoskeleton network is an essential structure regulating the transport of viral DNA to the nucleus in gene delivery. We clarified the mechanism behind the effect of the cell culture medium. The results suggested that the optimized culture medium mediates the reorganization of the vimentin network in the cells which leads to improved access of baculoviral nucleocapsids into the nucleus.

Baculovirus-mediated gene expression is transient. Baculoviruses have many advantages compared with viral vectors capable for long-term transgene expression. Baculoviruses are neither replicative nor pathogenic for vertebrate cells and have a large capacity for incorporation of foreign genes. Therefore, we generated a baculovirus-Epstein-Barr hybrid vector in order to achieve sustained transgene expression in mammalian cells.

In conclusion, novel baculoviruses were developed resulting in increased and prolonged gene expression in vertebrate cells. In addition, this thesis provides new insights concerning the nuclear entry of baculoviruses in mammalian cells.

Universal Decimal Classification: 577.218, 578.841

National Library of Medicine Classification: QZ 52, QU 470, QU 475, QW 51, QW 165, QU 350

Medical Subject Headings: Gene Therapy; Gene Transfer Techniques; Genetic Vectors; Transduction, Genetic; Baculoviridae; Gene Expression; Transgenes; Recombinases; Integrases; Herpesvirus 4, Human; Hepatitis B Virus, Woodchuck; Regulatory Elements, Transcriptional; Culture Media; Cytoskeleton; Intermediate Filaments; Vimentin; Nucleocapsid; Butyric Acid

Mähönen, Anssi J. Haasteena tehostettu ja pitkäkestoinen bakulovirusvälitteinen geenien ilmentyminen nisäkässoluissa. Itä-Suomen yliopiston julkaisuja. Terveystieteiden tiedekunnan väitöskirjat, 5. 2010. 111 s.

ISBN: 978-952-61-0021-0 (painettu)
ISBN: 978-952-61-0022-7 (pdf)
ISSN: 1798-5706 (painettu)
ISSN: 1798-5714 (pdf)
ISSNL: 1798-5706

TIIVISTELMÄ

Geeniterapian pyrkimyksenä on siirtää geneettistä materiaalia soluihin tai kudokseen ja näin hoitaa tai lievittää sairauden aiheuttamia oireita. Geeninsiirtoon käytetään sekä viruksiin pohjautuvia että synteettisiä vektoreita eli geeninkuljettimia. Tässä väitöskirjassa olen tutkinut hyönteisviruksen (bakulovirus) toimintaa nisäkässoluissa ja pyrkinyt muokkaamaan siinä olevan siirtogeenin ilmentymistä ja ilmentymisen kestoa paremmin geeninsiirtoon soveltuvaksi.

Väitöskirjan ensimmäisessä osatyössä havaittiin korkean ja pitkäkestoisen Cre-rekombinaasin ilmentymisen olevan toksista nisäkässoluille. Lisäksi ongelmaksi muodostui Cre:tä ilmentävän nisäkässoluissa toimivan viruspromoottorin (CAG) toimiminen myös bakteerisoluissa (*E.coli*). Tämä aiheutti vakavia ongelmia vektoreiden tuottamisessa. Nämä ongelmat ratkaistiin optimoimalla Cre:n ilmenemiskasettia siten, että ilmentyminen tapahtui vain nisäkässoluissa, ja sielläkin ilmentyminen oli väliaikaista ja rajoitettua. Tätä uutta ilmenemiskasettia voidaan käyttää kaikissa Cre-loxP teknologian sovellutuksissa. Tämä kasetti oli myös oleellinen osa neljännen osatyön bakulovirusvektoria.

Siirtogeenin ilmentyminen vaihtelee bakulovirusvälitteisessä geeninsiirrossa ja on yleensä heikompi muissa kuin maksaperäisissä soluissa. Toisessa osajulkaisussa geenin ilmentymistä voimistettiin liittämällä metsämurmelin hepatiittiviruksen post-transkriptionaalinen säätelyelementti (WPRE) osaksi bakuloviruksen ilmentymiskasettia. Lisäksi soluviljelymediumilla, jossa transdusoituvia soluja kasvatettiin, havaittiin olevan merkittävä vaikutus transduktiotehokkuuteen. Paras siirtogeenin ilmentyminen saatiin aikaan yhdistämällä optimoitu soluviljelymedium ja muokattu bakulovirus, joka sisälsi WPRE- säätelyelementin.

Nisäkässolujen sisältämä tukiranka on olennainen ja tärkeä osa solun toimintaa. Tämä tukiverkosto säätelee mm. virusten perimäaineksen kuljetusta solukalvolta tumaan. Kolmannessa osajulkaisussa tutkittiin syitä ja mekanismia soluviljelymediumin aiheuttamaan tehostuneeseen geeninilmentymiseen. Tulokset osoittivat, että optimoitu soluviljelymedium aiheuttaa solujen tukiverkoston (vimentiinin) uudelleenjärjestymistä ja helpottaa näin bakuloviruksen perimäaineksen pääsyä tumaan, mikä parantaa geeninsiirtotehokkuutta. Lisäksi havaittiin, että soluviljelymedium vaikuttaa positiivisesti myös muiden geeniterapiassa yleisesti käytettävien virusten tehoon.

Bakulovirusvälitteinen geeninsiirto johtaa lyhytkestoiseen siirtogeenin ilmentymiseen. Bakuloviruksella on kuitenkin monia etuja, jotka puuttuvat käytössä olevista siirtogeenin pitkäkestoiseen ilmenemiseen kykenevistä vektoreista. Neljännessä osajulkaisussa kehitettiin bakulovirus-Epstein-Barr – hybridivirus pitkäaikaista geeninilmentymistä varten. Tämä väitöskirjatyö edistää osaltaan bakulovirusten kehitystyötä geeniterapian tarpeisiin. Tuloksia voidaan soveltaa myös laajemmin.

Yleinen suomalainen asiasanasto: geeniterapia; geenitekniikka; geenit; bakulovirukset; Epstein-Barrin virus

To my dear wife
Tiina

And

To my children
Anniina and Miika

ACKNOWLEDGEMENTS

This study was done in the Department of Biotechnology and Molecular Medicine, A.I.Virtanen Institute, University of Eastern Finland and Arktherapeutics Oy.

I am deeply grateful to Professor Seppo Ylä-Herttuala for providing me with the opportunity to be involved in the exciting scientific projects in his group. I am thankful for his never-ending enthusiasm, patience and support during these many years. I admire his positive attidute and his miraculous way to find solutions and the right words should you feel you are in a blind alley.

I owe my sincere thanks to Professor Kari Airenne, who introduced me to the fascinating world of baculoviruses and gene therapy. His wide experience and extensive knowledge of viral vectors, especially baculoviruses, have made working with him such a pleasant and stimulating experience. Without his continuous support, steadfastness and constructive criticism, this study would never has been completed.

I am also very thankful to Dr. Olli Laitinen. Discussions with you have broadened my understanding of science, life and avidins. Your good sense of humor has been stimulating during these many years.

I am grateful to the official reviewers of this thesis, Docent Varpu Marjomäki and Docent Vesa Hytönen for the time they spent reviewing the manuscript and for their valuable comments. I express my warmest thanks to Dr. Ewen MacDonald for revision of the language of the thesis.

I am very thankful to Emilia Makkonen. I want to acknowledge your crucial role especially in our never-ending EBNA/oriP-project. Without your hard work and positive attitude, toward this project would not be in the form as it is now. I am also very grateful to Hanna Lesch and her crucial contribution to this project. In addition, I also want to express my sincere thanks to Miia Roshier, Sanna Purola, Karoliina Soininen, Justina Dimonaite, Pia Uhlbeck and Elina Malo who have provided essential help in my projects and whom I have had privilege to supervise. Furthermore, I want to thank Sari Kukkonen, Erik Peltomaa, Minna Kaikkonen, Mervi Riekkinen, Tommi Heikura and Kati Kinnunen for their invaluable help in my studies.

I grateful all my room-mates during these many years. Especially, I want to thank Helena Viita, Ann-Marie Määttä and Thomas Wirth for the interesting scientific and non-scientific discussions.

I am deeply indebted to our collaborators Professor Maija Vihinen-Ranta, Johanna Laakkonen, Teemu Ihalainen and Mirka Salminen. I am really grateful for your help in the confocal microscopy studies and for the possibility to visit your laboratory; I always remember its warm and sociable atmosphere.

Special thanks belong to Pauliina Lehtolainen-Dalkilic and Mikko Laukkanen, who introduced me to the world of science, monoclonal antibodies and atherosclerotic lesions during my master thesis work. This represented an important foundation for my scientific career.

Ville Harjulampi, Risto Feodoroff, Eero Paananen and Jani Räty are acknowledged for their help in computer issues. Jani Räty is also acknowledged for his valuable scientific contribution.

Without the technical assistance by Mervi Nieminen, Tarja Taskinen, Joonas Malinen, Riikka Eisto, Seija Sahrio and Anneli Miettinen, this study would not have been possible. I also wish to thank Anne Martikainen, Aila Seppänen, Jaana Siponen, Jaana Pelkonen, Sari Järveläinen and Mervi Huttunen.

I would also like to thank Helena Pernu and Marja Poikolainen for their invaluable help. Your experience especially coping with red tape routines, grant applications and EU-issues has been irreplaceable. In addition, special thanks for Ark's Admin&Facilities and Financial –teams, especially for Timo Ristola, Auli Harvima, Jenni Tuovinen, Saija Paukkunen, Johanna Pirinen and Katri Nikkinen.

I wish to thank all of the people working in the SYH-group and Ark Therapeutics for their help and for creating such a warm and stimulating atmosphere. Many of you have been essential pieces in my "thesis"-puzzle. Many "corridor"- discussions with you have cheered my gray days and given me belief in a better tomorrow.

I also wish to acknowledge Kuopio University Hospital and their really good doctors and physiotherapists who treated me and returned me back to health! A special thanks to my personal trainers, Anne and Matti Koskinen from ToriGym, who have kept me fit; with them I discovered the pleasure of physical exercise.

I want to express my gratitude to my parents Irja and Esa for all the support, help and guidance they have given me during my life. I am thankful to all my brothers Ari, Arto, Asko and their spouses who have helped and given me much valuable advice during these years. Many thanks to all my relatives, especially to Aino for her support during these years. I also want to thank my parents-in-law Sirpa and Pentti for support and relaxing vacations. Many thanks also belong to my sister-in-laws Kaisa, Hanna and Leena and their spouses. Many thanks to our family friends, especially to Johanna and Antti; with them I have had many relaxing board game sessions.

Finally, many thanks to God for my family. Grateful thanks for my children Anniina and Miika. You have been sunshine and the most valuable things in the world. Finally, I wish to say my sincerest thanks to my wife Tiina for her love, support and patience during these many years.

The author presents the warmest gratitude to all contributors to the thesis.

Kuopio, January 2010

Anssi Mähönen

This study was supported by Ark Therapeutics Oy, European Union (LHSB-CT-2006-037541) and grants from the Academy of Finland, Aarne and Aili Turunen Foundation, Sigrid Jusélius Foundation and Finnish Foundation for Cardiovascular Research.

Many thanks also to Ordior Oy, Immuno Diagnostic Oy, Biofellows Oy and Oligomer Oy.

LISTS OF ORIGINAL PUBLICATIONS

This study is based on the following original articles, which are referred to in the text body by the corresponding Roman numerals (I – IV):

I. Mähönen AJ, Airenne KJ, Lind MM, Lesch HP, Ylä-Herttuala S.
Optimized self-excising Cre-expression cassette for mammalian cells. *Biochem. Biophys. Res. Commun. 2004: 320(2):366-71.*

II. Mähönen AJ, Airenne KJ, Purola S, Peltomaa E, Kaikkonen MU, Riekkinen MS, Heikura T, Kinnunen K, Roschier MM, Wirth T, Ylä-Herttuala S.
Post-transcriptional regulatory element boosts baculovirus-mediated gene expression in vertebrate cells.
J Biotechnol. 2007: 131(1):1-8.

III. Mähönen AJ, Makkonen K-E, Laakkonen JP, Ihalainen TO, Kukkonen SP, Kaikkonen MU, Vihinen-Ranta M, Ylä-Herttuala S, Airenne KJ.
Culture medium induced vimentin reorganization associates with enhanced baculovirus-mediated gene delivery.
J Biotechnol. 2010: 145(2):111-119.

IV. Mähönen AJ, Makkonen K-E, Lesch HP, Purola S, Räty JK, Peltomaa E, Ylä-Herttuala S, Airenne KJ.
Prolonged transgene expression by Baculovirus-Epstein-Barr hybrid virus.
Manuscript

TABLE OF CONTENTS

ABBREVIATIONS

AcMNPV	*Autographa californica* multiple nucleopolyhedrovirus
Ad	Adenovirus
ADA	Adenosine deaminase
AIDS	Acquired immune deficiency syndrome
ATP	Adenosine triphosphate
BEBV	Baculovirus-Epstein-Barr hybrid virus
BKV	BK virus, member of polyomavirus family
BPV	Bovine papilloma virus
CAG	CMV early enhancer/chicken β actin promoter
CAR	Coxsackie-virus and adenovirus receptor
cDNA	Complementary DNA
CMV	Cytomegalovirus
DNA	Deoxyribonucleic acid
DMEM	Dulbecco′s Modified Eagle′s medium
DS	Dyad symmetry
DsRed	Red fluorescent protein
EBV	Epstein-Barr virus
EF1α	Elongation factor 1 alpha
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme linked immuno-sorbent assay
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
FR	Family of repeats
GFP	Green fluorescent protein
GV	Granulosis virus
HAC	Human artificial chromosome
HBV	Hepatitis B virus
HCV	Hepatitis C virus
hGH	Human growth hormone
HIV	Human immunodeficiency virus
HIV-1 PR	HIV-1 protease
HPRT	Hypoxanthine phosphoribosyltransferase
HSV	Herpes simplex virus
HUVEC	Human umbilical vein endothelial cell
IF	Intermediate filaments
kb	Kilo-base pair
kDA	Kilo dalton
lacZ	Gene encoding β-galactosidase
LCR	Locus control region
LTR	Long terminal repeat
MFs	Microfilaments
MFI	Mean fluorescence intensity
MOI	Multiplicity of infection
mRNA	Messenger RNA
MEM	Modified Eagle′s Medium
MTs	Microtubules
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MTOC	Microtubule organization center
NPC	Nuclear pore complex
NPV	Nuclear polyhedrosis virus
ODV	Occlusion-derived virion
PCR	Polymerase chain reaction
pfu	Plaque forming units
PIB	Polyhedral inclusion body
RGD	Arginyl-glycal-aspartic acid
RNA	Ribonucleic acid
RNAi	RNA interference
RPMI	Medium named after Roswell Park Memorial Institute
RSV	Rous sarcoma virus
RT-PCR	Reverse transcriptase polymerase chain reaction
SCID	Severe combined immunodeficiency
shRNA	Short hairpin RNA
S/MAR	Scaffold/matrix attachment region
VEGF	Vascular endothelial growth factor
VSVG	Vesicular stomatitis virus glygoprotein G
WPRE	Woodchuck hepatitis virus post transcriptional element

1 INTRODUCTION

In 2007 alone, 7.9 million (around 13 % of all deaths) individuals died from cancer. Moreover, the number of new cases is expected to increase over next decade reaching 12 million people in year 2030. In Finland, cancer was responsible for the deaths of 11 000 (22 % of all deaths) and cardiovascular diseases killed more than 21 000 (42 %) people in 2005 (http://www.who.int/en/). These alarming numbers are the basis for identifying new methods to cure these diseases in conjunction with conventional treatments. One new such promising technology is gene therapy.

Gene therapy is the application of molecular medicine to treat a disease. The basic idea of gene therapy is simple: to introduce nucleic acids into target cells or tissues resulting in either a full recovery from the disease or a slowdown in the progression of the disease. The major limiting factors in gene therapy are safety, efficacy, specificity and immune response against a vector.

More than 66 % of all vectors used in gene therapy trials are based on viruses (http://www.wiley.com/legacy/wileychi/genmed/clinical/). Viruses possess an inherent tendency for gene delivery and consequently the most popular gene therapy vectors are genetically modified human viruses such as adenoviruses (Ads) and retroviruses. These virus-based vectors have their own characteristic benefits, drawbacks and preferred applications. Therefore the search for potential new virus vectors is still on-going.

Baculoviruses are a large group of insect infecting DNA viruses characterized by the rod-shaped morphology of their virion. They have been used as biopesticides and as tools for efficient recombinant protein production in insect cells. Baculoviruses are also capable of entering into a wide spectrum of vertebrate cells making them a potential new tool for gene delivery.

In this work, we developed novel baculoviral vectors for enhanced and long-term gene delivery. We studied the feasibility of post-transcriptional regulatory element and optimized culture conditions in order to enhance transgene expression of baculovirus. The mechanism behind the positive medium effect is probably due to the reorganization of intermediate filaments (vimentin), which leads to improved access of baculoviral capsids into the nucleus. In addition, we created a baculovirus-Epstein-Barr

hybrid virus which combines the advantages of the baculovirus vector and prolonged episomal gene expression achieved by the Epstein-Barr virus-based episome.

2 REVIEW OF THE LITERATURE

2.1 Gene therapy

Gene therapy was first defined as being a technique for correcting defective gene or genes responsible for disease development. Nowadays it is defined as a delivery of genetic material to somatic cells in order to treat, prevent or slow down disease. Several approaches can be used in gene therapy: replacement of a mutated gene with a healthy copy of the gene, inactivation of the mutated gene or introduction of a new gene into the body to combat disease.

The first clinical gene therapy trial was started in 1989 (Rosenberg et al., 1990). In this study, the patients with an advanced cancer were given a retrovirus containing neomycin as a marker transgene. The first successful and encouraging clinical gene therapy treatment in order to treat adenosine deaminase (ADA) deficient in a four-year old patient occurred on September 14 in 1990 at the U.S. National Institutes of Health (Blaese et al., 1995). Retroviruses were used to carry the correct human ADA gene to the T-lymphocytes. Since then, the number of clinical trials has increased and today more than 1579 approved gene therapy trials have been registered. The greatest proportion of these trials (over 64 %) is cancer related and often carried out using terminally-ill patients (www.wiley.co.uk/genmed/clinical). Most clinical trials are in phase I or II, less than 3.5 % being in the Phase III. Most gene therapy trials are performed in USA (64.8 %) and Europe (29 %), but also Asia (3.4 %) is a new growing player in this field (Jia and Kling, 2006).

The gene therapy field has also encountered some failures (Hughes, 2007). In 1999, an 18-years old youth, Jesse Gelsinger, died in a gene therapy trial of ornithine transcarbamylase deficiency (OTCD) at the University of Pennsylvania. Gelsinger's death was proven to be caused by the Ad used in the trial (Raper et al., 2003). In 2003, French scientist reported that three children of nine developed leukemia in a trial of severe combined immunodeficiency (SCID) trial (Hacein-Bey-Abina et al., 2003a). The

leukemia was demonstrated to have been caused by vector (Moloney murine leukemia virus) integration leading to the activation of the growth-promoting proto-oncogenes which caused clonal T-cell proliferation (insertional mutagenesis) (Hacein-Bey-Abina et al., 2003a; Hacein-Bey-Abina et al., 2003b). Later, a fourth case of leukemia was observed in the same trial and since then one of these children has died (Hacein-Bey-Abina et al., 2008). In addition, one out of 10 children developed leukemia in a similar SCID gene therapy trial conducted in London (Howe et al., 2008). In this case, leukemia was caused by the activation of the proto-oncogene (LIM domain only 2; LMO2). This same gene was also dysregulated in a number of the severe adverse events in the French trial (Hacein-Bey-Abina et al., 2008). In 2007, a 36-year-old woman, Jolee Mohr, died in a gene therapy trial of rheumatoid arthritis in the University of Chicago Medical Center. Mohr's death was not linked to gene therapy; experts claim that the AAV-vector used in the trial was unlikely to be responsible. The recombinant DNA advisory Committee noted that Mohr apparently died from a fungal infection and a large blood clot. She was also taking anti-inflammatory drugs (Kaiser, 2007; Williams, 2008). However, these adverse events are rare when one takes into account the large number of on-going trials (1579 trials in December 2009). Indeed, gene therapy can be considered as a safe treatment compared with the conventional therapies. For example 2 million US citizens suffer drug interactions each year and about 5 % of these individuals die because of drug toxicity (Lazarou et al., 1998).

The first commercial gene-therapy drugs for anti-cancer gene therapy were launched recently in China (Guo and Xin, 2006). The first approved product was Gendicine (Shenzhen SiBiono GeneTech Co. Ltd.), which consists of a replication-defective adenoviral vector engineered to express p53, leading to the death of tumor cells (Peng, 2005). The second commercially available gene therapy vector is the oncolytic Ad H101 (Sanghai Sunway Biotech Co. Ltd.), which kills tumor cells oncolytically (Lu et al., 2004a). These first therapeutics indicate that gene therapy is becoming part of modern medicine and will soon represent an alternative to the present clinical treatments. The first western world approved novel gene therapy drugs are expected to enter the markets soon (Osborne, 2008).

2.2 Gene transfer vectors

2.2.1 Introduction

Gene transfer means the delivery of nucleic acid encoding for a therapeutic or marker protein (gene/s) to the target cells. The administration of gene/s requires a transportation vehicle, called a vector, which encapsulates the gene/s and carries the gene into the target cell. The vector binds to the target receptor and is internalized. The genome containing the desired gene/s is then transported into the cell nucleus, where it is expressed episomally or is integrated into the host cell genome. Every step in this entry process and the subsequent expression of the gene/s represent potential barriers to successful gene therapy (Figure 1).

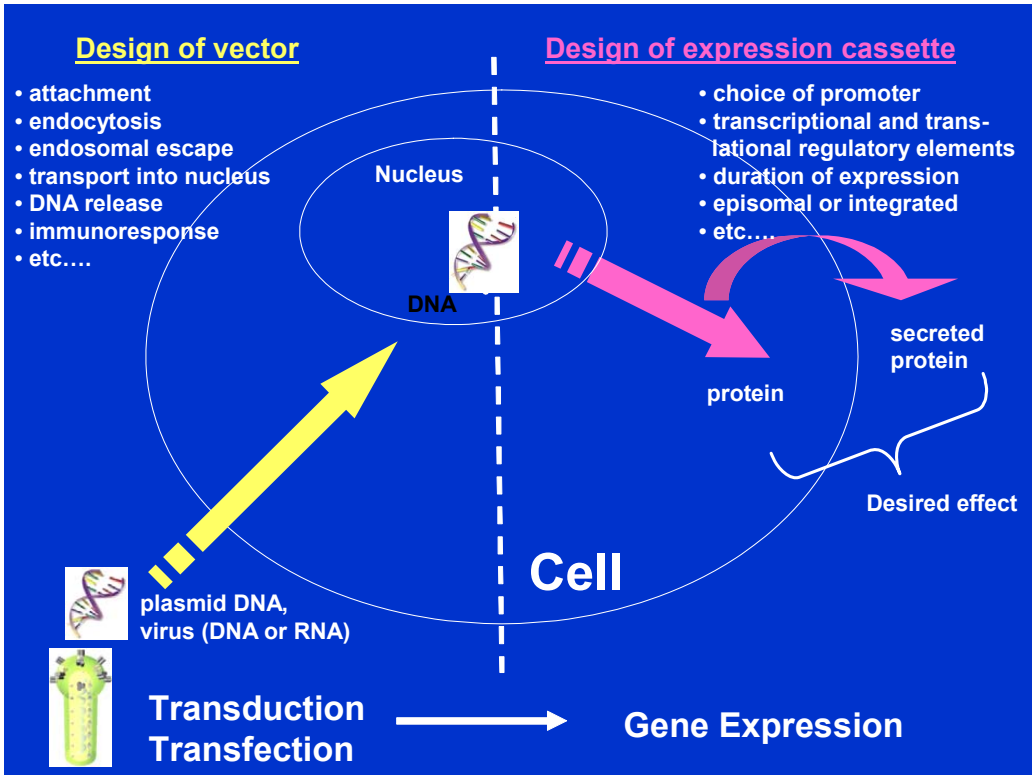


Figure 1. The challenge of gene delivery. The efficient gene transfer and transgene expression is a consequence of successful and accurate design of the vector and expression cassette.

The ideal gene transfer vector would target to a desired tissue at an adequate transduction efficiency and causes transient or stable therapeutic gene expression without any side effects. None of currently used vectors fit fully into a definition of ideal vector: they have their own advantages, weaknesses and applications. A comparison between baculovirus and the most commonly used gene transfer vectors is shown on the table 1.

Table 1. Characteristics of the commonly used gene transfer vectors compared to baculovirus (Thomas et al., 2003; Waehler et al., 2007; Schaffer et al., 2008).

	Size (nm)	Capacity (kb)	Duration of expression	Advantages	Disadvantages	Clinical Applications
Baculovirus (AcMNPV)	25-50 x 250 - 300	> 100	Transient	High titers Large capacity Non-human pathogen Easy production	Large size, Immunogenic	Protein and vaccine production
Ad	70 – 100	< 8	Transient	High titers Broad tropism High expression profile Safe, well tolerated	Pre-existing immunity Targeting Risk for replication competent virus formation	Cancer Cardiovascular disease
Retrovirus (MuLV) and Lentivirus (HIV)	100	< 10	Stable	Stable gene expression	Risk of insertional mutagenesis Risk for replication competent virus formation	Genetic diseases Central nervous system Hematopoietic system
AAV	18 – 26	< 5	Stable	Non-pathogenic Small Stable gene expression	Production difficult Risk of insertional mutagenesis Small capacity	Cystic fibrosis, Hemophilia B, central nervous disease
Non-viral	Variable	Unlimited	Transient	Low toxicity Simple production No insert limit	Low efficiency,	Melanoma

Abbreviations: MuLV, murine leukemia virus.

Many vectors, viral and non-viral, are used in gene therapy studies and clinical trials (Figure 2). Currently the most popular vectors in clinical trials are adeno- and retroviruses representing 44.7 % of all vectors used. Gene types transferred in gene therapy trials are mostly antigens or cytokines, representing 38.2 % of all used genes with most common indications addressed by gene therapy clinical trials being cancer diseases, representing 64.5 %.

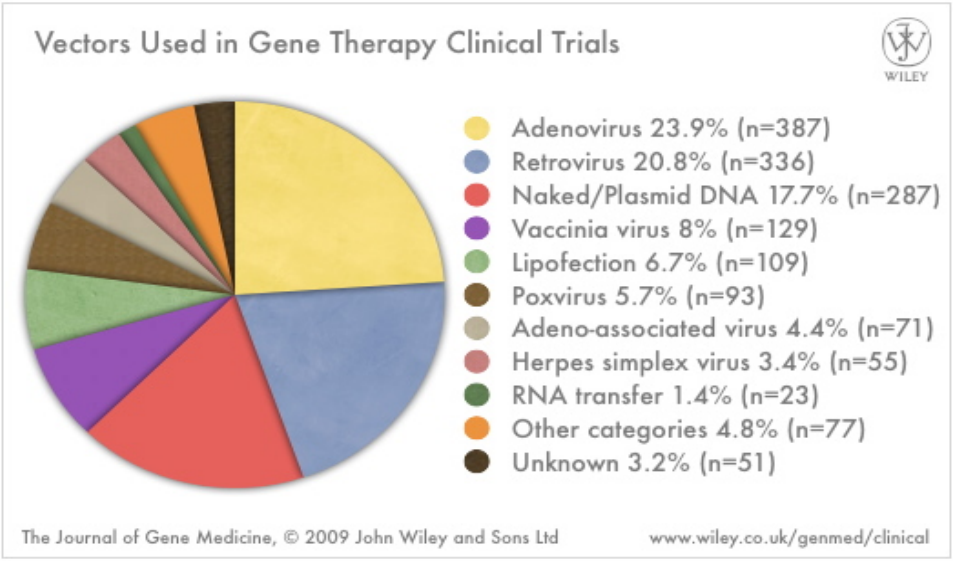


Figure 2. Vectors used in clinical gene therapy trials (http://www.wiley.co.uk/genmed/clinical/).

2.2.2 Adenoviruses

Human Ads belong to *Adenoviridae* family (DNA virus, genome of 35 kb), which consists of more than 50 serotypes (6 subgenera: A through F) (Figure 3). They can infect a wide range of organs such as the respiratory tract, eye, urinary bladder, gastrointestinal tract and liver. Wild type virus causes infections in humans i.e. respiratory tract infections, conjunctivitis, cystitis and gastroenteritis (Horwitz, 2001; Douglas, 2007). Ads enter the host by clathrin-mediated endocytosis (Leopold and Crystal, 2007), but some other routes have also been suggested (Meier and Greber, 2004). Infection is mediated by binding of the fiber knob region to a receptor on the target cell. The receptor is the Coxsackie–Ad receptor (CAR) for most serotypes (e.g. Ad 5) (Roelvink et al., 1998). Internalization of the virus is mediated by the interaction of a penton-base Arg–Gly–Asp (RGD) motif and cellular $\alpha v\beta$ integrins, which leads to endocytosis of the virion via clathrin coated pits (Wickham et al., 1994). The next steps in Ad entry are acidification of the clathrin-coated vesicles, dismantling and endosomal escape of capsids to cytosol and intracellular movement toward nucleus using microtubules and dynein motors. In the late stage of infection, the capsids dock at the nuclear pore complex (NPC) and the viral DNA is carried into the nucleus (Medina-Kauwe, 2003).

Ad vectors used in gene therapy are replication deficient (deletion of their E1A and E1B essential genes for replication) and most vectors are derived from Ad serotype 5 (subgenera C) (Graham and Prevec, 1995); however, other Ad vectors like Ad2, Ad7 and Ad4 as well as nonhuman Ads have also been studied (Stone and Lieber, 2006; Stone et al., 2007). Transgenes of up to 7.5 kb can be incorporated into these vectors. With so-called gutless vectors (all Ad genes deleted), the capacity is over 30 kb (Schiedner et al., 1998; Alba et al., 2005).

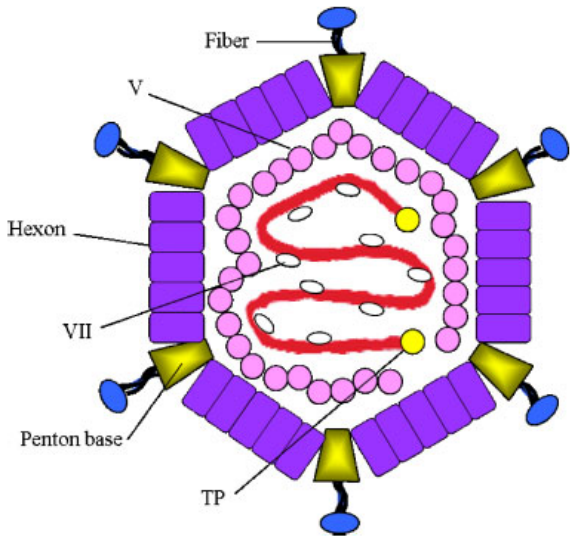


Figure 3. Schematic representation of adenoviral particle. Viral DNA associated with pV, pVII and TP proteins (Sonabend et al., 2006). Abbreviations: pV, protein V; pVII, protein VII; TP, terminal protein.

Ad vectors have been shown to mediate efficient gene delivery to the vasculature, cardiac myocytes, liver, lung, central nervous system and skeletal muscle (Kozarsky and Wilson, 1993; Huard et al., 1995). The major advantages of Ads are easy generation of high titer viral stocks (10^{12} – 10^{13} virus particles per ml), ability to infect post mitotic cells, broad host cell range, efficient gene delivery and the fact that they do not integrate into the host genome. The greatest limitation of Ad-mediated gene transfer is transient gene expression, which is a too short to cure genetic diseases. In addition, a pre-existing or an acquired immune response against the vector or transduced cells could be harmful (Bessis et al., 2004; Nazir and Metcalf, 2005). A number approaches have been devised in attempts to reduce the immune response to Ad vectors, prolong

transgene expression and enhance the efficiency of readministration. By changing the serotype (Stone and Lieber, 2006), minimizing gene content (gutless Ad) (Alba et al., 2005), creating chimeric vectors (Tsuruta et al., 2007) or modified tropism/targeting (Wickham, 2000), these challenges may be overcome.

Ads have already shown their capability for gene therapy. Replicating human Ads were used as an anti-cancer agent in 1956 to treat patients with advanced epidermal carcinoma of the cervix (Huebner et al., 1956). Subsequently non-replicating adenoviral vectors have been used in different kinds of gene therapy trials (http://www.wiley.com/legacy/wileychi/genmed/clinical/). In 2009, Ad vectors have been employed in one-fourth of clinical trials worldwide (387 of 1579) with most of these Ad applications being in the field of cancer or cardiovascular disease. The lack of sustained expression is not problematic in these applications. Conditionally replicative Ads, which exhibit tumor specific amplification and lysis of the cancer cells, were also shown to have potential in treating certain human tumors (Sonabend et al., 2006). The first commercial adenoviral gene-therapy drugs for anti-cancer gene therapy were recently launched (Gendicine and Oncorine) (Guo and Xin, 2006) and several (e.g. Cerepro, Advexin and TNFearade) Ad based vectors are in the final clinical phase (Phase III) and close to marketing approval outside of China (Osborne, 2008; Duzgunes, 2008).

2.2.3 Retro- and lentiviruses

Retroviruses belong to *Retroviridae* family (linear single stranded-RNA genome, 7-11 kb) consisting seven different genuses (alpha retro-, beta retro-, gamma retro-, delta retro-, epsilon retro-, lenti- and spumavirus) (Baum et al., 2006). They can infect a wide range of vertebrates. This large group of viruses causes a wide variety of different diseases including an array of malignancies, immunodeficiencies (e.g. AIDS) and neurologic disorders (Goff, 2001).

Retroviruses are enveloped viruses having a single stranded RNA genome and they replicate via a DNA intermediate. The DNA genome can then be integrated into the host genome with an integrase enzyme and they replicate as part of the cell's DNA. A viral genome comprises genes for viral structural proteins (gag, pol and env) and more complex retroviruses contain also some regulatory/accessory genes (tat, rev, tev, vif, nef, vpr and vpu) (Baum et al., 2006). Transduction begins by the recognition of a surface receptor (e.g. HIV recognize multiple receptors: heparin sulfate proteoglycan,

CD4, chemokine receptors) that allows fusion with the cell membrane (e.g. membrane fusion for HIV) or receptor-mediated endocytosis in the case of pseudo typed viruses (Anderson and Hope, 2005). Subsequently, the ribonucleoprotein complex is released into the cytoplasm (Mondor et al., 1998; Freed and Mouland, 2006). In the cytoplasm, the viral RNA genome is reverse transcribed into cDNA within a reverse transcription complex (RTC) and the resulting cDNA within a preintegration complex (PIC) is transported via microtubules (dynein) into the nuclear envelope. The preintegration complex is then transported through NPC into the nucleus wherein the viral cDNA integrates into the cellular genome (Anderson and Hope, 2005).

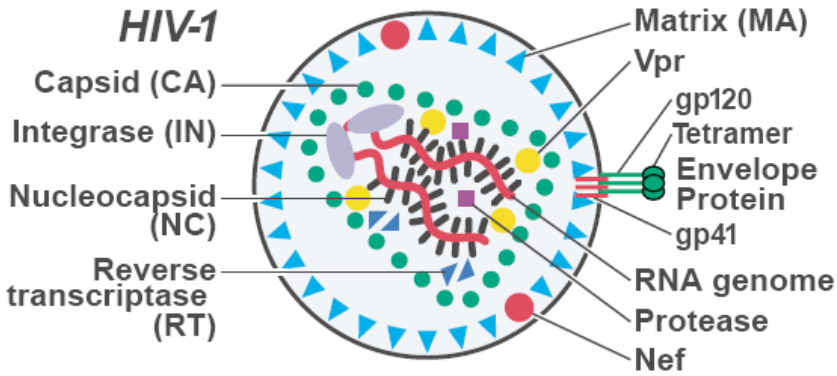


Figure 4. Schematic presentation of HIV-1 (Greene and Peterlin, 2002).

Unlike other retroviruses, lentiviruses are capable of infecting non-dividing cells. Most of the lentiviral vectors used are derived from the human immunodeficiency virus-1 (HIV-1) (Figure 4), but non-human simian, equine, bovine and feline immunodeficiency viruses have also been investigated. These replication-deficient virus vectors contain less than 5 % of the original viral genome. Long-term transgene expression has been obtained in brain, liver, hematopoietic stem cells, lymphocytes (T-cells) and muscle (Felder and Sutton, 2009). The safety of vectors is improved by the development of self-inactivating (SIN) vectors which avoid possible insertional activation of cellular oncogenes (Yu et al., 1986; Dull et al., 1998).

Lentiviruses possess several useful features, such as the capability of transducing quiescent cells, and long-term transgene expression, that make them attractive as a gene transfer vector. The greatest limitations of lentivirus-mediated gene transfer are the preparation of high titers and safety concerns like insertional mutagenesis and their

derivation from the pathogenic HIV-1 (Hacein-Bey-Abina et al., 2003a). One of the major challenges is to control the integration risk of lentiviral vectors (e.g. by directed integration by fusion protein or using nonintegrating vector) (Vargas, Jr. et al., 2004; Ciuffi et al., 2006).

In the very first clinical gene therapy treatment conducted in 1990, retroviruses (gamma-retrovirus) were used for gene transfer (Blaese et al., 1995). The ADA gene was introduced into the T-lymphocytes of ADA-deficiency patients (SCID-ADA). Subsequently gamma retroviruses treated hematopoietic stem cells have been successfully used to cure also X-linked severe combined immunodeficiency (SCID-X1) and chronic granulomatous disease patients (Cavazzana-Calvo et al., 2000; Aiuti et al., 2002; Ott et al., 2006; Cavazzana-Calvo and Fischer, 2007; Kohn, 2007).

Lentiviruses have been used to combat HIV by a variety of approaches such as inducible suicide gene, antisense RNA and RNAi therapy (siRNA/shRNA) (Dropulic et al., 1996; Novina et al., 2002; Poluri et al., 2003; Lu et al., 2004b) but also against disorders affecting hematopoietic and neuronal cells (Schambach and Baum, 2008). The first clinical trial using a lentiviral vector against HIV was started in USA in 2003. The results from the phase I trial indicated that the procedure was well tolerated and no adverse effects were detected (Levine et al., 2006; Dropulic and June, 2006). Two other clinical trials using lentiviral vecotors have been conducted to treat patients suffering from β-thalassemia (Bank et al., 2005) and adrenoleukodystrophy (Cartier, 2001; Cartier and Aubourg, 2008). Furthermore, two clinical trials (against SCID-X1) using safety-improved retro- and lentiviruses are starting soon in USA (Williams, 2009). The first clinical trial using a non-HIV based lentiviral vector (equine immunodeficiency virus) for the treatment of Parkinson's disease (http://www.oxfordbiomedica.co.uk/) has also been conducted (Azzouz et al., 2002).

2.2.4 Adeno-associated viruses

Adeno-associated viruses (AAV) belong to the *Parvoviridae* family within the genus of *Dependovirus* (single- stranded DNA genome, 4.7 kb). AAV was originally observed as a contaminant of a laboratory preparation of Ads and later isolated also from humans (Atchison et al., 1965; Grossman et al., 1992). AAV requires helper virus (Ad or herpervirus) functions to replicate. They are non-pathogenic to humans and can transduce a wide range of cells e.g. muscle, liver, brain and vasculature, providing long-term expression of the transgene (Carter et al., 2009). In order to achieve cell entry,

AAV-2 can use several receptors like heparin sulfate proteoglycans, $\alpha_v\beta_5$ integrin and human fibroblast growth factor receptor 1 (Summerford and Samulski, 1998; Summerford et al., 1999; Qing et al., 1999). AAV2 is endocytosed via clathrin-dependent receptor-mediated endocytosis (Ding et al., 2006). There is also some evidence that AAV may utilize multiple mechanism for cell entry (Duan et al., 1998).

There are 11 known serotypes of AAV and more than 100 human and nonhuman capsid sequence variants have been characterized (Wu et al., 2006). Serotype 2 (AAV2) has been most extensively examined in gene therapy thus far. AAV2 has a natural tropism towards certain tissues e.g. muscle, lung, retina, neurons, vascular smooth muscle cells and hepatocytes (Wu et al., 2006). AAV-5 has tropism for airway epithelial cells, neurons and astrocytes and AAV-6 and AAV-8 for most muscle groups (Carter et al., 2009). AAV-9 has been used to prevent hereditary cardiomyopathy (Goehringer et al., 2009).

The advantages of AAV are long-term gene expression (either integrated or episomal) and non-pathogenic in humans (Nakai et al., 2001; Goncalves, 2005). There is also some limitations like pre-existing immunity, challenge in producing high titer viral preparations without helper virus contaminations, low transgene capacity (4.9 kb) for some applications, risk for insertional mutagenesis and neutralizing antibodies against AAVs (Goncalves, 2005; Mingozzi and High, 2007; Williams, 2008). One of the major development areas in AAV technology is to create a more sophisticated production system for AAV vectors and applying these different serotypes appropriately for gene therapy applications (Negrete and Kotin, 2007).

AAV vectors have been used in treating a wide variety of diseases e.g. for treatment of cystic fibrosis, hemophilia B, arthritis, HIV (as a vaccine), muscular dystrophy, Parkinson's disease and prostate cancer (Carter, 2005). The first AAV vector used in a clinical trial was the AAV-2 vector expressing the cDNA of cystic fibrosis transmembrane conductance regulator gene to treat cystic fibrosis patients (Wagner et al., 2002). Most of the clinical trials have used AAV-2 but interest in serotypes AAV- 2.5 and AAV-1 is increasing (Carter, 2005).

2.2.5 Non-viral systems

Non-viral gene delivery systems can be divided into those using physical methods, synthetic vehicles and other means of delivery (Gao et al., 2007). Physical methods (carrier free method) use naked plasmids which are delivered by methods such as injection, gene gun, hydrodynamic delivery, electroporation, sonoporation, laser irritation or magnetofection (Mehier-Humbert and Guy, 2005; Dobson, 2006). In synthetic vehicles, DNA is shielded and packaged into several different natural or synthetic compounds (carriers) to facilitate cellular uptake and intracellular release (Figure 5) (Karmali and Chaudhuri, 2007; Wolff and Rozema, 2008; Ruponen et al., 2009). The synthetic vehicles are further divided into lipoplexes (cationic lipid/DNA complex) (Felgner et al., 1987), polyplexes (cationic polymers/DNA complex) (Wu and Wu, 1987) or lipid-polymer hybrids. These lipid-polymer hybrids include DNA precondensed with polycations, then coated with either cationic liposomes, anionic liposomes or amphiphilic polymers w/o helper lipids (Gao et al., 2007). These vectors try to mimic viral vectors in terms of assembly and cellular delivery.

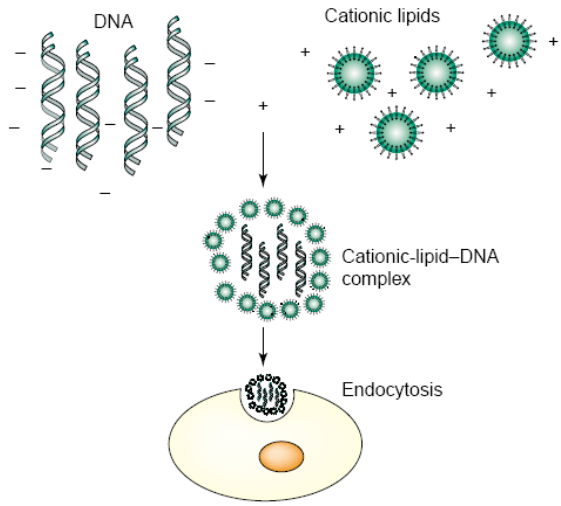


Figure 5. Cationic carrier. Cationic lipids and DNA are mixed to form complexes that can enter cell by endocytosis (Schmidt-Wolf and Schmidt-Wolf, 2003).

Non-viral vectors have many advantages over viral vectors such as easy large-scale production, large transgene capacity, safety and simplicity. Although significant progress has been made in the field of non-viral systems, the majority of these systems are still less efficient than viral vectors, especially for *in vivo* gene delivery. The major bottlenecks are the release of DNA-complexes from the endosomes, toxicity and immune responses against different complexes (Ruponen et al., 2003; Wolff and Rozema, 2008). Attempts have been made to circumvent these problems by the use dioleyl phosphatidylethanolamine (DOPE) (Farhood et al., 1995), polyethyleneimine (PEI) (Boussif et al., 1995) or polyethyleneglycol (PEG) (Kichler, 2004). Inclusion of dioleyl phosphatidylethanolamine is believed to increase membrane fluidity and facilitate lipid exchange and membrane fusion between lipoplexes and the endosomal membrane (Litzinger and Huang, 1992), whereas polyethyleneimine raises the osmotic pressure of the edosomes causing these vesicles to swell and rupture ("proton sponge hypothesis") (Sonawane et al., 2003).

Even though most clinical trials use viral vectors, as many as 17.7 % of all clinical trials have been carried out with naked/plasmid DNA. Ten of these trials are in the final clinical phase III (http://www.wiley.co.uk/genmed/clinical/). One of the most promising approaches (against metastatic melanoma) is a plasmid/lipid complex encoding human histocompatibility leukocyte antigen (HLA-B7) and ß2 micro globulin, which are believed to cause an immune response against metastatic tumors (Bedikian and Del, 2008).

2.3 Baculoviruses

2.3.1 General aspects

Baculoviruses (Latin *baculum* means 'stick') are large, double-stranded DNA (80-180 kb) containing insect viruses. More than 600 species are known, which infect permissively only arthropod hosts (insects, arachnids, crustaceans and others). Baculoviruses are common in nature and the food that we consume. Baculoviruses have a long history having been recognized for hundreds of years. As early as 1856, polyhedral inclusion bodies were observed when insect tissues was examined under the microscope (Cornalia, 1856; Summers, 2006). At present, no diseases have been linked to baculoviruses in any organism outside the phylum *Arthropoda* (Miller, 1997).

Baculoviruses have been studied since 1920s as biopesticides (Black et al., 1997) and therefore a considerable amount of data is available about their biology (Miller, 1997) and biosafety (Burges et al., 1980). The baculovirus expression vector system (BEVS) became a popular choice for recombinant protein production during the late 1980s and early 1990s with a large number of commercially available reagents (O'Reilly et al., 1994). Nowadays, many pharmaceuticals and biotechnology companies are utilizing baculovirus based protein production systems in their drug discovery process. After 1985, when the first successful *in vitro* gene transfer by a recombinant baculoviruses was accomplished (Carbonell et al., 1985), baculoviruses started to be examined more as a gene therapy vector. The first *in vivo* gene transfer attempts into the liver of rats and mice were conducted in 1995 (Sandig et al., 1996).

The prototype of the family *Baculoviridea* and the most extensively studied baculovirus is the *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV). Its genome (~134 kb) has been sequenced and predicted to contain 154 open reading frames (Ayres et al., 1994). Although the 3-dimensional structure remains to be determined, the components of its cigar-shaped loosely enveloped virion are fairly well known and have been extensively studied (Funk et al., 1997; Slack and Arif, 2007). The Vp39, p80, and p24 represent the major capsid proteins of AcMNPV (Figure 6). The vp39 is known to be randomly distributed over the capsid surface. The major envelope glycoprotein of the budded and occlusion derived virion form of baculovirus are gp64 and p74, respectively. The gp64 is believed to be responsible for the formation of peplomer structures at one end of the virion.

2.3.2 Structure

The double-stranded circular DNA genome (80-180 kb) of baculoviruses is condensed into a nucleoprotein structure known as a core. The core is located within a flexible rod-shaped capsid, averaging 25-50 nm in diameter and 250-300 nm in length and can expand relatively freely to accommodate even very large recombinant molecules. The core and the capsid are known collectively as the nucleocapsid. Membrane enveloped nucleocapsids are referred to as virus particles or virions (Figure 6).

Traditionally baculoviruses are divided into two morphologically distinct genera: nuclear polyhedrosis viruses (NPVs) and granulosis viruses (GVs). In the NPV group, virions that obtain an envelope from nuclear membrane are occluded within a

paracrystalline protein matrix (occluded virions), forming large (1-15 μm) polyhedral inclusion bodies (PIBs) containing multiple virions. NPVs are further distinguished on the basis of whether they contain a single nucleocapsid (single nucleopolyhedrovirus; SNPV) or multiple nucleocapsids (multiple nucleopolyhedrovirus; MNPV) per envelope in the polyhedrin matrix. In contrast to NPVs, GVs have only a single virion embedded in a very small inclusion body (O'Reilly et al., 1994).

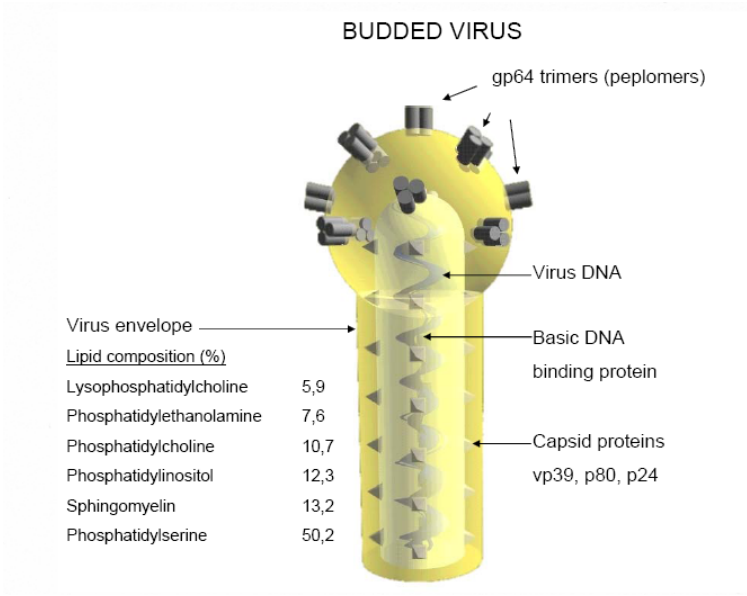


Figure 6. The schematic structure of budded virus (Airenne et al., 2009).

2.3.3 Baculovirus life cycle

Baculoviruses exist in two distinct forms in their life cycle that play different roles during the natural life cycle of the virus: Occlusions derived virions (ODV) and budded viruses (Figure 7). ODVs mediate primary infection and are also responsible for horizontal transmission between insect hosts. The systemic spread within the insect and propagation in tissue culture is dependent on budded viruses. Structurally, budded viruses and ODVs differ by the origin and composition of their envelopes. Budded viruses acquire their envelopes by budding through the plasma membrane whereas envelopes of ODVs are derived from the nuclear membrane of the insect cell. They also

differ in the mechanisms by which they enter the host cells. Budded viruses enter the cells by adsorptive endocytosis, but ODVs enter the midgut epithelial cells via direct membrane fusion at the cell surface (Summers, 1971; Granados, 1978b; Blissard, 1996; Wang et al., 1997).

Infection starts when larva eats plants contaminated by PIBs formed during the late phase of natural infection (Figure 7). In PIBs, virions are embedded in the crystalline protein matrix, which is composed mostly of polyhedrin protein which protects the virions from environmental factors. The crystalline polyhedron matrix of PIB is solubilized in the alkaline midgut of the larva and the ODVs enter the midgut cells by fusion with the microvilli membrane (Granados and Williams, 1986). Nucleocapsids from the endosomes and are transported to nucleus. Viral transcription and replication occur in the cell nucleus and new baculovirus particles are budded out from the basolateral side. Infection spreads within the insect from the midgut, through tracheal cells, to most tissues via the hemolymph (Keddie et al., 1989; Federici, 1997).

Baculovirus infection can be divided into early, late, and very late phases. Biologically these phases correspond to reprogramming the cell for virus replication (budded virus and ODV production). In the early phase (viral synthesis phase, the first 6 h), the virus prepares the infected cell for viral DNA replication (O'Reilly et al., 1994). Virus-specific RNAs can be detected in the cells at 30 min postinfection (pi) (Chisholm and Henner, 1988). The late phase (viral structural phase) extends from 6 h pi to approximately 20 to 24 h pi. During this phase, late genes are expressed and the production of budded viruses starts around 12 h pi. Progeny nucleocapsids leave the nucleus and are transported onto the plasma membrane where they acquire their envelope. Very late phase (the occlusion-specific phase) begins around 20 h pi. Production of infectious budded viruses decreases and occlusion of virus particles into polyhedrin matrix begins followed by cell lysis. Eventually the larva dies and PIBs are released into the environment, and a new cycle begins again. Most of the naturally occurring baculoviruses kill their target host within 4 to 7 days (O'Reilly et al., 1994).

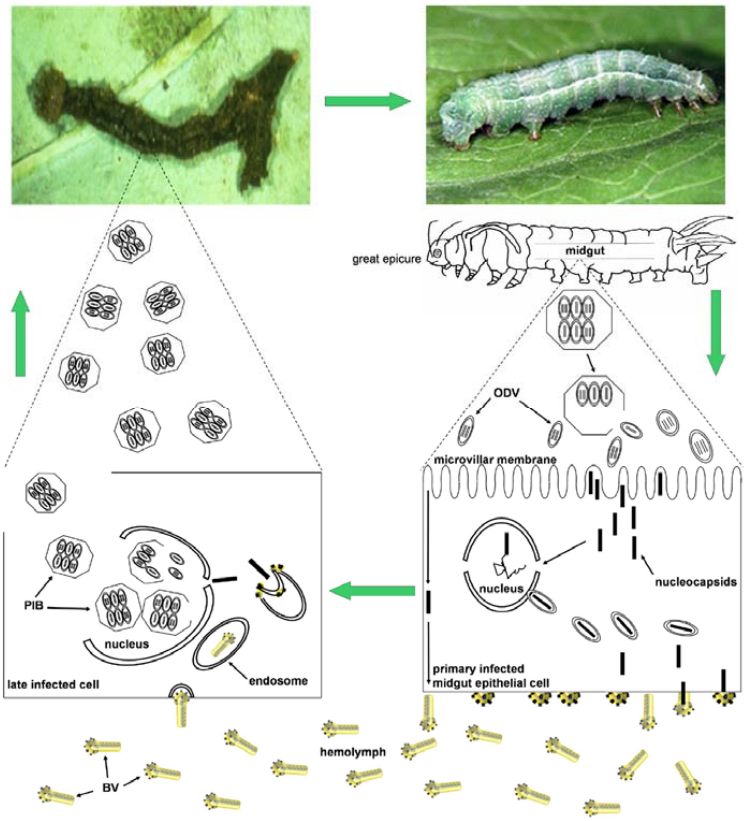


Figure 7. Baculovirus cycle. The primary infection on the right and the secondary infection on the left. In the primary infection PIBs are broken down in the alkalic midgut and released ODVs infect primary epithelia of the gut wall. In the secondary infection, replicated budded viruses (marked as a BV in the figure) spread everywhere through the hemolymph and cause infections in different tissues. Later virions are packed into PIBs, which are released to the environment when the larva dies (Airenne et al., 2009).

2.3.4 Baculovirus-mediated transduction of vertebrate cells

The safety of baculoviruses has been studied in the past for their ability to infect non-target cells with regard to their use as biological pesticides. Volkman and Goldsmith demonstrated that baculoviruses were able to enter certain cell lines derived from vertebrate species (Volkman and Goldsmith, 1983). Thirty-five nontarget host cell lines, 23 of human and 12 of nonhuman vertebrate origin, were exposed to AcMNPV. However, no evidence of viral gene expression was obtained. This study was in

accordance with the earlier reports showing uptake of AcMNPV by several vertebrate cell lines with no evidence of viral replication (McIntosh and Shamy, 1975; Granados, 1978a).

In the early 1980s, it was discovered that baculoviruses could penetrate into nontarget cells including many human cell lines (Carbonell et al., 1985). In their study Carbonell et al.(Carbonell et al., 1985) used a recombinant baculovirus bearing a RSV promoter (Rous sarcoma virus long terminal repeat promoter) and a marker gene as part of the recombinant baculovirus genome. Since a low level of marker gene expression was observed in the studied cells, it was claimed that marker protein was carried into the cells with the virus and not actively expressed in the cells (Carbonell and Miller, 1987a; Carbonell and Miller, 1987b). Ten years later Hofmann et al.(Hofmann et al., 1995) confirmed the initial results and also revealed that baculovirus could cause efficient gene expression when the marker gene is under a mammalian promoter. They were also the first to propose baculovirus-mediated gene therapy. During the late 1990s, the concept of baculovirus-mediated gene transfer was further verified and today the list of suitable target cells continues to increase (Airenne et al., 2009).

The first studies that baculovirus can cause efficient transgene expression in mammalian cells were performed in hepatocytes. Hofmann et al.(Hofmann et al., 1995; Hofmann et al., 1998) demonstrated that recombinant AcMNPV virus containing the luciferase gene under a cytomegalovirus (CMV) promoter could efficiently infect human hepatocytes (Huh7 and HepG2) as well as primary hepatocytes of human and rabbit origin. Boyce et al.(Boyce and Bucher, 1996) confirmed these results and showed that a virus carrying *lacZ* reporter gene under the control of RSV promoter led to a high-level expression of the marker gene in human hepatocellular carcinoma line HepG2 as well as in primary rat hepatocytes. Primary rat and human hepatic stellate cells were also shown to be highly susceptible to baculovirus-mediated gene delivery (Gao et al., 2002). All these findings supported the potential use of baculovirus vectors for achieving liver-directed gene therapy.

Further studies have indicated that a high-level expression of marker gene can be achieved not only in hepatic but also in a wide range of other cell lines (Shoji et al., 1997; Yap et al., 1997; Barsoum et al., 1997; Hofmann et al., 1998; Zhu et al., 1998; Condreay et al., 1999). Shoji et al.(Shoji et al., 1997) transduced a panel of mammalian cells with a baculovirus vector carrying a marker gene in comparison with a replication-defective

Ad vector. A high-level luciferase activity was detected in this study in monkey kidney cells (COS7), porcine kidney cells (CPK) and human cervix carcinoma cells (Hela). Condreay et al.(Condreay et al., 1999) demonstrated that recombinant baculoviruses containing green fluorescent protein (GFP) gene under the CMV promoter could transduce a wide range of mammalian cell types originating from different tissues. Cell lines of hepatic origin were transduced efficiently, as described earlier, but notable gene expression was detected also in cell lines derived from kidney tissue (Cos-7, BHK, CV-1, 293), some other non-hepatic cell lines like keratinocytes (W12, primary human keratinocytes), bone marrow fibroblasts and osteosarcoma cells (MG-63). Moreover, baculoviruses were also shown to transduce efficiently stem cells (Ho et al., 2005; Ho et al., 2006; Chuang et al., 2007; Chuang et al., 2009; Tsai et al., 2009). The lowest efficiencies of transduction and levels of GFP expression were seen in cell lines of hematopoietic origin, such as THP-1, U937, K562, Raw264.7 and P388D1. Efficient gene transfer has also been obtained in several other cell lines, which are summarized on Table 2.

Transduction of chicken and duck cells was examined with the AcMNPV construct. Ping et al. showed that chicken primary myoblasts and embryonic fibroblasts were more than 80 % positive after marker gene transduction (Ping et al., 2006). Primary embryonic cells (liver, heart and lung) from chicken and duck could also be efficiently transduced after baculovirus treatment. The transduction efficacy of duck cells was higher than that of chicken cells (Song et al., 2006). In addition, fish cells were also examined, because fish are susceptible to many diseases and baculovirus-mediated vaccination could be used to prevent infections. Tilapia, carp and salmon cells could be transduced with VSVG-pseudotyped baculovirus, except for rainbow trout gonad cells (Leisy et al., 2003). Zebra fish were also shown to be permissive for baculovirus transduction (Wagle and Jesuthasan, 2003; Wagle et al., 2004). Fruit fly cells (Drosophila S2 and Tribolium) (Oppenheimer et al., 1999; Lee et al., 2000) and honey bees (Ando et al., 2007) were shown also to be permissive for baculovirus-mediated gene delivery.

Table 2. Examples of cell lines of different species transduced with baculoviruses.

Species	Examples of cell lines	References	Primary cells	References
Human	Huh-7, HepG2 FLC4, 293T, Hela, A549, Ramos, Jurkat, Saos-2, MG-63, SKOV3, MRC-5, ECV-304	(Hofmann et al., 1995; Shoji et al., 1997; Zhu et al., 1998; Condreay et al., 1999; Kost and Condreay, 2002)	Stellate cells, Neural cells, Bone fibroblasts, Keratinocytes, Pancreatic β-cells Hepatocytes Mesenchymal stem cells and progenitor cells Embryonic stem cells Foreskin fibroblasts HUVEC	(Gao et al., 2002), (Sarkis et al., 2000), (Condreay et al., 1999), (Condreay et al., 1999), (Ma et al., 2000), (Boyce and Bucher, 1996), (Hofmann et al., 1995) (Ho et al., 2005; Chuang et al., 2007; Chuang et al., 2009; Tsai et al., 2009) (Ho et al., 2006; Lee et al., 2007b) (Zeng et al., 2007) (Kronschnabl et al., 2002) (Kronschnabl et al., 2002)
Rabbit	RaaSMC	(Airenne et al., 2000)	Hepatocytes Intervertebral Disc cells Chondrocytes	(Boyce and Bucher, 1996) (Liu et al., 2006b) (Sung et al., 2007; Chen et al., 2009)
Monkey	COS-7, CV-1, Vero	(Yap et al., 1997; Condreay et al., 1999; Aoki et al., 1999a; Tani et al., 2001)	Marmoset hepatocytes	(Martyn et al., 2007)
Rodent	CHO, BHK, RGM-1, PC12, N2a, L929	(Boyce and Bucher, 1996; Shoji et al., 1997; Condreay et al., 1999; Aoki et al., 1999a; Sarkis et al., 2000; Tani et al., 2001)	Hepatocytes of rat Condrocytes of rat Stellate cells of rat Kidney cells of mouse Myoblasts of rat Schwann cell of rat Pancreatic cells of mouse	(Boyce and Bucher, 1996) (Ho et al., 2005), (Lee et al., 2007a) (Gao et al., 2002) (Liang et al., 2004) (Shen et al., 2008) (Kenoutis et al., 2006) (Ma et al., 2000)
Pig	CPK, FS-l3, PK-15	(Aoki et al., 1999a)	N/A	
Bovine	MDBK, BT	(Aoki et al., 1999a)	N/A	
Sheep	FLL-YFT	(Aoki et al., 1999a)	N/A	
Birds	N/A		Embryonic cells and myoblasts of chicken Embryonic cells of chicken and duck (liver, lung, heart)	(Ping et al., 2006) (Song et al., 2006)
Fish	EPC, TO-2, CHH-1, CHSE-214	(Wagle and Jesuthasan, 2003; Leisy et al., 2003; Wagle et al., 2004)	N/A	

2.3.5 Optimization of baculovirus transduction

There are several ways to enhance baculovirus-mediated transduction and gene expression in vertebrate cells. The addition of histone deacetylase inhibitors, such as sodium butyrate or trichostatin A, following transduction has been shown to markedly increase transgene expression/transcription (Condreay et al., 1999; Airenne et al., 2000). Microtubule depolymerizing agents also improve baculovirus-mediated gene delivery since cytoplasmic transport and nuclear entry of the baculovirus nucleocapsid is

restricted by the presence of an intact microtubule network (van Loo et al., 2001; Salminen et al., 2005). However, both these strategies are limited by their cytotoxic effects to the cells (Hunt et al., 2002; Salminen et al., 2005). Transduction efficiency may be enhanced by extending standard transduction times (1-2h) under suitable transduction conditions (Cheng et al., 2004) and conducting the protocol at under 37°C (Hsu et al., 2004). Repeated transduction of cells (supertransduction) may also be used, since baculoviruses are not toxic to vertebrate cells (Wang et al., 2005a). Other options include pseudotyping with VSVG (Barsoum et al., 1997; Pieroni et al., 2001; Tani et al., 2001; Tani et al., 2003), VSV-GED (Kaikkonen et al., 2006), the RGD-motif (Matilainen et al., 2006), tumor-homing peptides (Makela et al., 2006), hepatitis virus S protein (Tani et al., 2001), extra copy of gp64 (Tani et al., 2001), avian influenza virus hemagglutinin (Matilainen et al., 2006), short peptide motif from gp350/220 of Epstein-Barr virus (Ge et al., 2007), biotin acceptor peptide (Kaikkonen et al., 2008), lymphatic homing peptide (Lyp-1) (Makela et al., 2008) or avidin display on the viral envelope (Raty et al., 2004). Furthermore, the choice of a promoter may also make a difference. For example, in some cell lines, the chicken β-actin promoter (CAG) is more efficient than the CMV promoter for transgene expression (Shoji et al., 1997). Also, cell-type specific gene expression may be achieved with tissue-specific promoters (Park et al., 2001; Li et al., 2004; Li et al., 2005; Wang and Wang, 2006). In addition, the homologous region sequence (hr1) of AcMNPV was shown to affect transcription from non-baculoviral promoters in mammalian cells (Viswanathan et al., 2003). A cytomegalovirus enhancer (Ong et al., 2005; Li et al., 2005; Wang and Wang, 2006) and a transcriptional activator (Ramos et al., 2002; Liu et al., 2006a) have also been used to enhance transgene expression.

2.3.6 Baculovirus hybrid vectors

The extraordinary capacity of AcMNPV to carry foreign DNA (>100 kb tolerated) allows construction of recombinant viruses bearing also large expression cassettes (Fipaldini et al., 1999; Cheshenko et al., 2001). This is an advantageous property for hybrid vector construction, as shown by several recent reports. Palombo et al.(Palombo et al., 1998) constructed a baculovirus-adeno-associated virus (Bac-AAV) hybrid vector to prolong the transient baculovirus-mediated transgene expression. The concept was to use the natural integration capacity of AAV to carry the transgene cassette into a defined region

(chromosome 19) of the host cell genome. Integration into the genome of 293 cells was shown to take place at a significant frequency, but the problem was nonspecific integration and multiple insertions of the marker gene. Subsequently, a similar method was used to construct Bac-AAV hybrid vectors to improve and extend gene expression in neural cells (Wang and Wang, 2005; Wang and Wang, 2006; Wang, 2008), to treat glioma (Wang et al., 2006) and to transduce human embryonic stem cells (Zeng et al., 2007).

The baculovirus-adenovirus hybrid vector (Bac-Ad) was created for the production of gutless adenovirus vectors (FD-AdVs) that do not require helper Ads (Cheshenko et al., 2001). High titer FD-AdV virus preparations (10^8 pfu/mL) were attained. However, the system has to be further improved to quarantee that there will not be any generation of replication-competent viruses during a large-scale production.

The baculovirus-lentivirus hybrid vectors (BAC-gag-pol, BAC-VSVG and BAC-REV) were created in order to obtain better and scalable lentivirus production systems (Lesch et al., 2008). The new production system was functional and the titers were comparable to the titers of the conventional four-plasmid method.

A recombinant baculovirus carrying a cDNA of the bacteriphage T7 RNA polymerase under the control of the CAG promoter was constructed and used together with a plasmid bearing the entire poliovirus genome to produce a high-titer type I poliovirus (Yap et al., 1997). In order to efficiently propagate and study biology of hepatitis B virus (HBV) and hepatitis C virus (HCV) in cultured cells, recombinant baculoviruses were created carrying the HBV (Delaney and Isom, 1998; Delaney et al., 1999; Delaney et al., 2001; Abdelhamed et al., 2002; Heipertz, Jr. et al., 2007; Lucifora et al., 2008) and HBC (Fipaldini et al., 1999; Street et al., 2005; McCormick et al., 2006). Hepatitis-baculovirus hybrid vectors represent a simple and highly flexible system for studying the effects of antivirals and/or cytokines on HBV (Shaw et al., 2006) and HCV production, and for the understanding of their replication and pathogenesis at the molecular level. Biology of the human cytomegalovirus (HCMV) was investigated by Ba-HCMV hybrid vector (Dwarakanath et al., 2001). In addition, a Ba-WHV hybrid vector was created in order to study woodchuck hepatitis virus (WHV) replication (Zhu et al., 2004).

The first recombinant baculoviruses carrying genetic elements from Epstein-Barr virus (EBV), *OriP* and *EBNA-1*, which are essential for episomal maintenance of EBV genome, were constructed (Shan et al., 2006; Wang et al., 2008; Lo et al., 2009; Suzuki et al., 2009). These baclovirus-EBV hybrid vectors were capable of persisting in a

significant proportion of infected mammalian cells. Transgene expression lasted up to 60 days in HEK293 cells with markedly enhanced expression level (Shan et al., 2006; Lo et al., 2009).

2.3.7 Entry mechanism of baculovirus

The budded form of AcMNPV enters cells by adsorptive endocytosis in a multistep process including virus attachment, cellular entry (endocytosis), vesicular transport, endosomal escape, intracytosolic transport, nuclear entry, capsid disassembly and gene expression (Figure 8). The exact molecular mechanism(s) leading to infection of insect cells, or transduction of vertebrate cells, remains still poorly understood, but the mechanism of the viral capsid transport into the nucleus before uncoating is apparently identical to that occurring in insect cells (Granados and Lawler K.A., 1981). Results from both the insect and vertebrate cells ascribe the virion uptake by clathrin-mediated endocytosis (Wickham et al., 1992a; Wang et al., 1997; van Loo et al., 2001; Matilainen et al., 2005), macropinocytosis (Matilainen et al., 2005; Long et al., 2006) or some kind of phagocytosis-like entry (Laakkonen et al., 2009). The presence of specific binding sites for baculovirus uptake has been proposed (Wickham et al., 1992b; Wang et al., 1997). Candidate receptors were postulated to be general cell surface molecules like phospholipids or heparin sulfate proteolygans (Hynes, 1992; Duisit et al., 1999; Tani et al., 2001).

After endocytosis, AcMNPV infection is continued by pH-dependent fusion of the envelope with endosomes (van Loo et al., 2001). The nucleocapsids are transported via actin filaments toward the nucleus (van Loo et al., 2001). Disruption of microtubules by nocodazole or vinblastine enhanced baculovirus-mediated gene delivery in vertebrate cells, suggesting that microtubules may be a mechanical barrier for nucleocapsid movement towards and entry into the nucleus (van Loo et al., 2001; Salminen et al., 2005). The nucleocapsids are transported through the nuclear pore into the nucleus, where disassembly of the viral nucleocapid and exposure of nucleprotein core take place (Kukkonen et al., 2003; Salminen et al., 2005). In the nucleus of human liver cells, viral capsids were seen in large, discrete foci and they also induced remodeling of the host cell chromatin (Laakkonen et al., 2008). Although AcMNPV does

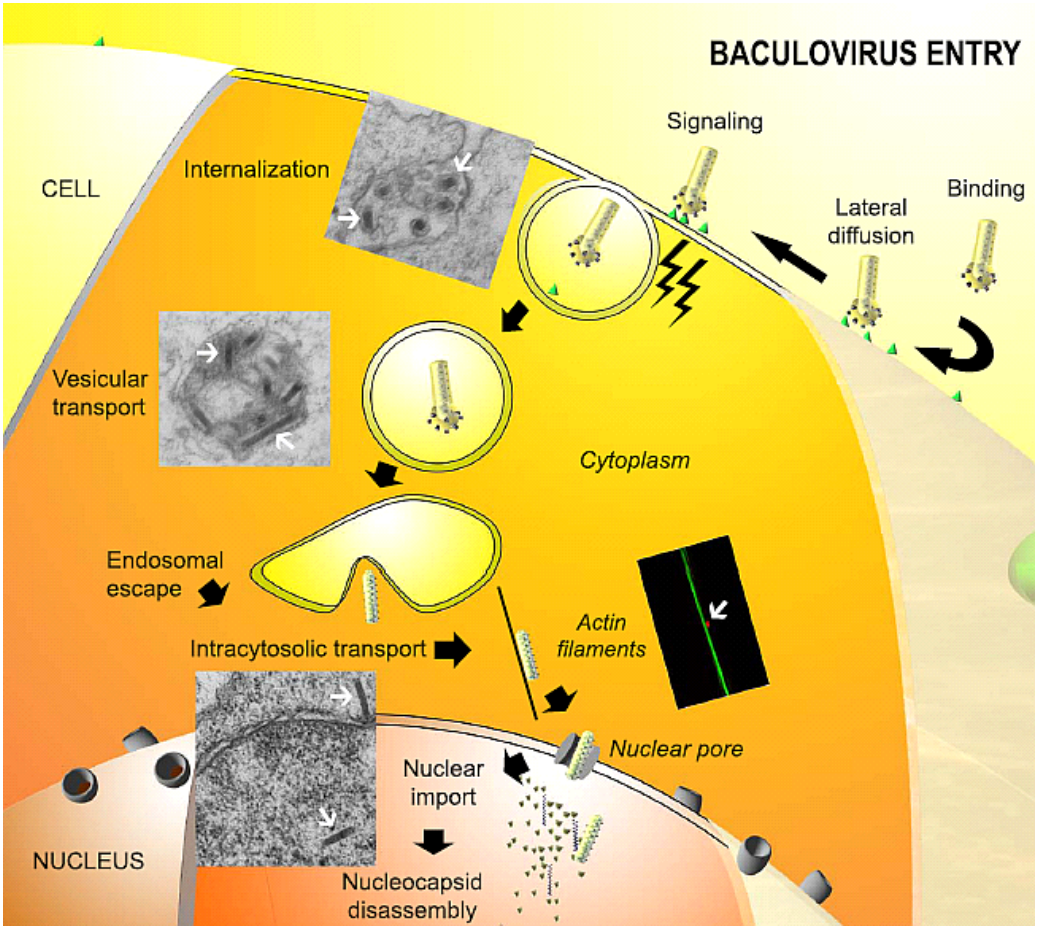


Figure 8. The entry mechanism of baculovirus in mammalian cells (Airenne et al., 2009).

not replicate in mammalian cells, some transcription products of early genes (IE-1 and IE-2) and translation of IE-2 were detected in mammalian cells (Hoopes, Jr. and Rohrmann, 1991; Murges et al., 1997; Dai et al., 2004; Laakkonen et al., 2008). Baculoviruses can transduce a wide range of mammalian cells, but in certain cell lines (e.g. Ea.hy 926, MG-63 and NHO) the transgene expression is weak. It has been suggested that this is due to the impaired intracytoplasmic movement and restricted entry of nucleocapsids from cytoplasm into the nucleus (Kukkonen et al., 2003; Kitajima et al., 2006).

2.3.8 Baculoviruses as a therapeutic vector

The safety and large cloning capacity make baculovirus a very attractive vector for gene therapy. The other benefits are that baculoviruses can easily be produced in high titers (up to 10^{11} pfu/ml) and they transduce also non-dividing cells, have a long history with intensive safety studies, they do not replicate in mammalian cells and are non-toxic even at high MOIs (Airenne et al., 2009). The *In vivo* gene transfer studies performed this far with baculoviruses are shown in Table 3.

The first *in vivo* gene transfer with baculovirus was performed into the liver parenchyma of rats and mice by directly injecting virus into the systemic and intraportal circulation (Sandig et al., 1996). These experiments resulted in undetectable transgene expression, pointing to virus inactivation by the complement system (Sandig et al., 1996), more exactly by activation of classical (Hofmann and Strauss, 1998) and alternative pathways (Hoare et al., 2005). Hofmann et al. studied baculoviruses in immune-compromised animals and obtained some transgene expression in the liver parenchyma of C5-deficient immunocompromised mice and in the Huh7-derived human hepatocarcinomas in nude mice (T cell deficient) (Hofmann et al., 1998). Systemic baculovirus gene delivery into complement deficient Neuro2a tumor-bearing A/J mice resulted in transgene expression in the tumor but also in liver, spleen, and kidney (Kircheis et al., 2001). Direct injection of recombinant baculoviruses into the quadriceps femoris muscle of BALB/c and C57BL6 mice resulted in a transient expression of β-galactosidase and this was more sustained when C5-deficient mice was used (Pieroni et al., 2001).

Table 3. *In vivo* gene transfer studies performed with baculoviruses.

Species	Tissue	Transgene	Reference:
Mice	Brain	GFP, Luc	(Sarkis et al., 2000; Tani et al., 2003)
	Testis	GFP, Luc	(Tani et al., 2003)
	Eye	GFP	(Haeseleer et al., 2001)
	Abdomen	GFP	(Huang et al., 2008)
	Muscle	LacZ, EPO, HA, E2/HCV, CS	(Pieroni et al., 2001; Abe et al., 2003; Facciabene et al., 2004; Strauss et al., 2007)
	Liver	Luc	(Hofmann et al., 1998; Kircheis et al., 2001; Nishibe et al., 2008)
	Lung	GFP	(Kim et al., 2006)
	Systemic administrarition	Luc, LacZ, GFP	(Kircheis et al., 2001; Hoare et al., 2005; Kim et al., 2006; Yang et al., 2009)
	Nasopharyngeal carcinoma	RTA	(Wang et al., 2008)
Rat	Brain	GFP, LacZ, Luc,	(Sarkis et al., 2000; Lehtolainen et al., 2002; Li et al., 2004; Laitinen et al., 2005; Wang and Wang, 2005; Ong et al., 2005; Li et al., 2005; Wang et al., 2006; Wang and Wang, 2006; Raty et al., 2006; Kaikkonen et al., 2006; Liu et al., 2006a; Boulaire et al., 2009)
	Liver	hFIX	(Hüser et al., 2001)
	Eye	Luc, GFP	(Li et al., 2005; Luz-Madrigal et al., 2007)
	Dorsal root ganglia	Luc	(Wang et al., 2005b)
	Biodistribution	LacZ	(Raty et al., 2007)
Rabbit	Artery	LacZ	(Airenne et al., 2000)
	Muscle	LacZ	(Kaikkonen et al., 2006)
	Disc	GFP	(Liu et al., 2006b)
	Chondrocytes (*ex vivo*)	BMP-2	(Chen et al., 2009)
	Eye	LacZ, VEG-$D^{\Delta N\Delta C}$	(Kinnunen et al., 2009)
Honeybee	Several tissue	GFP	(Ando et al., 2007)
Fish	Embryo	LacZ, GFP	(Wagle and Jesuthasan, 2003)

Abbreviations: BMP-2; Bone Morphogenetic protein-2, RTA; immediate–early proteins of EBV, E2/HCV; E2 glycoprotein of hepatitis C virus, CS; Plasmodium falciparum circumsporozoite, HA; influenza virus hemagglutinin, EPO; erythropoietin, Luc; luciferase, LacZ; gene encoding β-galactosidase, GFP; green fluorescent protein, VEGF-$D^{\Delta N\Delta C}$; vascular endothelial growth factor, hFIX; human Factor IX.

The inhibition of the complement system or the usage of a complement protected virus was shown to be effective in baculovirus-mediated gene therapy. Pegylation of baculovirus enabled transgene expression in brain, liver, spleen, lung, heart, and kidney of BALB/c mice (Kim et al., 2006). In addition, polyethylenimine (PEI) coating is shown to protect baculoviruses against human and rat serum-mediated inactivation (Yang et al., 2009). Intraportal application with a complement inactivator (sCR1) led to some hepatic expression of *Lac Z* marker gene in MF-1 mice (Hoare et al., 2005). Injection of decay acceleration factor -modified complement-resistant baculovirus vector into the liver parenchyma of complement-sufficient neonatal Wistar rats were also shown to enhance expression of the marker gene (Hüser et al., 2001). In addition, the synthetic protease inhibitor (FUT-175) and VSVG-pseudotyping prevented complement activation (Tani et al., 2003).

Delivery methods that allow gene transfer in the absence of serum, or to those sites where viruses are not exposed to complement, have led to more successful experiments in immune-competent animals. BALB/c mice, nude mice, BDIX rats and Sprague-Dawley rats were injected with recombinant baculovirus directly into the striatum of brain (Sarkis et al., 2000; Lehtolainen et al., 2002; Kaikkonen et al., 2006). Marker gene expression was detected in the striatum, the corpus callosum, and choroid plexus cells, evidence of the ability of baculoviruses to transduce neural cells *in vivo*. Subsequently, recombinant baculoviruses with tissue specific promoters were tested in brain also with promising results (Li et al., 2004; Wang and Wang, 2006; Liu et al., 2006a). Boosted expression was achieved with an expression cassette flanked with ITRs of AAV (Wang and Wang, 2005) or by a chimeric transactivator strategy (Liu et al., 2006a). Suppressed malignant glioma development was achieved by using a virus expressing the A-chain of diphtheria toxin (Wang et al., 2006). Efficient transduction into the intervertebral disc, an encapsulated and avascular immune-privileged tissue, was reported in rabbits (Liu et al., 2006b). In another study, lumbar intrathecal injection into the cerebrospinal fluid was used to transduce rat dorsal root ganglia cells (Wang et al., 2005b). In a different approach, carotid arteries of New Zealand White rabbits were successfully transduced with recombinant baculoviruses using a collar device. This system allowed gene delivery with minimal exposure to the complement system (Airenne et al., 2000). Transient expression in the adventitial cells was observed with an efficacy and duration comparable to Ads. The ocular tissue contains also immune priviledged areas and therefore is a potential target for baculovirus-mediated gene therapy (Haeseleer et al., 2001; Kinnunen et al., 2009). High gene delivery efficacy was observed also in immune privileged mice testis (Tani et al., 2003).

Gene silencing by RNA interference (RNAi) is a powerful approach to study function of genes. Baculoviruses have been used to deliver U6-based short hairpin RNA (shRNA against lamin A/C) into Saos2, Hepg2, Huh7, and primary human hepatic stellate cells (Nicholson et al., 2005). Baculovirus were also used to suppress transgene expression in rat brain (Ong et al., 2005). Suppression of porcine arterivirus, HBV, HCV, HIV-1 and influenza virus A and B replication by baculovirus-mediated shRNA delivery provides the proof of principle of baculovirus as a potential vehicle for antiviral therapy (Suzuki et al., 2005; Lu et al., 2006; Kaneko et al., 2006; Starkey et al., 2009).

Baculoviruses, expressing and/or delivering antigens, could also be used to obtain a valuable vaccine vehicle against infectious diseases. Baculoviruses exhibit a strong adjuvant property inducing proinflammatory cytokines and a type I interferon

response (Hu et al., 2008; Tani et al., 2008). Furthermore, baculoviruses have been shown be potentially useful in a vaccine production (van Oers, 2006; Hu et al., 2008). A cervical cancer vaccine, Cervarix (GlaxoSmithKline PLC), against the two most prevalent cancer-causing types (16 and 18) of the human papillomavirus, was approved for clinical use in woman aged 10 to 45 years old in Australia, Europe and Philippines in year 2007 (Harper et al., 2004). Other examples of advanced baculovirus based vaccines are the influenza vaccines, FluBlok and Panblock (Protein Sciences Corporation, see: http://www.proteinsciences.com), which have already shown efficacy in clinical trials (Safdar et al., 2006; Treanor et al., 2006; Treanor et al., 2007).

2.4 Site-specific recombinases

The endogenous mammalian repair system, homologous recombination (HR), has been widely used for the modification of murine embryonic stem cells (Glaser et al., 2005). Illegitimate recombination, inefficiency and laborious screening of correctly recombined cell clones are the major drawbacks of HR. Endogenous DNA recognizing zinc finger domains fused to an endonuclease were generated to create zinc finger nucleases (ZFNs). These nucleases were used for the repair of point mutations and for gene addition into a pre-determined locus in human cells (Moehle et al., 2007; Miller et al., 2007). In addition to endogenous mammalian repair systems, heterologous site-specific recombinases (SSR)-based targeting strategies have been widely used for DNA manipulation (Kolb, 2002; Akopian and Marshall, 2005; Wirth et al., 2007).

In site-specific recombination, a DNA strand is rearranged by enzymes (recombinase) which recognize and bind to specific DNA sequences (sites). Recombinase evokes the excision of the DNA segment between flanked two specific sites and then to the integration or inversion of the orientation of the flanked DNA segment (Figure 9). In intramolecular recombination, the recombination takes place between two sites which are in the same molecule, whereas in intermolecular recombination, the sites are situated on two different molecules. Site-specific recombination differs from general recombination i.e. these short sites must be present before recombination can be initiated (Grindley et al., 2006).

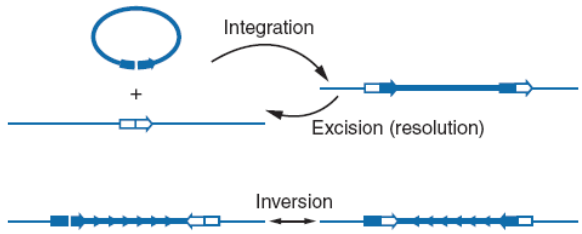


Figure 9. Three possible outcomes of site-specific recombination (Grindley et al., 2006).

Based on the amino acid sequence homology and biochemical features, most site-specific recombinases are grouped into one of two families: the tyrosine recombinase family or the serine recombinase family (Grindley et al., 2006). The names originate from the conserved nucleophilic amino acid residue that they use to attack the DNA and to which it becomes covalently linked during strand exchange. Tyrosine recombinases break and rejoin single strands in pairs to form a Holliday junction intermediate. In contrast, serine recombinases cut all strands in advance of strand exchange and religation (Stark et al., 1992).

Typical examples of tyrosine recombinases are the well known enzymes such as Cre (from the PI phage), FLP (from yeast *S. cerevisiae*) and λ integrase (from lambda phage) (Ghosh and Van Duyne, 2002; Chen and Rice, 2003). Commonly used serine recombinases include : gamma-delta resolvase (from the Tn*1000* transposon), Tn3 resolvase (from the Tn3 transposon) and ϕC31 integrase (from the ϕC31 phage) (Smith and Thorpe, 2002).

There are numerous applications of site-specific recombination. A wide variety of commercially available cloning vectors are based on recombinases (e.g. λ integrase in Gateway Technology and BaculoDirect, Invitrogen). Many viral vectors rely on recombinase-mediated construction steps (Aoki et al., 1999b; Cheshenko et al., 2001; Nakano et al., 2005). A large variety of approaches have been used to modify plants (Gilbertson, 2003) or the mammalian genome for gene activation, inactivation, deletion, integration, genome targeting, mutagenesis and for selection marker removal (Sauer, 1998; Nagy, 2000; Kolb, 2002). In addition, techniques called recombinase-mediated cassette exchange (RMCE) were established which involve the exchange of a chromosomally placed DNA cassette for a cassette located in an incoming plasmid (Bode et al., 2000b; Sorrell and Kolb, 2005). Since most of these strategies require the use of ES cells, *in vivo* experiments have typically only been performed in mice.

The limitation of almost all of the recombinase strategies is that the target of recombinase has to be first inserted into the genome using a classical homologous recombination step. Therefore, strategies using designer recombinases, that are able to recognise predetermined unique natural choromosomal sites, have been developed. These new recombinases were screened using multiple cycles of DNA shuffling/mutagenesis and selection (Collins et al., 2003). New variants of Cre, FLP and ϕC31 integrase with altered sequence recognition specificity were also developed (Sclimenti et al., 2001; Buchholz and Stewart, 2001; Santoro and Schultz, 2002; Voziyanov et al., 2003). Another strategy is to change the site specificity of the enzyme by replacing its DNA binding domain with another binding domain e.g. with a Zn-finger that recognizes a new target sequence (Akopian et al., 2003; Akopian and Marshall, 2005).

2.4.1 Cre recombinase

Cre recombinase (38 kDa) is a tyrosine recombinase from P1 bacteriophage that catalyzes site-specific recombination of DNA between two *loxP* site (Hamilton and Abremski, 1984). The reaction does not require any energy factors and reaches equilibrium between substrate and reaction products. In the bacteriophage P1, the function of Cre recombinase is to circulate the P1 genome during infection and maintain the genome in the monomeric state for cell division (Hamilton and Abremski, 1984).

The *LoxP* site (locus of X over P1), which is recognized by Cre recombinase, is a 34 bp long consensus sequence, consisting of a core spacer sequence of 8 bp and two 13 bp palindromic flanking sequences (Figure 10) (Hoess et al., 1982). A single recombinase molecule binds to each palindromic half of a *loxP* site, and then the recombinase molecules form a tetramer, thus bringing two *loxP* sites together. The post-recombination loxP sites are composed of the two complementary halves of the pre-recombinations sites (Sorrell and Kolb, 2005).

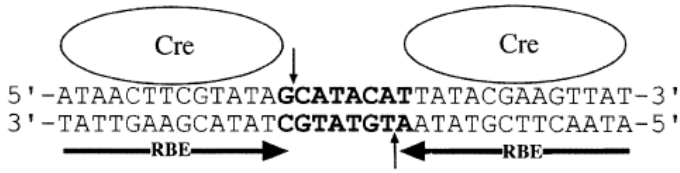


Figure 10. Cre binds to the loxP site. Cre specifically recognizes the recombinase binding elements (RBEs), which are arranged as inverted repeats surrounding a central 8-bp crossover region (shown in boldface). Cleavage sites are indicated by vertical arrows (Ghosh and Van Duyne, 2002).

The result of recombination depends on the location and orientation of the *loxP* sites. The orientation is defined by the asymmetric core sequence of *loxP* sites. They can be in a parallel or antiparallel orientation. In the case of parallel localization, the outcome is insertion of one DNA into another molecule, translocation between two molecules or excision of a *loxP* flanked DNA, whereas in the antiparallel orientation, this causes inversion of a *loxP* flanked sequences (Figure 11) (Aranda et al., 2001). One of the major advantages of cre recombinase is that there is no need for additional co-factors or sequence elements for effective recombination.

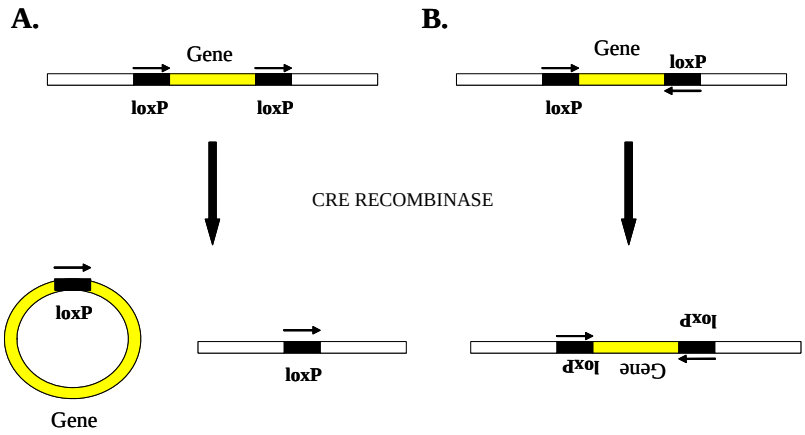


Figure 11. Schematic of *Cre*-mediated recombination of a target gene flanked by *loxP* sites. A) The parallel *loxP* sites. B) The antiparallel *loxP* sites.

Protein engineering has also led to the availability of improved second-generation Cre recombinases with novel properties. These changes include codon-improved Cre recombinases with better in vivo efficiencies (Koresawa et al., 2000; Shimshek et al., 2002), engineered recombinases with novel recognition sites (Buchholz

and Stewart, 2001; Santoro and Schultz, 2002), and Cre recombinases capable of permeating cell membranes (Jo et al., 2001; Peitz et al., 2002).

Cre recombinase is a powerful tool which has many applications. However, one major limiting factor is the potential toxicity of Cre. The toxicity of Cre has been demonstrated in mammalian cells (Schmidt et al., 2000; Silver and Livingston, 2001; Loonstra et al., 2001; Pfeifer et al., 2001; Heidmann and Lehner, 2001). Post meiotic spermatids of transgenic mice (Schmidt et al., 2000), mouse embryonic fibroblasts (MEFs) (Silver and Livingston, 2001; Loonstra et al., 2001; de, I et al., 2001), and NIH 3T3 cells (Silver and Livingston, 2001) were shown to be sensitive to the continuous Cre expression. In addition, in human kidney cell line 293 (Silver and Livingston, 2001), human osteosarcoma cell line U2OS (Silver and Livingston, 2001), Drosophilia cells (Heidmann and Lehner, 2001) and plants (Coppoolse et al., 2003) Cre toxicity has been observed. The toxicity of Cre depends upon strand cleavage (Silver and Livingston, 2001) of genomic DNA at sites that share homology with the *lox* sites of bacteriophage P1 (pseudo-*lox* sites). This cleavage causes genomic DNA strand breaks and chromosomal rearrangements (Silver and Livingston, 2001; Loonstra et al., 2001). These pseudo-*lox* sites have been found in human, mouse, yeast, and *E.coli* genomes (Sauer, 1992; Sauer, 1996; Thyagarajan et al., 2000). The toxicity in mammalian cells is attenuated by limiting the intensity and duration of cre expression, minimizing the chances for recombination or allowing repair of DNA damages (Silver and Livingston, 2001; Loonstra et al., 2001).

One alternative strategy to Cre is the Flp system derived from *Saccharomyces cerevisiae*. However, Flp has been reported to be less efficient than Cre (especially in ES cells) (Schaft et al., 2001). In addition, it also has poor thermal stability, its function being optimal at 25-30 °C and significantly reduced at 37 °C, whereas the optimal temperature for Cre is 37-39 °C which is more suitable for *in vivo* experiments (Buchholz et al., 1996b). Some optimized versions of Flp have been launched (Raymond and Soriano, 2007).

2.4.2 Applications of Cre recombinase

Soon after the first studies of Cre recombinases were done in tissue culture and in transgenic animals, the idea of conditional transgenesis was launched (Lakso et al., 1992). In the first applications of conditional transgenesis, the promoter and the coding region for the gene of interest were separated by a loxP-flanked STOP region. This region does not allow any transcription initiation from the promoter (transcriptional STOP). Cre expression causes removal of the transcriptional STOP and then the gene of interest will be expressed (Lakso et al., 1992).

The cre system is used to modify genes and chromosomes (deletion, inversion, translocation of desired DNA fragments) *in vitro* and *in vivo* (Nagy, 2000; Garcia-Otin and Guillou, 2006). The different kinds of alterations can be limited to certain cell types [tissue-specific knockout, see special issues of Genesis vol 26, issue 2, 2000 (Porret et al., 2006)] or they can be activated in a time-controlled fashion by drug/ligand administration [inducible knockout (O'Neal and Agah, 2007)] in a number of transgenic species. The availability of transgenic lines with tissue specific or inducible Cre expression makes it possible to inactivate or activate a gene of interest simply by breeding a "floxed" animal (an animal containing a desired DNA sequence between two *loxP* sites) to pre-existing Cre-transgenics (see: http://www.mshri.on.ca/nagy/).

2.5 The role of the cytoskeleton during viral infection

The cytoskeleton forms a 3D-network which is regulated by many accessory proteins. It mediates physical robustness, cell-cell contact, cell crawling, cell division, organelle or RNA transport during interphase and chromosome movement during mitosis and meiosis. Viruses utilize the cytoskeleton for intracellular transport either hijacking the cytoplasmic membrane traffic or directly interacting with the cytoskeletal transport machinery (Ploubidou and Way, 2001; Dohner and Sodeik, 2005; Radtke et al., 2006; Marsh and Helenius, 2006).

Within the eukaryotic cytoplasm, organelles, solutes, and a complex lattice-like mesh of microtubule, actin, and intermediate filament networks effectively restrict free diffusion of molecules larger than 500 kD (Seksek et al., 1997). The movements of large complexes, like membranous organelles and also ribonucleoprotein particles e.g. in developing oocytes are dependent on active processes and generally involve either actin

or microtubule cytoskeleton (Grunert and St, 1996; Hirokawa, 1998; Holleran et al., 1998; Mermall et al., 1998).

2.5.1 Microtubules

Microtubules (MTs) are found in all eukaryotes and they have important roles in organizing the spatial distribution of organelles throughout interphase as well as in the chromosomes during cell division. Microtubules are long (from 200 nm to 25 μm, diameter 25 nm), hollow tubes assembled from heterodimers of α-tubulin and β-tubulin and microtubule-associated proteins. The tubulin dimers polymerize leading to the formation of profilaments, which are then bundled into hollow cylindrical filaments. This structure is polaric with a dynamic fast-growing plus-end and a less dynamic minus-end. Microtubules are nucleated and organized by the microtubule organization centers (MTOCs), such as centrosomes and basal bodies (Wade, 2007). The major microtubule motor proteins are kinesin (movement toward the cell membrane) (Hirokawa and Noda, 2008) and dynein (movement toward the nucleus) (Hook and Vallee, 2006).

Most viruses take advantage of microtubule cytoskeleton-dependent movement for intracellular traffic. Ads (subgroup C) binds to microtubules through interaction with cytoplasmic dynein (Suomalainen et al., 1999; Kelkar et al., 2004), which drives motility toward the microtubule organizing center and the nucleus. Some data is also available that the subgroup C Ad might have some interactions with kinesin motor protein (Suomalainen et al., 1999). HIV-1 also uses dynein motors and the microtubule network for trafficking. Viral particles of HIV accumulate into MTOC and dynein together with microtubules facilitate the delivery of virus genome into the nucleus (McDonald et al., 2002; Anderson and Hope, 2005). The trafficking of AAV from the cell periphery to a perinuclear region is incompletely understood. A relative of AAV, canine parvovirus, utilizes the dynein machinery for its transport to the perikaryon (Suikkanen et al., 2003). Recent studies have shown that somewhat unexpectedly AAV2 can transduce cells independently of dynein function and intact microtubules (Hirosue et al., 2007). Endosomes containing influenza viruses were transported to the perinuclear region via a dynein-directed movement on a microtubules (Lakadamyali et al., 2003). Simian virus 40 (SV-40) utilize calveolae-dependent endocytosis and use also microtubules for viral transport into the ER (Pelkmans et al., 2001).

2.5.2 Microfilaments

Microfilaments (MFs), which are also often referred to as actin filaments, are solid rods made of globular proteins called actin. These thinnest filaments are common to all eukaryotic cells. MF range from 5 to 9 nanometers in diameter and are designed to bear large amounts of tension. In association with myosin (motor protein of actin), MFs help to generate the forces used in cellular contraction and basic cell movements. They also determine cell shape, strength, contraction and allow cells to form more elaborate structures like microvilli, lamellipodia, ruffles, cortical actin and filopodia. In MFs, long polymerized chains of the molecules are intertwined in a helix, creating a filamentous form of the protein (F-actin). The globular form of actin is called G-actin. Each microfilament exhibits polarity and therefore the two ends of the filament are distinctly different. In the trafficking process, cargo can travel along actin filaments using myosin, which generally moves toward the plus end of actin filaments (Revenu et al., 2004).

Numerous viruses interact with actin during their life cycles, both disrupting and rearranging the actin cytoskeleton to their own advantage (Cudmore et al., 1997). The Ad interaction with target cells induces polymerization of cortical actin filaments and an intact actin cytoskeleton is required for virus entry into the cells (Li et al., 1998), whereas in late state of ,natural infection, actin filaments are destroyed/rearranged (Staufenbiel et al., 1986; Mangel et al., 2003). The HIV movement inside the cell depends on both the actin and microtubule networks (McDonald et al., 2002). Actin is known to be necessary for efficient infectivity of HIV (Bukrinskaya et al., 1998; Iyengar et al., 1998). Depolymerization of the actin cytoskeleton reduced Gag release and viral infectivity in T-cells, and actin and tubulin inhibitors reduced Env incorporation into virions (Jolly et al., 2007). In addition, HIV-1, simian immunodeficiency virus, and intracellular mature vaccinia virus were sensitive to inhibition of the actin polymerization mediator (Arp2/3), but not MLV, HSV-1, and VSVG-pseudotyped HIV-1 (Komano et al., 2004). Some contradictory results were also published where actin was considered to be a barrier for HIV-1 infection: In particular, in cases where virions are lacking a functional Nef protein or the membrane does not contain a pH-dependent envelope protein from VSV or ebola (Campbell et al., 2004). Actin filaments are also important for vaccinia virus motility (Cudmore et al., 1995).

2.5.3 Intermediate filaments

Intermediate filaments (IF) are 10-12 nm in diameter and so intermediate in size compared with the other cytoskeletal components. IFs are coded by more than 70 different genes and are divided into five different types. Types I to IV are cell specific and cytoplasmic. Types I and II are the acidic and basic keratin expressed in epithelial cells. Type III includes vimentin, desmin, glial fibrillary acidic protein, syncoilin and peripherin, which are found, respectively, in mesenchymal cells, muscle cells, astrocytes, and some neurons. Type IV includes neurofilament proteins (L, M and H), nestin, synemin and α-internexin, which are found in most neurons, except for nestin found in neuroepithelial cells and synemin found in muscle cells. Finally, type V corresponds to the ubiquitous nuclear laminins (A/C, B1 and B2), which form a nuclear lamina underlying the nuclear membrane (Omary et al., 2006; Minin and Moldaver, 2008). The function of IFs has been studied by gene knockout experiments (Magin et al., 2004). These experiments provided the evidence that IFs were involved in cellular resilience and maintenance of tissue integrity (Galou et al., 1997). IFs have many organelle location specific (e.g. mitochondrial and golgi positioning) and protein-targeting (subcellular and membrane targeting of proteins) functions (Toivola et al., 2005) (see special issue of Experimental cell research concerning intermediate filaments, June 2007, volume 313, Issues 10, pages 1979-2282).

Many viruses cause rearrangements of intermediate filaments. IF network may play a role, in the early step of Junin virus replication. Subsequent to virus entry, the integrity of IF network is necessary for the normal replication of Junin virus in neural and fibroblast cells as well as in the Vero cell line (Cordo and Candurra, 2003). Polioviruses cause rearrangement of IF network during infection (Doedens et al., 1994). Vif of HIV is shown to colocalize with IFs (vimentin, keratin) and causing reorganization of IF (Karczewski and Strebel, 1996). Theiler's virus binds specifically to the IFs (vimentin and desmin), and following infection, the IF network becomes rearranged into a shell-like structure which surrounds a viral inclusion (Nedellec et al., 1998). Human respiratory syncytial virus causes biochemical and morphological changes in cytoskeletal IFs: The amount of vimentin and other cytokeratins are reduced because of proteolytic degradation (Garcia-Barreno et al., 1988). Frog virus 3 reorganizes vimentin-type IFs to surround the virus's cytoplasmic assembly sites (Chen et al., 1986; Murti et al., 1988). Vaccinia virus causes a marked reorganization of IFs around the nucleus of infected cells and an increase in the phosphorylation level of IF proteins vimentin and desmin (Ferreira et al., 1994). One strain of influenza A avian

virus induces morphological modification of IFs, changing their physiological radial pattern into an array surrounding the nucleus (Arcangeletti et al., 1997).

2.5.3.1 Vimentin

Vimentin (54 kDa, type III) is the major intermediate filament protein of mesenchymal cells (e.g. fibroblasts, epithelial cells, muscle cells, blood cells and leukocytes). It shows a high degree of sequence homology throughout all vertebrates from fish to humans (Nelson and Traub, 1982; Schaffeld et al., 2001) and its expression is developmental stage dependent (Herrmann et al., 1989). There are no diseases that are associated to vimentin (Omary et al., 2004). The protein is physiologically important even though vimentin -/- mice developed and reproduced without any obvious disturbed phenotype (Colucci-Guyon et al., 1994). The function of viementin has long remained an enigma, but recent studies have revealed that loss of vimentin can lead to morphological changes in glia cells, impaired wound healing, defects in capacity of fibroblast to migrate, decreased flow-induced dilation of resistance arteries, distributed homing of leukocytes to lymph nodes and lack of integrity in vascular endothelium (Ivaska et al., 2007). Vimentin is an organizer of many of the key proteins involved in cell adhesion, attachment, migration and cell signaling (see Table 4.).

Several viruses have been reported to cause major changes in vimentin during infection. Ads were shown to activate nonviral proteolytic enzyme which cleaves vimentin in the early stage of infection. The vimentin processing is serotype related and shown to be important for Ad2, Ad5, Ad4 and Ad9 (Belin and Boulanger, 1987). Retroviruses were also shown to cause cleavage of vimentin (Snasel et al., 2000; Naghavi and Goff, 2007). Human immunodeficiency virus type 1 protease (HIV-1 PR) is responsible for cleavage of vimentin and liberation of vimentin peptides, which cause changes in nuclear architecture and chromatin distribution (Shoeman et al., 1990; Shoeman et al., 2001). In addition, some other viruses rearrange vimentin like African swine fever virus (Stefanovic et al., 2005), vaccinia virus, bluetongue virus (Bhattacharya et al., 2007), frog virus 3 (Chen et al., 1986) and rotavirus (Weclewicz et al., 1994).

Table 4. Vimentin functions in different physiological processes and tentative or identified molecular targets involved in the observed effects. Table modified from Ivaska et al. (Ivaska et al., 2007).

Cellular function	Target of regulation/ action	Effect	Reference
Structural integrity of cells and tissue	Endothelial cell junctions, ECM interactions	Integrity of cell layers and tissues	(Nieminen et al., 2006)
Cell adhesion and migration	Integrins, cell adhesion molecules, cytoskeletal cross linking proteins	Formation, regulation and turnover of vimentin associated matrix adhesion (VMA)	(Homan et al., 1998; Gonzales et al., 2001; Kreis et al., 2005)
	Leukocyte attachment and migration	Transcellular migration	(Dejana, 2006)
Organization of cell membrane complexes	β-adrenergic receptor (βAR)	Modulates lipolysis	(Kumar et al., 2007)
	SNARE-receptor	Intracellular vesicular transport	(Faigle et al., 2000)
	Sodium-glucose cotransporter (SGLT1)	Glucose metabolism	(Runembert et al., 2004)
Association with other filaments	Actin filaments	Effect on mechanical features of network	(Esue et al., 2006)
	MTs	Moves along MTs	(Clarke and Allan, 2002)
Regulation of DNA	Genomic DNA, satellite DNA, telomere DNA, retroposons, mitochondrial DNA, MARs	DNA recombination, repair and transcription	(Traub, 1995; Tolstonog et al., 2000) (Tolstonog et al., 2001)
A scaffold/modulator for protein kinases	Different kinases	Regulates their distribution and ability to phosphorylate	(Perlson et al., 2005) (Pallari and Eriksson, 2006)
Cell stress and virus infection	Virus entry, heat shock proteins, caspase, p53, monocytes, macrophages	Tolerate different forms of stress (virus, heat, mechanical), apoptosis, cell death, immune response	(Byun et al., 2001; Belichenko et al., 2001; Mor-Vaknin et al., 2003; Yang et al., 2005; Zhang et al., 2006; Garg et al., 2006)

Abbreviations: ECM, extracellular matrix; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; MAR, matrix attachment region.

Phosphorylation is a key regulator of IF dynamics, modulating the organization of IF networks and the subcellular distribution of proteins (Sihag et al., 2007). Vimentin has a complex phosphorylation pattern with sites and kinases specific for different states (mitosis, differentiation and stress) (Omary et al., 2006). Most of the functions of vimentin are regulated by phosphorylation. Phosphorylation sites are clustered in the head and tail domains and some of these are highly conserved (Figure 12).

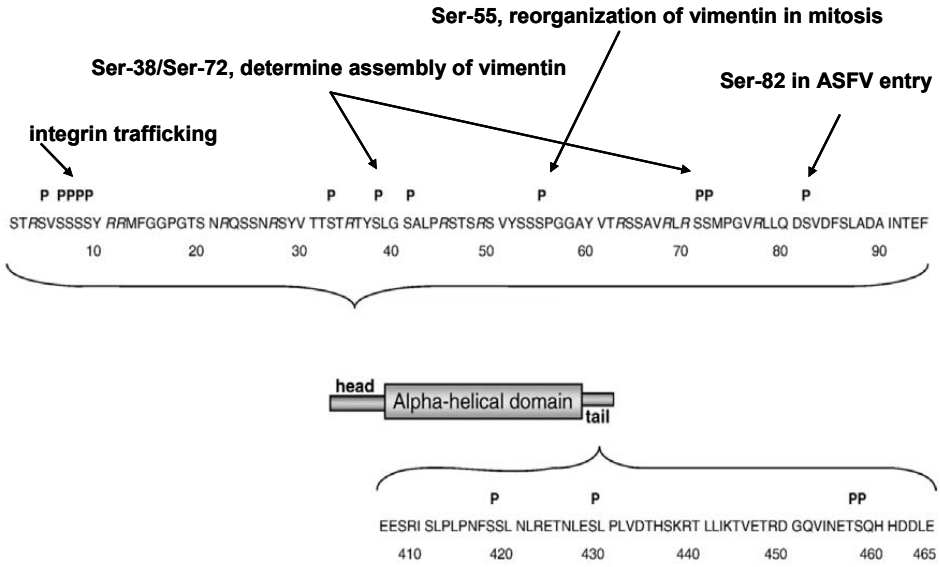


Figure 12. Phosphorylation sites of vimentin. Phosphorylaton of N-terminal phosphorylaton sites (4, 6-9) of vimentin regulates integrin traffic and cell motility (Ivaska et al., 2005). Ser-38 and -72 are important in determining the structure and assembly state of vimentin (Eriksson et al., 2004). The phosphorylation of vimentin at ser-55 has been shown to be essential for the disassembly of vimentin IFs during mitosis (Chou et al., 2003). African swine fever virus (ASFV) causes rearrangement of vimentin by phosphorylating Ser-82 (Stefanovic et al., 2005). The figure is modified from Ivaska et al. (Ivaska et al., 2007).

2.6 Factors influencing transgene expression in virus-mediated gene delivery

Viruses are obligatory intracellular parasites. Their transgene expression and the therapeutic consequences of transduction, depend on the ability to transmit their genomes into the nucleus of transduced cells. Virus entry is a multi step process and if it is successful, all of the steps have to occur at the right place and at the right time (Marsh and Helenius, 2006). In addition, viruses have to maintain their genes/transgenes and expression cassettes in such a form that natural transcriptional and translational machinery can use them as a template for gene expression (Makrides, 1999).

2.6.1 Efficiency of virus transport into nucleus

The first barriers encountered by incoming viruses are the layer of glycoconjugates (glycoproteins, glycolipids and proteoglycans) covering the external surface of cells, the plasma-membrane (Poranen et al., 2002), cortical actin (Gruenheid and Finlay, 2003) and the crowded cytoplasmic compartment (Luby-Phelps, 2000). In addition, the endosomal escape (Gruenberg and van der Goot, 2006) and traveling through the nuclear envelope (Whittaker, 2003) are also challenging for their survival and completing their mission.

Viruses need an active transport mechanism once they gain access to the inside of the cell. The reason is the molecular crowding of cytoplasm as a result of the presence of organelles, cytoskeleton and high protein concentrations (up to 300 mg/ml) effectively restricting free diffusion of molecules larger than 500 kDa (Luby-Phelps, 2000). Diffusion of a 100-bp DNA fragment in the cytoplasm is approximately five times slower than diffusion in water and diffusion of a 250-bp fragment and a 2000-bp fragment is 17 and 100 times slower, respectively. In the nucleus, DNA fragments are immobile (Lukacs et al., 2000).

In order to infect or transduce, the virus must bind to the host cell surface. There are two types of surface factors to which viruses can bind: attachment factors and receptors. Distribution and behavior of these cellular components determines which cell types, tissues, or organisms a particular virus can infect. Attachment factors, which usually concentrate viruses on cell surface, are usually non-specific and involve interaction with general cell surface factors like heparin sulfate or other carbohydrate structures. Contrary to attachment factors, receptors are specific cell surface molecules

and actively promote viral entry. The virus-receptor interaction causes a conformational change in the virus particle, activates signaling pathway, promotes endocytic internalization and determines which cell types and species are permissive for the virus. Hundreds of receptors and attachment factors have been determined and usually viruses can use more than one type of receptor (Young, 2001; Marsh and Helenius, 2006). For example immunodeficiency virus type I (HIV-1) uses heparin sulfate proteoglycans as their attachment factor, CD4 as a receptor, and chemokine receptors (CXCR4, CCR5) as coreceptors (Mondor et al., 1998; Berger et al., 1999). Ads use CAR-receptor for the attachment. Internalization of Ad is mediated by the interaction via $\alpha v\beta$ integrins, which leads to endocytosis of the virion via clathrin coated pits (Wickham et al., 1994).

The virus-induced signaling cascades are important in both earlier entry steps and in activation of the cytoskeleton/endocytic machinery. Ads are a good example of this kind of cell signaling (Nemerow and Stewart, 1999). The interaction between adenoviral pentons and integrins activates several signal cascades e.g. serine/theorine, tyrosine and PI kinases, phosphatases and PI 3-kinase (Greber, 2002; Pelkmans et al., 2005). The importance of signaling for viral entry emphasizes the fact that the activation of clathrin – and caveolae/raft –mediated endocytosis can be regulated by at least five different kinases (Pelkmans et al., 2005).

Many viruses enter inside the cell via endocytic vesicles. These existing pathways are designed for nutrient uptake, receptor down regulation and signaling. By using these pathways viruses can move deep into the cytoplasm and bypass many of the barriers like membrane context and cytosolic crowding. Viruses are known to use several different pathways for the endocytic internalization into mammalian cells (Figure 13). The most common and studied uptake pathway is the clathrin-mediated pathway (Greber and Way, 2006; Ungewickell and Hinrichsen, 2007), which is a continuous, efficient and rapid gateway into the cell. Viruses are transported together with their receptors into early or late endosomes. The incoming viruses are often exposed to the acidic environment of endosomes within minutes after internalization. Those viruses, which do not use clathrin-mediated uptake, use other routes (see Figure 13, examples of viruses are mentioned in the figure text). Some viruses use several different pathways to infect efficiently different cell types e.g. SV40 can use different pathways in order to efficiently infect different cell types and cells at different physiological states (Marsh and Helenius, 2006).

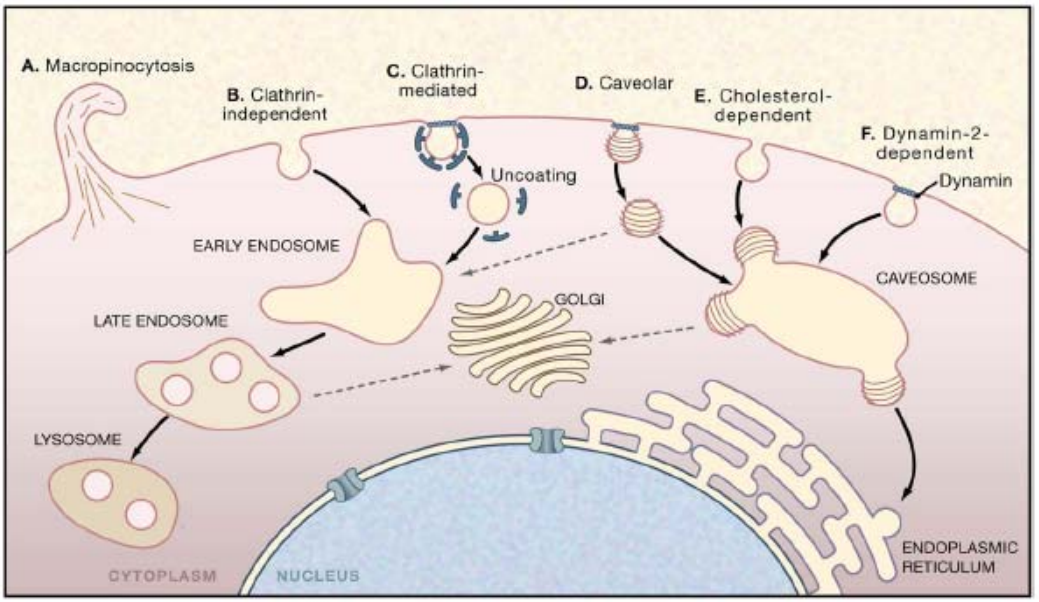


Figure 13. Endocytic pathways used by viruses (Marsh and Helenius, 2006; Mercer and Helenius, 2009). **A.** Macropinocytosis (e.g. CBV, Ad3, HIV-1, EV1, HSV, VV), **B.** Clathrin-independent or nonclathrin/non-caveolar (e.g. influenza virus), **C.** Clathrin-mediated (e.g. Ad2, Ad5, SFV, HIV-1), **D.** Caveolar (e.g. SV-40, BKV), **E.** Cholesterol-dependent/lipid raft-mediated (e.g. polyomavirus, SV-40) and **F.** Dynamin-2-dependent (e.g. Echo virus 1) pathways. Phagocytosis (e.g. HSV-1) is also one route, not shown in this figure. Abbreviations: CBV, coxsackie B virus; EV-1, echovirus; VV, vaccina virus; SFV, semliki forest virus; SV-40, simian virus 40.

Most endocytotic pathways terminate at the lysosomes and therefore viruses have developed different strategies to escape from endocytotic vesicles or to be able to pass through cellular membranes. The transfer of viral genome (DNA or RNA) and accessory proteins through the cellular membrane into the cytosol is called penetration. For the enveloped viruses, the penetration from endocytotic vesicles involves membrane fusion and for non-enveloped viruses means pore formation or membrane lysis. The fusion of the enveloped viruses with the endosomal membrane (pH-dependent or -independent) causes the release of the nucleocapsids into the cytoplasm (Earp et al., 2005; Marsh and Helenius, 2006; Weissenhorn et al., 2007). For example, the presence of adenoviral particles together with low endosomal pH cause endosomal disruption and escape to the cytosol (Meier and Greber, 2004). After penetration, the viral genome together with the accessory proteins is released into the cytoplasm where

most RNA viruses start replication. DNA viruses transport their capsid/genomic DNA to the nucleus for transcription (Marsh and Helenius, 2006).

Incoming plasmids and capsids are transported to the correct site within the cytosol or nucleus. For the transport, viruses usually use cytoskeleton, especially microtubules and different transport machineries (see paragraph 2.5).

The nucleus is a very restricted area and therefore viruses need a specific mechanism to import their genome into the nucleus (Whittaker, 2003). The import of virus and viral genomes occurs usually through NPCs. Viruses use nuclear localization signals and cytosolic import receptors to go through NPC. Viruses have four different ways to enter into the nucleus: 1) genomic DNA or RNA is released into cytoplasm from where it is actively imported into nucleus, 2) capsid binds to the cytosolic side of the pore where it is opened and the DNA passes through the pore, 3) capsid binds to pore, becomes disassembled and DNA is released into nucleus or 4) the capsid can pass through the pore in an intact form (Whittaker, 2003). Some viruses, like retroviruses, do not use the NPCs for nuclear entry. These viruses can enter only during mitosis when the nuclear envelope is temporally absent (Lewis and Emerman, 1994).

Capsids of some viruses enter into nucleus in an intact form. Good examples are Parvoviruses and Hepatitis B viruses whose disassembly takes place in the nucleus (Chapman and Rossmann, 1993; Kann et al., 1999). In addition, nucleocapsids of baculovirus are actively transported through the nuclear pore into the nucleus, where disassembly of the viral nucleocapid and exposure of nucleprotein core take place (Kukkonen et al., 2003; Salminen et al., 2005).

2.6.2 Transcriptional regulation

One of the major obstacles in gene delivery has been maintenance and regulation of transgene expression. In the cells bearing foreign DNA and expressing foreign proteins, there is a tendency for promoter elements controlling transgene expression to be silenced by methylation (Jones et al., 1998). Therefore the correct choice of appropriate promoters and surrounding DNA sequences are essential for attaining the desirable transgene expression (Makrides, 1999). In general, viral promoters are recognized as "foreign" in eukaryotic cells and therefore shut down. For example, the commonly used CMV IE promoter, which gives strong expression in most cell types, becomes down-regulated over time in most mammalian cells (Mehta et al., 2009). However, after the addition of a muscle cell specific enhancer element (Dai et al., 1992) or skeletal actin

promoter sequence (Hagstrom et al., 2000), the CMV promoter shutdown can be prevented. In addition, modifications like inclusion of an endogenous promoter to direct the expression of marker genes (Vile et al., 1994) or incorporation of a stable marker gene (Artelt et al., 1991) or usage of internal ribosome entry segments (IRES) (Attal et al., 1999) or inactivation of the 3′ LTR in SIN retrovirus vectors (Deglon et al., 2000), have had an effect on the transgene expression.

The controls that act on gene expression are more complex in eukaryotes than in prokaryotes. The main difference is the presence of a nuclear membrane in eukaryotes which prevents the simultaneous transcription and translation that occurs in prokaryotes. Transgene expression is controlled by promoters, enhancers, introns, polyadenylation signals (polyA), transcription terminators, Kozak sequence, UTRs, termination codon and codon usage (Makrides, 1999). Some comparative analyses have been done between different combinations of promoters, enhancers, introns and polyA sequences (Xu et al., 2001; Xu et al., 2002). *In vitro* and *in vivo* experiments revealed that an optimized expression cassette (composed of CMV enhancer, chicken β-actin promoter, β-actin intron and SV-40 polyA or composed of CMV enhancer, CMV promoter, intron A, SV-40 polyA) drove highest levels of expression (Xu et al., 2001). Later studies showed that Ad vectors containing the largest intron of CMV and bovine growth hormone polyA are powerful elements for efficient transgene expression (Xu et al., 2002).

Promoters, defined as the region of DNA that facilitates the transcription of a particular gene, are divided into universal or tissue specific promoters. Universal promoters are transcriptionally active in a wide range of cell types and tissues. Due to the capability of achieving a high level of gene expression within several DNA constructs (e.g. viruses, plamids, etc.), these promoters are still used widely throughout the scientific community (Papadakis et al., 2004). The widely used universal promoters are cytomegalovirus (CMV or CMV-IE), elongation factor 1α (EF1α), long terminal repeat (LTR), phosphoglycerate kinase (PGK), SV-40 and RSV promoter. EF1α promoter has been shown variously to exceed and outlast CMV-mediated expression from several vectors, including Ad, AAV and plasmid vectors, *in vitro* and *in vivo* (Nakai et al., 1998; Ye et al., 1998). The choice of the right promoter is important; e.g. chicken β-actin promoter has been shown to be more active than the CMV promoter in baculoviral vectors (Shoji et al., 1997).

Promoters can be also divided into those that function constitutively and those that are regulated by an inducer or a derepressor (Papadakis et al., 2004). Inducible

promoters are suitable for production of proteins which are toxic for cells, and for studying gene regulation during development. These kinds of regulated promoters are divided into ligand-inducible systems or stimulus-inducible systems. The most prominent ligand-inducible system has been tetracycline (tet) on/off system, which is based on sensitive prokaryotic tetracycline resistance operon (Gossen et al., 1995). Stimulus-inducible systems are activated by different factors e.g. by heat (Lee et al., 2001b), radiation (Marples et al., 2000) or metal-response elements (Steinwaerder and Lieber, 2000). Hypoxic response element (hypoxia-inducible factor 1; HIF-1) have triggered significant interest in the field of ischemic diseases and cancer therapies (Shibata et al., 2000; Su et al., 2002).

Tissue specificity of a promoter is of particular interest in gene therapy applications (transcriptional targeting). Endogenous eukaryotic promoters are typically inferior to viral promoters in terms of expression intensity (Papadakis et al., 2004). However, if one uses tissue specific promoters then the transgene expression was longer as compared to viral promoters, e.g. the apolipoprotein (apo) A-I promoter in conjugation with its intron and enhancers in Ad vector was able to drive transgene expression for up to six months (De Geest et al., 2000). The list of tissue- and disease-selective eukaryotic promoters is still expanding and also genome-wide endogenous promoter extraction and analysis are being evaluated (Xuan et al., 2005).

Introns are sections of DNA that will be spliced out after transcription of RNAs. About 95 % of human genes harbor introns. The average human gene contains 5-6 introns. The average length of introns is 2100 nucleotides (nt), but they can be as long as 100 000 nt. A number of elements regulating gene expression has been found within intronic sequences (Fedorova and Fedorov, 2003). Genomic constructs were shown to be expressed more efficiently than identical constructs lacking introns (Palmiter et al., 1991; Choi et al., 1991). Although many cDNA constructs lacking introns can be expressed efficiently, it has been shown that inclusion of introns can lead to a 10- to 20-fold increase in transgene expression (Buchman and Berg, 1988) and certain mammalian transcription units fail to produce RNA if they lack an intron; e.g. intronless β-globin gene is a good example (Buchman and Berg, 1988). In addition, the placement of introns has been shown to be essential for efficient gene expression; e.g. the placement of the small-t intron downstream of a cDNA leads to aberrant splicing within the gene (Huang and Gorman, 1990). The intron from the SV40 late gene (VP1) and the synthetic introns (SIS) (Ad splice donor and an immunoglobulin G splice acceptor) were shown to cause efficient transgene expression in different cell types (Petitclerc et al., 1995).

Eukaryotic mRNA contains a poly(A) tail (n ≈ 200) at their 3′ ends, which is added during a complex process of transcription (Proudfoot et al., 2002). The poly(A) tail is important for mRNA stability and translatability (Jackson and Standart, 1990; Gray and Wickens, 1998) The most commonly used poly(A) signals are derived from bovine growth hormone, mouse β-globin, the SV-40 early transcription unit, and herpes simplex virus thymidine kinase gene (Makrides, 1999). In lentivirus-based gene therapy vectors, the transgene expression cassettes are usually cloned without polyadenylation. The lack of the polyadenylation signal, however, has been shown to reduce transgene expression, e.g. the addition of SV40 early polyadenylation signal into the lentivirus backbone increased transgene expression 3 – 6.5-fold (Hager et al., 2008).

Transcription terminators have many beneficial effects on gene expression of prokaryotes by increasing mRNA stability, minimizing background transcription and increasing the level of protein production (Makrides, 1996). In both eukaryotes and prokaryotes, promoter occlusion has been shown to be a problem if transcription terminators are omitted. Continued transcription from an upstream promoter through a second transcription unit can inhibit the function of a downstream promoter (Proudfoot, 1986).

Chromatin is the physical structure of DNA in the nucleus. The eukaryotic chromosomes consist of DNA and proteins. The modulation of the structure of the chromatin is critical for the regulation of gene expression since it determines the accessibility of underlying DNA. Heterochromatin is dense chromatin structure and inaccessible for RNA polymerases, whereas euchromatin is open and actively transcribed. The structure of chromatin in the integration site is especially critical for integrating viruses. Several factors have been implicated in the regulation of transcription from chromatin (Owen-Hughes, 2003). These factors can be classified as enzymes that function as post-translational modifiers of the chromatin structure (covalent modifications), and those that manipulate chromatin structure non-covalently by driving ATP-dependent alterations to chromatin folding (Owen-Hughes, 2003). This mode of regulation has been referred to as epigenetic regulation which denotes an inherited state of gene regulation (Bernstein et al., 2007).

In covalent modifications, the histone proteins are subjected to a range of post-transcriptional modifications including acetylation, phosphorylation, methylation, ubiquitinylation and ribosylation. This part of epigenetic code is called the “histone code” (Strahl and Allis, 2000). The histones are subjected to more than 100 different posttranslational modifications. This additional information contained within the

chromatin proteins associated with DNA (the epigenome) has been shown to be inherited in each cell cycle (Turner, 2000).

Non-covalent ATP-dependent alterations of chromatin folding, such as nucleosome sliding, play also an integral role in gene regulation. Chromatin modelers are specialized multi-protein complexes that enable access to nucleosomal DNA by altering the structure, composition and positioning of nucleosomes (Saha et al., 2006). All eukaryotic cells contain at least five families of choromatin remodelers, which are specialized for altering the structure, composition, and positioning of nucleosomes (Saha et al., 2006).

Eukaryotic chromosomes are typically arranged as a 50- to 100 kb loop domains that are attached at their bases to the intracellular framework by non-histone proteins. Scaffold/matrix attachment regions (S/MARs) are sequence elements that define the borders of these chromatin domains containing functional segment of a gene or gene clusters. S/MARs are AT-rich DNA sequences with a high unwinding propensity that mediate the structural organization of the chromatin within the nucleus and have an important role in different aspects of chromatin function. These elements constitute anchor points of the DNA for the chromatin scaffold and serve to organize the chromatin into structural domains (Chattopadhyay and Pavithra, 2007). The inclusion of S/MARs into episomal vectors augment transcription/long-term gene expression (Bode et al., 2000a).

Locus control regions (LCRs) modulate the chromatin structure and have a strong transcription-enhancing activity of genes and gene clusters. They are operationally defined by their ability to enhance the expression of linked genes to physiological levels in a tissue-specific and copy number-dependent manner (Li et al., 2002). DNase I hypersensitive sites (HS sites), which are located in transcriptionally active sites, are thought to be controlled by LCRs and contain components of LCRs. The first identified LCR was the human β-globin locus (Grosveld et al., 1987).

Chromatin insulators are DNA areas between genes defining independent expression profiles for different genes (Valenzuela and Kamakaka, 2006; Bushey et al., 2008). They prevent the interaction between regulatory elements of neighboring gene loci. The presence of chromatin insulators can improve the expression performance of a widely used class of integrating vectors by protecting these vectors from chromosomal position effects, e.g. inclusion of the HS4 chromatin insulator from the chicken β-globin LCR has been shown to increase expression and the proportion of positive cells with retrovirus transduction (Emery et al., 2000).

2.6.3 Translational regulation

The optimal translation initiation sequence, which occurs on eukaryotic mRNAs, is called the Kozak sequence (gccPURINEccAUGg) (Kozak, 1984; Kozak, 1987a; Kozak, 1987b). The purines A or G in position -3 (three nucleotides upstream from the AUG codon) and G immediately following the AUG codon are crucially important for optimal translation initiation. Initiation by ribosomes will start at the first AUG codon, but if there is a weak or no Kozak consensus sequence some ribosomes bypass this part and scan downstream until another AUG start codon can be located (leaky scanning) (Kozak, 2005). It has been reported that the insertion of a Kozak sequence can significantly increase transgene expression (Olafsdottir et al., 2008).

5′- and 3′- untranslated regions (5′UTR, 3′UTR) of mRNA contain motifs capable of regulating many aspects of mRNA function and can thereby influence gene function (Hughes, 2006; Mariati et al., 2010). They can affect mRNA nuclear export, cytoplasmic localization, translational efficiency and stability. These UTRs are binding sites for regulatory proteins and RNAs. In addition, they can form different secondary structures influencing the access of translational machinery to the mRNA (Pelletier and Sonenberg, 1987). At least 10 % of human 5′-UTRs contain upstream open reading frames (uORFs) (Pesole et al., 2001). These frames are short potential reading frames and they can inhibit translation by restricting the access of ribosomes to the correct start codon. The 3′ UTR has been shown to influence the stability of mRNA (Ross, 1995). One determinant for the stability of eukaryotic mRNA is an AU-rich sequence in the 3′-UTR (Chen and Shyu, 1995). Kakoki et al. have demonstrated that the production of a transgene, *in vitro* and *in vivo*, can vary over a 100-fold range due to differences in the 3′-UTR in an otherwise identical expression cassette (Kakoki et al., 2004).

The Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) is a powerful cis-acting RNA element present in the viral 3′-UTR. It improves gene expression at the post-transcriptional level by modifying RNA polyadenylation, export and/or translation (Donello et al., 1998). The WPRE exhibits partial homology to the post-transcriptional regulatory element of HBV. Both post-transcriptional regulatory elements contain functionally important conserved RNA stem-loop structures (Smith, III et al., 1998). However, the WPRE contains an additional γ-element, which makes it particularly effective (Smith, III et al., 1998). It has been shown that insertion of the WPRE into the 3′-UTR of transgenes carried by adeno-, AAV-, retro- or lentiviral vectors may increase and extend their expression rate *in vitro* (Zufferey et al., 1999; Loeb

et al., 1999; Xu et al., 2003) and *in vivo* with a transgene, promoter and vector independent manner (Lipshutz et al., 2003; Xu et al., 2003). In lentiviruses, WPRE has been shown to increase titers and expression rate. The mechanism behind this effect is thought to be improved vector genomic transcript termination and not due to increased nuclear mRNA export, increased rate of transcription or increase in mRNA half-life as proposed earlier (Higashimoto et al., 2007).

In the genetic code, a stop codon (or termination codon) is a nucleotide triplet (UAA, UGA or UAG) within messenger RNA that signals a termination of translation. Analysis of mammalian genes has revealed that the base following the stop codon influences the efficiency of translation termination (G and A are preferred to C and U as a 4th base) (McCaughan et al., 1995).

Prokaryotic and eukaryotic genes exhibit a non-random usage of synonymous codons (Sharp et al., 1988). Usage of codons may affect translation efficiency of heterologous genes. For example, the jellyfish *Aequorea victoria* green fluorescent protein (GFP) is widely used as a reporter gene. However, the expression of the jellyfish GFP reporter gene was unsuccessful because of poor translation efficiency of the mRNA in the human cell environment, which is characterized by a set of isoacceptor tRNAs that are different from those used in the jellyfish. Using the newly designed codon-optimized ("humanized") GFP, efficient GFP expression could be achieved (4- to 10-fold increase) (Zolotukhin et al., 1996) .

2.7 Long-term gene expression

Viral vectors can be divided into integrating and non-integrating vectors. Integration of gene therapy vectors into the host genome in theory provides long lasting expression of the transgene, but the integration event is random (Naldini et al., 1996). Integration may result in mutagenic interruption and/or transcriptional activation/silencing of tumor suppressor- or oncogenes (Hacein-Bey-Abina et al., 2003a). In addition, the situation is complicated by the fact that retroviruses and lentiviruses tend to integrate in the coding or regulatory regions of transcriptionally active genes (Bushman et al., 2005). One way to achieve high transgene expression levels without affecting the cellular genome is to use episomal plasmid vectors which replicate extrachromosomally (Lufino et al., 2008).

2.7.1 Integrating vectors

Integrating vectors can provide the long-term transgene expression in many gene therapy applications. The most widely studied integrating vectors are retro- and lentiviruses (see chapter: Gene transfer vectors). One common property of retroviruses is their ability to reverse transcribe the viral RNA to linear double stranded DNA and to induce integration of this DNA into the host genome resulting in stable expression (Greene and Peterlin, 2002; Baum et al., 2006). Lentiviruses were also developed to be non-integrating in order to prevent insertional mutagenesis. In these approaches, the integrase gene (IN) is mutated which allows formation of circular episomal genomes in the nucleus of the transduced cells (Philippe et al., 2006). This defective integrase together with SV40 promoter/origin of replication also allows sustained gene expression (Vargas, Jr. et al., 2008). Furthermore, the development of lentiviruses, which are capable of site-specific integration, is under intensive research e.g. the wild type integrase has been fused to zinc finger proteins, which direct integration into some desired genomic area. Even though integration is at least partly directed to the desired areas, non-specific integrations can still occur (Tan et al., 2004).

Adeno-associated viruses are also capable for long-term gene expression. The wild type AAV, which contains the rep gene, can integrate into chromosome 19, whereas the AAV vectors used in gene therapy lack the rep gene and they mainly generate episomal head-to-tail concatamers (Nakai et al., 2001; Schnepp et al., 2003).

2.7.2 Episomal vectors

Genetic engineering of episomal (extrachromosomal) vectors offers an interesting alternative to circumvent many of the problems of insertional mutagenesis. Episomal vectors persist in multicopies in the nucleus, resulting in amplification and replication of the gene of interest without any rearrangement of the cellular DNA (Van Craenenbroeck et al., 2000; Lufino et al., 2008). Episomal plasmids can be divided into viral episomal vectors [e.g. developed from several DNA viruses, including Epstein-Barr virus (EBV), bovine papilloma virus (BPV) and BK virus (BKV)] or chromosome-based episomal systems (e.g. S/MAR vectors and human arficial chromosomes) (Table 5.). Viral episomal systems contain a viral origin of DNA replication and a virally

encoded gene that transactivates the viral origin and allows the episome to replicate in the host cells. Chromosome based episomal systems contain parts of genomic areas like S/MARs or functional parts of natural chromosomes which enable long-term episomal gene expression.

Episomal vectors offer several advantages over integrating vectors. They persist in the nucleus in an extrachromosomal state, do not lead to cell transformation, persist in multiple copies per cell, result in high expression of the transgene and have a high insert capacity.

Table 5. The properties of episomal vectors. Table modified from Lufino et al. (Lufino et al., 2008).

Episomal vector	Insert capacity	Copy number	Retention during cell cycle (%)	Comments
Viral episomal vectors:				
SV40	< 17 kb	1-100 000	NA	Transient, cause cell death, cell cycle independent replication, oncogenic
BKV	6 kb	20 -150	NA	Widespread in humans, latent in kidney and lymphocytes, oncogenic
BPV-1	9 kb	15- 80	NA	Cell cycle independent replication, oncogenic
EBV plasmid	20-30 kb	5 – 30	94 – 98	Widespread in humans, latent in lymphocytes, cell cycle dependent replication
EBV-BAC	20-300 kb	2 – 17	92 – 98	-
Lenti/mutated IN	< 10 kb	NA	NA	Transient, low level expression
Lenti/mutated IN/ SV-40	< 10 kb	NA	NA	For malignancies caused by SV40
AAV	< 5 kb	NA	NA	Parts of AAVs are integrated
Chromosome-based episomal vectors:				
S/MAR-plasmid	20 - 30 kb	4 -13	98	Delivery challenges
S/MAR-BAC	135-300 kb	1 – 4	97.5 – 99.8	Delivery challenges
Minichromosomes	Unlimited	1 -2	94 -100	Delivery challenges
HAC	300 kb	1 – 2	98 – 100	Needs optimization; safety, stability, production

Abbreviations: BKV, BK virus; BPV, bovine papilloma virus; EBV, Epstein - Barr virus; BAC, bacterial artificial chromosome; IN, integrase; S/MAR, scaffold/matrix attachment region; HAC, human artificial chromosome; NA, not analyzed.

2.7.2.1 Epstein-Barr virus (EBV) based vectors

EBV is a human gamma herpes virus that latently infects a large proportion of the human population. EBV contains a linear double-stranded DNA of 172 kb inside an enveloped capsid (Lindahl et al., 1976). The two main cellular targets of EBV are B lymphocytes and epithelial cells. About 90 % of the world population is estimated to be infected with EBV during early childhood (Cohen, 2000). The infection cycle of EBV is divided into two stages: lytic cycle and latent cycle (Mattia et al., 1999; Tsurumi et al., 2005). During latency, about 10 of the 100 genes are expressed, whereas during the lytic cycle most genes are expressed. The infection is usually asymptomatic, but can cause infectious mononucleosis in some, usually adolescent people. In a minor fraction of humans, EBV can contribute to several cancers e.g. Burkitt's lymphoma, some T-cell lymphomas, Hodgkin's disease, post-transplant lymphoproliferative disease, nasopharyngeal carcinoma and gastric carcinoma (Cohen, 2000).

Two viral elements of EBV are responsible for the extrachromosomal maintenance: latent replication origin (OriP) (Yates et al., 1984) and EBV nuclear antigen 1 (EBNA-1) (Yates et al., 1985; Rawlins et al., 1985; Ambinder et al., 1991). These elements are necessary for the efficient replication of circular episomes and, together with EBNA-1, allow for segregation of the replicated genomes to daughter cells (Lupton and Levine, 1985). Replication is cell cycle controlled and occurs only once per cell cycle (Yates and Guan, 1991).

EBNA-1 is a 641 amino acid long phosphoprotein (76 kDa). EBNA-1 is a nuclear DNA-binding protein which is divided into several domains associated with various functions (Ambinder et al., 1991). The N-terminus of the protein contains DNA linking activity areas whereas C-terminus of the protein contains dimerization/DNA binding activity areas (Figure 14). The nuclear localization signal is located in the middle of the protein and its main function is to activate the replication of OriP containing episomes during every cell cycle by binding to dyad symmetry (DS) and family of repeats (FR) areas of OriP (Frappier and O'Donnell, 1992). EBNA-1 acts also as a transcriptional regulator (Sugden and Warren, 1989). Since it can bind to metaphase chromosomes and interphase chromatin, especially at newly replicated regions. This interaction is considered to facilitate the partition of oriP plasmids into the daughter cells during mitosis, ensuring high mitotic stability of EBV-based plasmids (Ito et al., 2002). Different

cellular factors have also been shown to participate in this replication process (Deng et al., 2003). One very interesting feature of EBNA-1 is the capability of the Gly-Ala repeat to prevent cytotoxic T lymphocyte -mediated immune attack (Ossevoort et al., 2003).

OriP (1.8 kb), a cis-acting element identified as the origin of DNA replication (Figure 14), is composed of two noncontiguous regions: FR and DS (Yates et al., 2000). The FR region consists of 20 tandem imperfect copies of 30-bp repeat sequences. These 30-bp repeats contain the consensus sequence for EBNA-1 binding. DS contains four related copies of the 30-bp repeat (Rawlins et al., 1985; Reisman et al., 1985). The FR is responsible for DNA replication. Replication initiation is greatly stimulated by the FR, possibly by formation of a DNA loop between the FR and the DS sites. FR contains a replication fork barrier and therefore the replication from oriP proceeds mainly unidirectionally (Gahn and Schildkraut, 1989; Tsurumi et al., 2005). The DS (120-bp) region that can be divided into two halves, each containing two EBNA-1 binding sites, is separated by a 9-bp sequence. The main function of DS is to stimulate the initiation of replication and to work as a functional replicator. Replication of *oriP*-dependent plasmids occurs only once within a cell cycle (Yates and Guan, 1991). Replication is performed by the cellular replication machinery under cell cycle control (Adams, 1987). For initiation of DNA synthesis in the presence of EBNA-1, DS mediates recruitment of the human homologues of eukaryotic initiation factors like origin recognition complex (ORC) and replication licensing factor (MCM) (Tsurumi et al., 2005).

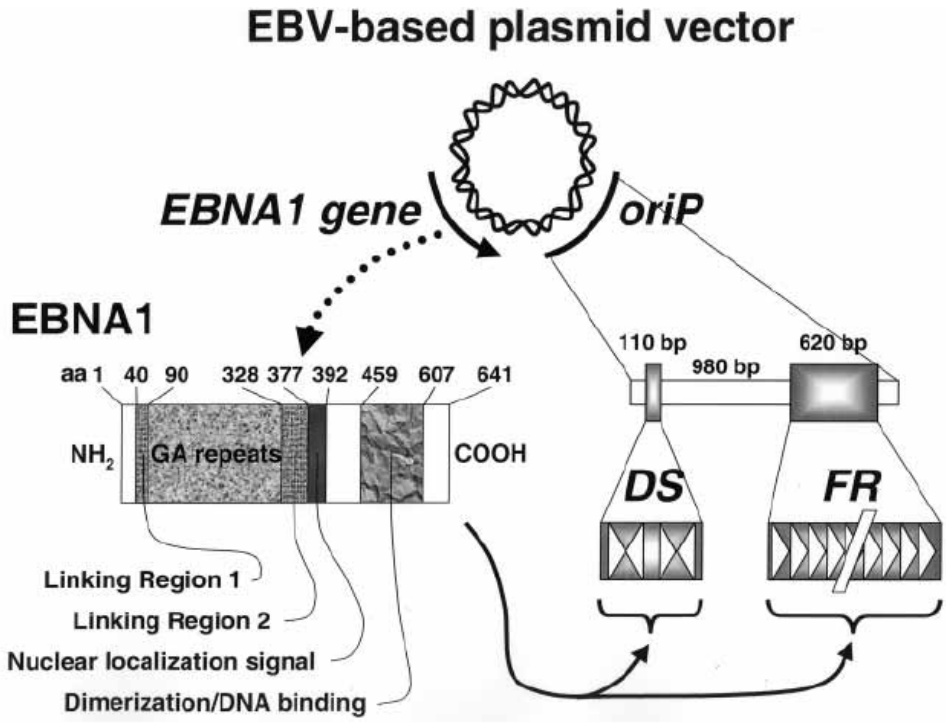


Figure 14. The structure of EBNA-1 and oriP in EBV-based vectors (Mazda, 2002). See the text for the details.

2.7.2.2 EBV based vectors in gene therapy

The typical EBV-based plasmid consists of the oriP and EBNA-1 sequences, the expression cassette for eukaryotic selection (e.g. hygromycin B), and sequences necessary for the maintenance and amplification in bacteria, and the transgene of interest under the desired promoter (Van Craenenbroeck et al., 2000). These elements are sufficient to ensure the retention and replication of EBV vectors in many different cell lines like fibroblasts, peripheral blood cells, bone cells lymphoma cells, monkey cells, dog cells (Yates et al., 1985; Van Craenenbroeck et al., 2000). EBV-based vectors can also replicate in rodent cells such as rat and mouse cells (Mizuguchi et al., 2000), but the presence of the human helper protein (eukaryotic rRNA processing protein EBP2) seems to be essential in some murine cell lines (Habel et al., 2004). In addition, EBV

vectors have been successfully applied also in primary cells (Min et al., 2003; Zhang et al., 2003) and stem cells (Ren et al., 2005; Ren et al., 2006; Thyagarajan et al., 2009).

EBV-derived expression vectors have already been successfully investigated in several studies, including expression of cytokine genes, cytokine receptors, growth factors and amino-acid transporter (Mackey and Sugden, 1999; Van Craenenbroeck et al., 2000). Table 6. summarizes *in vivo* experiments done with EBV-vectors.

The large DNA capacity of EBV-based vectors has encouraged attempts to deliver large (> 100 kb) genomic loci (White et al., 2002; Wade-Martins et al., 2003). The entire human β-globin gene locus (185 kb) was cloned into an EBV-vector and maintained in murine fibroblast cells as an episome for a period of three months (Black and Vos, 2002). In addition, a 117 kb episome was retained in human cells for 15 months and could be shuttled unrearranged from human cells into bacterial cells (Wade-Martins et al., 1999).

EBV-derived vectors have many advantages such as stable maintenance of large DNA fragments and avoiding the tendency for chromosomal integration. However, some integration of the vector in the host chromosome, rearrangements of EBV-derived vectors, toxicity in epithelial cells and regulation of proto-oncogenes have been reported (Van Craenenbroeck et al., 2000; Jones et al., 2003). EBNA-1 has been suggested to be oncogenic in mice (Wilson et al., 1996; Schulz and Cordes, 2009), but also an opposite result has been published (Kang et al., 2008). It is known that only a small percentage of the *oriP* episomes introduced into human cells is able to form a stable replicon. Therefore, most of the studies have been done under selective pressure. The reasons for the unstability of replicons without selection are partly unknown, but epigenetic events (Leight and Sugden, 2001), such as methylation (Kameda et al., 2006) have been suggested. In addition, insufficient expression of EBNA-1 is also known to influence the maintenance of replicons (Kreppel and Kochanek, 2004; Shan et al., 2006).

Table 6. Some *in vivo* gene therapy experiments performed with EBV-vectors.

Vector	Animal/tissue	Administration	Transgene	Duration	Reference
EBV-plasmid	Mice	i.v. injection	LacZ, Luc, angiostatin, p53, hFIX	> 8 weeks	(Tu et al., 2000; Cui et al., 2001) (Sclimenti et al., 2003)
EBV-plasmid	Rat/heart	gene gun	LacZ	1 – 6 weeks	(Tomiyasu et al., 1998; Nishizaki et al., 2000)
EBV-plasmid, EBV/minichromosomes	Mice/muscle	Injection, electroporation	LacZ, human dystrophin, Luc, FVIII	> 5 -12 weeks	(Tsukamoto et al., 1999; Magin-Lachmann et al., 2003; Mei et al., 2006)
EBV-plasmid	Rat/kidney	Catheter	Luc	8 weeks	(Tsujie et al., 2001)
EBV-plasmid	Mice/Tumor	electroporation, injection, liposome,	IL-12, LacZ, Luc, TK, MDR1, IL-18 FasL	> 21 days NA > 7days < 25 days > 1 month NA NA	(Maruyama-Tabata et al., 2000; Kishida et al., 2001) (Otomo et al., 2001; Izumo et al., 2007) (Lee et al., 2001a; Nakanishi et al., 2003) (Iwai et al., 2002)
EBV-plasmid	Mice/lung	Systemic administration	Luc	> 3 weeks	(Zhang et al., 2003)
EBV-plasmid	Mice/liver,	i.v. injection, liposome	Luc, α_1-antitrypsin, LacZ	10 days – 9 months	(Saeki et al., 1998; Stoll et al., 2001) (Kaneda et al., 2000)
EBV-Baculovirus	Mice/tumor	Tumor injection	RTA	> 19 days	(Wang et al., 2008)
EBV-Ad *	Mice/liver	i.v. injection	EYFP	> 6 weeks	(Gallaher et al., 2009)

Abbreviations: i.v., intravenous; Luc, luciferase; GFP, green fluorescent protein; LacZ, gene encoding β-galactosidase; hFIX, human Factor IX; FasL, Fas ligand; TK, thymidine kinase; RTA, viral lytic gene of EBV; MDR1, multi-drug resistance gene; EYFP, enhanced yellow fluorescent protein; FVIII, coagulation factor VIII. * = human replication origin together with FR used.

2.7.2.3 EBV based hybrid vectors

Hybrid vectors have been created to combine desired features of different vectors. Several episomal hybrid vectors have been constructed e.g. for cases where longer expression times are needed. These vectors combine the advantages of episomal vectors with the features of a backbone vector (Van Craenenbroeck et al., 2000).

In order to obtain long-term Ad-based gene expression, an Ad-EBV hybrid vector was developed that could deliver an episome to the nucleus of the infected cells (Tan et al., 1999; Leblois et al., 2000; Dorigo et al., 2004; Kreppel and Kochanek, 2004; Gallaher et al., 2009). This hybrid system consisted of two different vectors. Upon coinfection of cells, Cre recombinase expression from one Ad vector induced the recombination of loxP sites on a second vector that contains all of the sequences necessary to generate an EBV episome (Tan et al., 1999; Leblois et al., 2000; Dorigo et al., 2004). The FLP-mediated circulation of the episome has also been used instead of the Cre/loxP-system (Kreppel and Kochanek, 2004).

Hybrid vectors consisting of EBV sequences together with retrovirus (Grignani et al., 1998), herpesvirus (Wang and Vos, 1996; Muller et al., 2005) and baculovirus (Shan et al., 2006; Wang et al., 2008; Lo et al., 2009) elements have been described. The baculovirus hybrid vector containing *EBNA-1* and *oriP* elements was capable of gene expression which persisted for 60 days in HEK293 cells without any selective pressure. The long episomal expression of baculoviral vectors was achieved by using the effective CMV promoter to express EBNA-1. This recombinant baculovirus was evaluated as outstanding as compared with control baculoviral vectors without CMV promoter driven EBNA-1 (Shan et al., 2006).

2.7.2.4 Bovine papilloma virus derived vectors

Bovine papilloma virus belongs to a group of DNA viruses (double stranded genome of 7.3-8.0 kb) of the family of *Papilloviridae* that are common in cattle. It causes warts in the epithelial tissues and different types of cancers (Lancaster and Olson, 1982).

BPV-1 is most intensively studied for the stable expression experiments. Double stranded circular genome consist of E (early) and L (late) regions (Van Craenenbroeck et

al., 2000). E-region is responsible for DNA replication, regulation of transcription and cellular transformation, whereas L-region codes structural proteins of virion. The minimal ori and minichromosome maintenance elements (MME) are required for stable extrachromosomal replication together with the functional domains of E1 (ATPase activity, DNA-binding domain and E2 interaction domain), E2 (transactivation domain and DNA-binding domain) and E3-E8. These essential elements represent 69 % of the viral DNA of BPV-1.

These BPV-1 based episomal vectors have been successfully used to express rat preproinsulin (Sarver et al., 1981), human growth hormone (Pavlakis and Hamer, 1983), influenza virus hemagglutinin (Sambrook et al., 1985) and vesicular stomatitits virus glycoprotein G (Lefkowitz et al., 1990). The major drawbacks of BPV-1 based vectors are a high frequency of DNA rearrangements, transforming capacity (Uemura et al., 1992) and the fact that they may integrate into chromosomal DNA (Ashman and Davidson, 1985; Waldenstrom et al., 1992).

2.7.2.5 BKV derived vectors

BKV is a member of human polyomavirus family. The BKV is widespread and 50 % of the 3-year-old children have got a primary infection. Primary infection of BKV is asymptomatic or causes only a mild respiratory infections or fever. Virus may be activated under conditions of immunosuppression causing upper respiratory or urinary tract disease (Van Craenenbroeck et al., 2000).

The genome of BKV is divided into an early region (coding the large T and the small t antigen) and a late region (coding viral capsid protein) and a non-coding regulatory region. The presence of the BKV early promoter, small t antigen and large T antigen are necessary if one wishes to create a typical BKV-expression vector (Seif et al., 1979; Van Craenenbroeck et al., 2000).

BKV has been used to express different reporter genes, the herpes simplex virus type I glycoprotein B and human serotonin receptor h5-HT_{1B} (Van Craenenbroeck et al., 2000). The major drawback is BKV's capability to cause transformation of the transfected cells by binding p53 in the cell (Harris et al., 1996). In addition, it has been shown that BKV is mutagenic and causes chromosomal aberrations in human cells (Trabanelli et al., 1998).

2.7.2.6 S/MAR based vectors

DNA replication occurs in association with the nuclear matrix in the mammalian cells. This proteinaceous framework is generally termed as S/MAR (Lufino et al., 2008). These regions, usually several hundred nucleotides in length, are typically AT-rich stretches and clusters of topoisomerase II cleavage sites and do not share a strictly conserved consensus sequence. It has been shown that 11 % of the conserved noncoding DNA consist of predicted S/MARs in human and mouse (Glazko et al., 2003; Girod et al., 2007). The comparison of seven different matrix/scaffold attachment regions showed that human β-globin S/MAR is one of the most effective (Kim et al., 2004). S/MARs have been found nearby to promoters, enhancers and origins of replication. A wide range of possible functions has been considered for S/MARs, including transcription activation by chromatin remodeling (Bode et al., 2000a) and insulation of genes from position effects (Allen et al., 2000).

The human interferon β-gene has been the most extensively studied S/MAR. It has been shown to be functional in CHO cells (Kim et al., 2004; Jenke et al., 2004; Lufino et al., 2007) and primary human cells (Papapetrou et al., 2006). A vector containing SV40 ori and S/MAR sequence isolated from the human β-interferon gene was stably retained in CHO cells without selection pressure for over 100 generations (Piechaczek et al., 1999). The S/MARs, which have been tested *in vivo*, are listed in table 7.

Table 7. S/MARs tested in *in vivo*.

Vector	S/MAR derived from	Target tissue	Gene of interest	Duration	Reference	Comments
pHM5/hFIXmg	Chicken lysozyme locus (ChMAR)	Mouse liver	Human coagulation factor IX (hFIX)	1 year	(Ehrhardt et al., 2003)	5-10 -fold higher expression levels
pEPI-EGFP	Human β-interferon	Pig Sperm	EGFP	Fetuses analyzed on day 70 of pregnancy	(Manzini et al., 2006)	Transgenesis
pLucA1	Human β-interferon	Mouse liver	Luciferase	> 6 month	(Argyros et al., 2008)	Liver specific promoter

2.7.2.7 Artificial chromosomes

The design of vectors resembling natural chromosomes is a very interesting approach and ideally these structures would behave like normal chromosomes. Artificial chromosomes are divided into three main classes: human artificial chromosomes (HAC), yeast artificial chromosomes (YAC) or bacterial artificial chromosomes (BAC or PAC). HACs are microchromosomes that can replicate and segregate as a normal chromosomes and they use human functional components for their replication and stable propagation. Yeast artificial chromosomes contain all sequences needed for maintenance in yeast cells, whereas BAC/PAC contains elements for replication and preservation in bacterial cells. Since artificial chromosomes are independent molecules/structures, they should be free of possible adverse effects such as insertional mutagenesis and positional effects (Grimes and Monaco, 2005).

Since the first prototype of HAC was launched in 1997, a number of artificial chromosomes have become available (Harrington et al., 1997; Macnab and Whitehouse, 2009). These HACs can be engineered in two different ways: de novo assembly of chromosomal components to form fully functional chromosomes or assembly of chromosomes through manipulation of endogenous chromosomes (Irvine et al., 2005). HACs have been developed for gene expression studies and targeting strategies e.g. HACs containing genomic fragment of 40-kb hypoxanthine guanine phosphoribosyltransferase (HPRT) locus were created (Mejia et al., 2001; Grimes et al., 2001). The expression of HPRT lasted for more than 60 days without selection and efficiently complemented the metabolic defect in HPRT-deficient HT1080 cells (Grimes et al., 2001). HACs are also very useful tools for elucidating human chromosome functions. Even though substantial progress towards the generation of HACs has been made, there are still several targets for future development e.g. achieving the feasible purification of large chromosomes, overcoming problems in transferring of HACs, improving their stability and safety (Larin and Mejia, 2002; Irvine et al., 2005).

3 AIMS OF THE STUDY

The aim of this thesis was to study baculoviruses as gene therapy vectors and to improve their transgene expression profile and duration. These aims were divided into specific tasks as follows:

(I) Could EBNA/oriP as a part of baculovirus be used to achieve long-term and persistent expression in vertebrate cells?

(II) Could an intron be used to inhibit leaky expression of cre-recombinase in *E.coli*? Could the modified *loxP*-sites flanking *Cre*-gene be used to self-inactivate cre-recombinase expression and to prevent the toxicity of Cre expression in mammalian cells? Would this self-inactivating Cre-recombinase still be functional?

(III) Could WPRE be used to achieve a better transduction efficiency and higher transgene expression level after baculoviral gene transfer?

(IV) Could different cell culture mediums have an impact on baculovirus-mediated transgene expression in non-permissive cells? What is the mechanism behind the possible medium effect and how is this associated with the intermediate filaments (vimentin)?

4 MATERIALS AND METHODS

The materials and methods used in the articles I - IV are summarized in the tables shown below and (Tables 8 -13) described in more detail in the relevant publications.

4.1 Methods

Table 8. Methods used in studies I-IV.

Methods	Description	Used in
DNA cloning	Vector construction	I – IV
Production of viruses	Concentration and production of baculoviruses and lentiviruses	II, III, IV
Analysis of viruses	Tittering and immunoblotting	II, III, IV
***In vitro* experiments**	Cell culture DNA transfection Transduction experiments	I – IV I, IV II, III, IV
Analytical experiments	Flow cytometry analysis Immunoblotting ELISA analysis PCR Real-time RT-PCR β-galactosidase enzyme assay Cytotoxicity assay (MTT)	II, III, IV I, II, III I I, II, IV II II IV
Microscopy	Light and fluorescent microscopy Confocal microscopy	I-IV III
Statistical methods	Mean±SEM, unpaired t-test	II, III, IV

4.2 Cell lines

Cell lines used for *in vitro* studies are listed in Table 9.

Table 9. Cell lines used in studies I-IV.

Cell line	Source	Description	Used in
293T	ATCC: CRL-11268	Human kidney carcinoma	I, II, III, IV
CHO-K1	ATCC: CCL-61	Chinese hamster ovary cells	I, IV
HepG2	ATCC: HB-8065	Human hepatocarcinoma cells	I, III, IV
Ea.hy926	University of North Carolina, Department of Pathology, NC, USA	Hybridoma of human airway epithelium and HUVEC cells	II, III
RaaSMC	(Yla-Herttuala et al., 1995)	Rabbit aortic smooth muscle cells	II, III, IV
MG-63	ATCC: CRL-1427	Human osteosarcoma cells	III
Hela	ATCC: CCL-2	Human cervical cancer cells	III
Sf-9	Invitrogen	Spodoptera frugiperda IPLB-Sf-21-AE cells	II, III, IV

4.3 Plasmids

Details of the plasmids used in the publications I – IV are shown in Table 10. These plasmids or parts of them were used to create final constructs used in the experiments.

Table 10. Plasmids used in studies I-IV.

Plasmid	Reference	Description	Used in
pBS185	Life Technologies	Source of *Cre*-gene	I
pAIV-11	(Airenne et al., 2000)	Source of intron Source of LacZ-gene	I II
pDsRed2-N1	Clontech	Source of *DsRed*-gene	I
pFlox	(Leppanen et al., 2006)	Used to test SSiCre function	I
pWPT-EGFP	Tronolab	Source of *WPRE*-gene Source of *EGFP*-gene	II II
pLodavin	(Lehtolainen et al., 2003)	Source of *Lodavin*-gene	II
pFastBac-1	Invitrogen	Backbone for BEBV	IV
pBVboostFGR	(Laitinen et al., 2005)	Backbone for WPRE constructs	II
pCAGGS	Generous gift from Dr Jun-ichi Miyazaki, Osaka, Japan	Source of *CAG*-promoter	IV
pEAK12	Edge Biosystems	Source of 1st generation episome	IV
pEGFP-N1	Clontech	Source of *EGFP*- gene	I, IV
pUNI/V5-His-TOPO	Invitrogen	Source of R6Kγ-ori	IV
pCEP4	Invitrogen	Source of 2nd and 3rd generation episome	IV
pMDLg/pRRE	Tronolab	Lentivirus production	III
pRSV-Rev	Tronolab	Lentivirus production	III
pCMV-VSVG	T.Friedmann, UCSD, La Jolla, CA, USA	Lentivirus production	III
LV1-GFP	(Makinen et al., 2006)	Lentivirus prodcution	III

4.4 Viral vectors

The characteristics of the viral vectors constructed in the studies are listed in Table 11.

Table 11. Viruses used in studies I-IV.

Vector name	Description	Used in
Ba-CAG-EGFP	baculovirus containing EGFP	II
Ba-CAG-EGFP/WPRE	baculovirus containing EGFP + WPRE	II, III
Ba-CAG-lacZ	baculovirus containing lacZ	II
Ba-CAG-lacZ/WPRE	baculovirus containing lacZ + WPRE	II
Vp39EGFP	(Kukkonen et al., 2003)	III
LV-GFP	LV VSVG-pseudotyped	III
LV-Gp64	LV Gp64-pseudotyped	III
EVO_{EGFP}	1st generation BEBV	IV
$KEVO_{EGFP}$	Control for 1st generation BEBV	IV
SiCreR6K+EBNA	2nd generation BEBV	IV
SiCreR6K-EBNA	Control for 2nd generation BEBV	IV

4.5 DNA oligomers

The oligomers used in cloning and sequencing are listed in Table 12.

Table 12. DNA oligomers used in studies I-IV.

Sequence	Used for	Used In
gttacgaattcgccaccatgtccaatttactgaccgt	Cre/intron cloning	I
cagccctctacttacctggtcgaaatcagtgcgtt	Cre/intron cloning	I
ttcttacctttctaggttcgttcactcatggaaaa	Cre/intron cloning	I
taagcagatctccatcgccatcttccagcaggc	Cre/intron cloning	I
actgatttcgaccaggtaagtagagggctgggctg	Cre/intron cloning	I
catgagtgaacgaacctagaaaggtaagaaaagtg	Cre/intron cloning	I
ggggacaagtttgtacaaaaaagcaggctgtcgccaccatggtgagcaag	EGFP/WPRE cloning	II
ggggaccactttgtacaagaaagctgggtctagctagctactagct	EGFP/WPRE cloning	II
ggggaccactttgtacaagaaagctgggtcccgatgcggggaggcggccc	EGFP/WPRE cloning	II
ggcatcgacttcaaggaggac	RT-PCR/EGFP	II
tgcttgtcggccatgatataga	RT-PCR/EGFP	II
aattccctaggatttaaatgctagcgtttaaacgatatcg	Gp64 cloning	III
aattcgatatcgtttaaacgctagcatttaaatcctaggg	Gp64 cloning	III
ctagacacgtgcttaagcagctgctgcaggtcgacatttaaatcccgggggcgcgcc	MCS cloning	IV
ctagggcgcgcccccggatttaaatgtcgacctgcagcagctgcttaagcacgtgt	MCS cloning	IV
ataacttcgtatagcatacattatacgaagttatgctagccag	pEAK12 cloning	IV
ctggctagcataacttcgtataatgtatgctatacgaagttat	pEAK12 cloning	IV
gatccctgcagataacttcgtatagcatacattatacgaagttata	pEAK12 cloning	IV
gatctataacttcgtataatgtatgctatacgaagttatctgcagg	pEAK12 cloning	IV
gttaccctaggaaccagataagtgaatcta	R6Kori cloning	IV
acacttaacggctgacatggttaattaatgtgggcggacaaaatagtt	R6Kori cloning	IV
aactattttgtccgcccacattaattaaccatgtcagccgttaagtgt	R6Kori cloning	IV
ccgcggctcttccgctgatctgttgatcagcagttcaacctgttga	R6Kori cloning	IV
ccatcgcgaataacttcgtatagcatacattatacgaagttatgatatatgg	loxP site/pCEP4	IV
ccagatatcataacttcgtataatgtatgctatacgaagttattcgcgatgg	loxP site/pCEP4	IV
gggctatgaactaatgaccccg	PCR primer	IV
gcttcccgttgggtaacata	PCR primer	IV
actgtgggcgatgtgcgct	PCR primer	IV
tgggaggggtggaaatggag	PCR primer	IV
cactgcattctagttgtgg	PCR primer	IV

4.6 Antibodies

The antibodies used in immunoblotting and confocal microscopy studies are shown in table 13.

Table 13. Antibodies used in studies I-IV.

Antibody	Source	Description	Used in
anti-gp64	Insight Biotechnology	to detect GP64	II, IV
anti-Cre	Novagen, Darmstadt,	to detect Cre	I
anti-β-galactosidase	Promega	to detect β-galactosidase	II
anti-avidin	(Laitinen et al., 2002)	to detect avidin	II
anti-vimentin	Novocastra	to detect total vimentin	III
anti-alpha-tubulin	Abcam	to detect microtubules	III
anti-nuclear lamins A/C	Novocastra	to detect nuclear lamins	III
anti-vimentin S72	Abcam	to detect phosphorylated S72 in vimentin	III
anti-vimentin S38	Abcam	to detect phosphorylated S38 in vimentin	III
anti-vimentin S82	Abcam	to detect phosphorylated S82 in vimentin	III
anti-beta actin	Cell Signaling	to detect beta actin	III

4.7 Statistical analysis

An unpaired *t*-test, performed with Prism™ (version 4 or 5) from GraphPad Software, Inc., was used to determine the statistical significance of the data (in Article II and III).

5 RESULTS AND DISCUSSION

The following chapter will go through the main results of the Studies I – IV. More details of these are available in the original publications (the publication as a Roman numeral/ reference). Some unpublished data is also presented.

5.1 Cre-recombinase is toxic for mammalian cells (Article I)

While cloning the Cre/*lox*P system into a transfer plasmid for BEBV-baculovirus preparation (IV), we discovered that Cre was toxic to 293T cells (I/Fig 3). The cells which were expressing Cre-DsRed fusion protein were rounded, unhealthy and detached away from the bottom of the wells. The red fluorescent protein (DsRed2) *per se* did not cause any such changes suggesting that the toxicity was Cre-dependent. This is in line with the observations by Silver and co-workers (2001). They showed cre toxicity in 293xLac (a derivative of human 293 cells) cells with a retrovirus (Silver and Livingston, 2001). The retrovirus-encoded Cre-recombinase led to cellular toxicity, while GFP-virus did not.

The toxicity of Cre is a consequence of its strand cleavage activity. This was demonstrated by Cre mutants which were defective in DNA cleavage activity, since these were not toxic when compared to the wild type Cre (Silver and Livingston, 2001). The toxicity could be avoided by limiting the intensity and duration of Cre expression in mammalian cells (Silver and Livingston, 2001; Loonstra et al., 2001). Silver et al. introduced a method in which Cre excises the gene directing its own synthesis once a critical level of expression required for the excision is reached. In their hands, this method efficiently reduced cre-mediated toxicity (Silver and Livingston, 2001). Another approach to solve this problem is to fuse Cre recombinase to a ligand-binding domain of normal cellular receptor. For example, Heidmann et al. and co-workers (2001) fused Cre to the human estrogen receptor, which enables its regulation by estrogen (Heidmann and Lehner, 2001). Another way is to fuse Cre to a mutated form of the estrogen receptor which binds to a synthetic ligand, tamoxifen, instead of endogenous estrogen (Feil et al., 1996). Other approaches are tetracycline-controlled or interferon-inducible cre expression systems (Kuhn et al., 1995; Utomo et al., 1999). These

subsequent studies have demonstrated that also the inducible forms of Cre can be toxic for mammalian cells and transgenic animals, if the dose of the inducer is too high or the treatment is too long (Naiche and Papaioannou, 2007; Higashi et al., 2009).

Even though Cre/loxP-technology is widely used to delete or to activate gene expression (temporal or tissue-specific manner) in transgenic animals, only a few reports have been published where Cre has shown toxicity or abnormalities in transgenic animals (Schmidt et al., 2000; Lee et al., 2006; Buerger et al., 2006; Naiche and Papaioannou, 2007; Higashi et al., 2009). One reason could be that those embryos in the founder generation which express high quantities of Cre would either die or be grossly abnormal. Another reason could be that the observed phenotypes are assumed to be consequences of the loss or gain of the studied gene/s, instead of the possible cre toxicity. Indeed, Naiche and co-workers (2007) showed that cre expression could generate spurious phenotypes and that these could can confound genetic analyses; they also claimed that most studies using cre-technology have inadequate controls to pinpoint Cre toxicity. They noted that ubiquitously expressed Cre caused dramatic developmental defects in transgenic mice, such as loss of hematopoietic activity (anemia) and it dramatically up-regulated apoptosis (Naiche and Papaioannou, 2007). These results were confirmed later by another group (Higashi et al., 2009). In addition, infertility has been reported in transgenic mice expressing Cre in postmeiotic spermatids (Schmidt et al., 2000). Dilated cardiomyopathy was observed in mice expressing Cre under the control of α-myosin H chain (Buerger et al., 2006). Lee and co-workers (2006) showed that the earlier results from cre-induced mice used to study glucose intolerance were biased and actually it was the Cre expression under the control of the insulin 2 promoter that caused glucose intolerance (Lee et al., 2006). Taken together, these publications suggest that uncontrolled Cre toxicity may be a source for many artifacts and adequate controls are required.

5.2 Optimized Cre-expression cassette (Ssi-Cre) is safe and functional (I)

In order to avoid Cre toxicity, we designed and generated the **S**ilent **S**elf-**i**nactivating **Cre** (SSi-Cre) cassette (I/Fig. 1) with modified *loxP* sites flanking Cre/Int/DsRed gene that also prevents the leaky cre-expression discussed in section 5.3. This system self-inactivates Cre expression soon after Cre production, to minimize the intensity and duration of the Cre expression. The excision of the Cre/int/DsRed fusion gene takes

place when a critical level of cre expression has been reached. The concept of self-inactivating Cre expression is similar to that described by Silver et al. (Silver and Livingston, 2001). The benefit compared to earlier constructs is that SSi-Cre system is in a universal and all-in-one format. Silver et al. generated a self-excising system which was functional only in retroviral vectors. This system contained one lox 511 site at the 3′ LTR U3 region of the virus genome. This *lox*P site will be duplicated during virus production and flanks the Cre/GFP fusion gene, causing negative feedback of the Cre expression. The SSi-Cre system contains both *lox*P sites and is therefore compatible with all vectors and applications. Comparison of the SSi-Cre with existing Cre-expression cassettes is shown in the table 14.

Table 14. Comparison and benefits of the SSi-Cre system compared to the other cre-expression cassettes (+ = included in cre-expression cassette, **-** = not included).

	Shine-Dalgarno Inactivated	Universal eukaryotic promoter	Reporter gene for cre	Cre interrupted by an intron	Cre expression totally inactivated	Modified *lox*P, not compatible with wild type *lox*P	All these features in the same expression Cassette
Ssi-Cre	+	+ (CAG)	+ (DsRed)	+	+	+ ($lox P_{FAS}$)	+
(Kaczmarczyk and Green, 2001)	-	- (Probasin)	-	+	-	-	-
(Zuo et al., 2001) (**Note:** only for plants)	-	- (O^{LexA}-46 for plants)	-	+	-	-	-
(Bunting et al., 1999)	-	- (tACE)	-	+	-	-	-
(Mlynarova and Nap, 2003) (**Note:** only for plants)	-	- (CaMV for plants)	-	+	-	-	-
(Silver and Livingston, 2001) (**Note**: only retrovirus compatible system)	-	+ (5'-UTR)	+ (GFP)	-	-	+ (*lox* 511)	-

Cre-recombinase recognizes a 34 bp *loxP*-site and mediates precise site-specific recombination between a pair of specific target sequences. In order to expand the utility of the Cre/loxP-system, new modified loxP-sequences were created and studied and their internal activity was compared to the wild type loxP (Siegel et al., 2001). Several non-compatible loxP pairs (e.g. WT/FAS, WT/2272 and 2272/5171) were formed which showed only a negligible amount of recombination between each other (Siegel et al., 2001). In the first article (I) we successfully used the wild-type *loxP* sites together with the $loxP_{FAS}$ sites without any detectable recombination between these sites.

Toxicity of the SSi-Cre system was tested by transfecting and observing human kidney cells (293T) (I/Fig. 3). Two days after transfection, the expression of the cre/int/DsRed fusion gene was observed as a faint red color in the transfected cells. As a result of the self-inactivation, the expression disappeared with time during culturing. Five days after the transfection, the red color was scarcely detectable indicating that the cre/int/Dsred fusion gene has been eliminated. No cre expression was detected by anti-Cre staning (data not shown). The pSSi-Cre cassette carrying plasmid also contained an EGFP expression unit (non-excisable) in addition to the SSi-Cre cassette in order to detect transfected cells. The green-colored cells appeared healthy, with no signs of toxicity. This same expression cassette was also used successfully in publication IV without any side-effects. All these results together demonstrate that the strategy for Cre expression from the SSi-Cre cassette is feasible and non-toxic. It would be intriguing to apply this Ssi-Cre technology, not only for Cre, but also for other toxic genes.

The functionality and compatibility of the pSSi-Cre in double-*lox*P experiments was investigated by co-transfecting the pSSi-Cre together with a pFlox (contains a wild type *lox*P-flanked STOP cassette) into CHO cells (I/Fig 4). Excision of the STOP cassette activates VEGF expression which can be detected by an ELISA assay. This transient Cre production was sufficient to efficiently catalyze VEGF production. These results show that the Ssi-Cre is functional and compatible with double-lox approaches. This lox/stop/lox/VEGF cassette was also utilized to generate a transgenic mouse. In this model, hVEGF-A_{165} expression was activated by Ad-mediated Cre-gene transfer (Leppanen et al., 2006). The transgene expression declined dramatically within a few weeks in the transgenic animals and Cre toxicity was believed as one possible reason for this effect. In the future, the usage of Ssi-Cre system could be more feasible system to achieve active VEGF-expression in mice and in that way, prevent possible cre-mediated side-effects.

5.3 SSi-Cre cassette inhibits leaky Cre-expression in E.coli (I)

Earlier reports have shown that *Cre* gene under the plant specific (O^{LexA}-46) and CMV promoters is expressed in *E.coli* (Zuo et al., 2001; Kaczmarczyk and Green, 2001). We found that CAG also drives the production of Cre in *E.coli,* causing serious problems in achieving an intact plasmid bearing both the Cre-recombinase under a mammalian promoter and the *loxP*-sites flanked DNA (I/Fig. 2). In agarose gel electrophoresis, we observed only cre-cut plasmids. Sequence comparison of the plasmid showed that a Shine-Dalgarno-like sequence (Kozak, 1999), which directs translation initiation in *E.coli*, was located just before the initiation codon of the Cre recombinase. To test the hypothesis that this was the reason for leaky cre-expression, we deleted the major part of the Shine-Dalgarno sequence. This deletion caused a significant decrease in the recombination of the *loxP* sites containing plasmid. Earlier reports have also shown that mutations in Shine-Dalgarno sequence affect the transgene expression e.g. one base mutation (GGAGG → AGAGG) caused a several fold decrease in β-galactosidase expression in *E.coli* (Velazquez et al., 1991). Although the leaky cre-expression was significantly reduced, a fraction of the plasmids were still lost. In an attempt to solve this problem, the Cre encoding sequence was interrupted by a short mouse protamine intron to disable bacterial expression of *Cre*. It is a known that bacteria cannot splice introns and the interruption of coding sequence by functional intron prevents in-frame translation of a target sequence (Bunting et al., 1999; Zuo et al., 2001; Kaczmarczyk and Green, 2001). Leaky cre-expression in *E.coli* was solved by this modification.

These findings highlight also the poorly-recognized ability of some mammalian promoters to drive gene expression in *E.coli*. Previously, chloramphenicol acetyltransferase (CAT) under the human cytomegalovirus immediate-early gene region 1 promoter-enhancer (CMV-IE) was demonstrated to be expressed in the HB101 *E.coli* strain (Davis and Huang, 1988). In addition, CMV and RSV promoters have been shown to direct protein synthesis in bacteria (Goussard et al., 2003) as well as avian tumor virus promoter (Mitsialis et al., 1981) or plant promoter (Zuo et al., 2001).

5.4 Post-transcriptional regulatory element enhances transgene expression in baculovirus-mediated transduction (II)

Baculoviruses are capable of entering into a wide spectrum of cells. However, the level of transgene expression varies and is generally lower in cell types other than those of hepatic origin. Therefore, several means have been introduced to enhance baculovirus-mediated transduction and gene expression in vertebrate cells e.g. envelope modifications, optimal promoter use, use of histone deacetylase inhibitors and enhancement of cytoplasmic transport by destroying microtubules (see paragraph 2.3.5). The earlier reports showed that WPRE improves transgene expression *in vitro* and *in vivo* with many viral vectors like retroviruses, Ads and AAV (Zufferey et al., 1999; Loeb et al., 1999; Paterna et al., 2000; Salmon et al., 2000; Lipshutz et al., 2003; Xu et al., 2003). Since this element had not been tested before in baculoviruses, we decided to clone it into the baculovirus system (II/Fig. 1). The inclusion of this element was successful and no negative effects were detected on baculovirus titers (e.g. Ba-CAG-EGFP 1.2 x 10^{11} pfu/ml and Ba-CAG-EGFP/WPRE 1.1 x 10^{11} pfu/ml, both 250 fold concentrated). Indeed, earlier studies have shown that WPRE might have a positive effect on viral titers e.g. the titers of retroviruses were shown to increase significantly when WPRE was used (up to 14-fold increase in titer) (Werner et al., 2004).

The new vector was tested in four different cell lines which have all been reported to represent differences in their susceptibility to baculovirus-mediated gene transfer. The order from highly susceptible to poor was HepG2, 293T, RaaSMC and EA.hy926. In the previous studies, Ea.hy926 was demonstrated to be a poorly permissive cell line for achieving baculovirus-mediated gene expression (Kukkonen et al., 2003), whereas HepG2 is known to be very permissive (Hofmann et al., 1995).

Enhanced green fluorescent protein (EGFP) expression was analyzed by fluorescence microscopy following transduction of HepG2, 293T and RaaSMC cells at MOI 250 and 1000 (II/Fig 2). The WPRE significantly increased the proportion of GFP positive cells as well as the intensity of green fluorescence in 293T and RaaSMC cells. An even more prominent effect was detected in 293T cells. In accordance to the high susceptibility of liver cells (HepG2) to baculoviruses, only a slight increase in the transduction efficacy was observed with the WPRE virus compared to the control virus. Both viruses showed a dose-dependent response. The earlier results with adeno-, AAV-,

retro- and lentiviral vectors, and now also conducted with baculoviruses, emphasize the vector independent action of WPRE (Zufferey et al., 1999; Loeb et al., 1999; Xu et al., 2003). However, the magnitude of the effect varied upon the specific context of the promoter, transgene and cells type (Salmon et al., 2000; Schambach et al., 2000; Klein et al., 2006; Boulos et al., 2006; Hermening et al., 2006). For example, lentiviral vector containing CMV promoter along with WPRE was superior in 293T cells and HT1080 fibrosarcoma cells, whereas CAG and EF1α together with WPRE gave the highest transgene expression in human hematopoietic stem/progenitor cells (HSPCs) (Ramezani et al., 2000). In addition, the other gene expression regulating elements like introns together with WPRE can cause a significant change in the magnitude of the effect. The construct containing WPRE and an intron was best (6-fold increases in expression compared to the vector without these elements) i.e. better than the construct containing only intron (3-fold increase in expression compared to the vector without intron and WPRE) (Wang et al., 2003). In general, WPRE result in a higher increase in transgene expression than an intron (Xu et al., 2003; Wang et al., 2003; Hermening et al., 2006). The studies, where other regulatory elements have been tested together with WPRE or compared with WPRE have shown that WPRE is one of the best potential regulatory elements to enhance gene expression (Schambach et al., 2000; Brun et al., 2003).

The microscopical results were confirmed by FACS. The WPRE equipped virus outperformed at different MOIs (10, 100, 250, 500 and 1 000) in a dose-dependent manner (II /Fig. 2B). The increase in the numbers of EGFP positive cells was most evident at lower MOIs in HepG2, RaaSMC and 293T cells being from four- to six-fold ($p<0.01$). However, the increase (10-fold at MOI 1000) was more evident at a higher MOI in the very poor baculovirus target cells (Ea.hy926). The most prominent increase (> 60-fold) was achieved with EA.hy926 when optimized cell culture medium (RPMI 1640) was used (see paragraph 5.6).

The advantage of the WPRE was further studied by measuring the mean fluorescence intensity (MFI) of the green fluorescing cells. In all cell lines and MOIs, the intensity of green fluorescence was best with the WPRE virus (II/ Fig. 2C). The highest increase was seen in RaaSMC (> 5-fold at MOI 1000). The increase in MFI was four-fold in 293T cells and three-fold in HepG2 cells. The quantification of the mRNA amount in the transduced cells was performed with real-time RT-PCR (II/Fig. 3A). A prominent elevation was seen in RaaSMC cells (11-fold). These results are consistent with the previous results showing that WPRE can elevate target gene mRNA levels which

correlates with the transgene translation (Zufferey et al., 1999; Higashimoto et al., 2007). Xu and co-workers showed that WPRE element between the luciferase gene and poly(A) in Ad increased transgene expression by 2- to 7-fold in different cells *in vitro* and by 2- or 50-fold more expression was seen in liver, kidney and lung of mouse *in vivo* (Xu et al., 2003) The corresponding expression cassette in a retroviral vector increased marker gene expression by 5- to 6-fold *in vitro* (Zufferey et al., 1999). One significant benefit from the increased transgene expression is the possibility to use lower doses and smaller volumes of vector and in that way to reduce the potential side-effects (Klein et al., 2002).

The exact mechanism of the WPRE-mediated expression enhancement is unknown, but increased mRNA export, increased rate of transcription or increase in mRNA half-life have been suggested (Zufferey et al., 1999). For example, Mastroyiannopoulos and co-workers showed that 85 % of the WPRE-containing transcripts were in cytoplasm, whereas the control transcripts without WPRE were mostly in the nucleus (only ~ 40 % in the cytoplasm)(Mastroyiannopoulos et al., 2005). The latest results with retroviral vectors showed that WPRE could improve also transcription termination and in this way, enhance transgene expression and increase viral titers (Higashimoto et al., 2007; Schambach et al., 2007).

The earlier results showed that the WPRE effect is transgene independent (Zufferey et al., 1999). In addition to EGFP, WPRE was also cloned here next to the *LacZ* and *Lodavin* genes (II/Fig. 4). In accordance with the EGFP results, WPRE markedly increased the number of β-galactosidase-positive cells and the intensity of blue-staining at MOI 250 and 1000. These results were further verified by luminescent β-galactosidase enzyme assay and by immunoblot analysis. According to the luminescent assay, the increase was 17-fold at MOI 250 and 34-fold at MOI 1000 in 293T cells. Immunoblot analysis showed more β-galactosidase and lodavin in cells transduced with the WPRE equipped viruses.

5.5 Sodium butyrate-like effect of WPRE on baculovirus transduction (II)

Earlier reports had shown that sodium butyrate could significantly enhance baculovirus-mediated gene expression in vertebrate cells (Condreay et al., 1999; Airenne et al., 2000). The exact mechanism of butyrate is unknown, but it is probably due to the more accessible expression cassette, leading to enhanced transcription (Berger, 2000). In order to compare the butyrate effect to that of WPRE, we transduced cells (virus ± WPRE element) with or without 2.5 mM sodium butyrate at MOI 250 (II/Fig. 3B-3D). Sodium butyrate substantially enhanced baculovirus-mediated gene expression in all of the studied cell lines (HepG2, 293T and RaaSMC). The strongest sodium butyrate-mediated increase was seen in Ba-CAG-EGFP treated 293T cells (11-fold). Interestingly, the difference between Ba-CAG-EGFP/WPRE transduced HepG2 cells (57.8 ± 4.9 %) and Ba-CAG-EGFP + 2.5 mM butyric acid treated cells (61.7 ± 8.4 %) was not statistically significant ($p>0.05$). A similar result was also observed in RaaSMC cells transduced with Ba-CAG-EGFP/WPRE (25.3 ± 4.6 %) or with Ba-CAG-EGFP + 2.5 mM butyric acid (37.4 ± 4.3 %, $p>0.05$), indicating that the WPRE-containing baculovirus can drive gene expression efficiently without sodium butyrate, at least in these two cell lines. The combination of transduction with Ba-CAG-EGFP/WPRE and 2.5 mM butyric acid further boosted the transduction efficacy.

5.6 WPRE effect is boosted by optimized cell culture medium (II)

When Ea.hy926 cells were cultured in RPMI 1640 medium instead of the normally used DMEM, remarkable increase in a number of EGFP positive cells was detected at different MOIs. The combination of optimized culture medium and a WPRE-containing baculovirus produced the most striking results (II/Table 1). At MOI 1000, the increase in the percentage of EGFP positive cells was more than 60-fold in favour of the WPRE virus.

In conclusion, WPRE is a simple way to enhance baculoviral transgene expression. The only concern is the potential oncogenic activity of WPRE. Its sequence (nt. 1093-1684; GenBank accession no. J02442, used e.g. in lentiviral vectors and in our baculoviral vector) contains a promoter of the woodchuck hepatitis virus X protein (WHX) and the sequence for the first 60 amino acids of the X-protein (150 aminoacids).

X-protein has been implicated in the development of liver tumours (Kingsman et al., 2005; Schambach et al., 2006; Zanta-Boussif et al., 2009). However, there is no evidence that 60 aa long polypeptide could cause cancer. Indeed, a lentiviral vector bearing either WPRE or WPRE mutated at the X gene did not develop tumors in mice (Themis et al., 2005). New mutated/truncated forms of WPRE have been successfully created to avoid unwanted X protein expression. These new forms have been shown to enhance gene expression as well as the original WPRE (Schambach et al., 2006; Zanta-Boussif et al., 2009).

5.7 Influence of different culture mediums on the baculovirus transduction efficiency (III)

Different cell lines are usually cultured in different culture mediums. The medium composition has been shown to have many effects e.g. on the phenotype and metabolism of cells (Doostdar et al., 1988; Jasmund et al., 2007). However, very little is known about the effects of medium on virus-mediated gene delivery. The earlier reports have shown that mouse cardiomyocytes are transduced better by the adenoviral vector in MEM than in M199 medium (90±8 % in MEM vs 5±1.2 % in M199). The preliminary results showed that the reason behind the effect was enhanced CAR expression in the cell membrane (Li et al., 2003).

We used six different cell lines (Ea.hy 926, HepG2, Hela, MG-63, RaaSMC and 293T) which were cultured in different cell culture mediums (RPMI 1640, MEM, McCoys 5A, Optimem, F-12 and DMEM) to investigate in detail the test medium effect on the baculovirus transduction. The effect of medium was prominent in all tested cell lines analyzed by fluorescent microscopy (III/ Fig. 1A). A remarkable increase in the number of *EGFP* expressing cells was observed in RPMI 1640 compared to DMEM medium, where hardly any green cells were detected. Even MG-63 and Ea.hy926 (usually cultured in DMEM) showed high transgene expression when cultured in RPMI 1640.

The comparison of different mediums showed that, in general, DMEM was the poorest and RPMI 1640 the best with respect to baculovirus transduction efficiency. This is in line with our preliminary results shown in Article II and by Hsu et al. who showed that usage of DMEM resulted in lower transduction efficiency and gene expression than insect culture medium (TNM-FH) or phosphate buffered saline (PBS) (Hsu et al., 2004).

Quantification by a flow cytometry confirmed the fluorescence microscope observations (III/ Fig. 1B). Improvement in transduction was evident with all MOIs and tested cell lines. Human liver cells (HepG2), were transduced 2.5-fold more efficiently in RPMI 1640 than in DMEM (76.4 ± 1.8 % vs. 29.7 ± 3.0, $p < 0.0001$, MOI 200). The corresponding improvement was also seen in Ea.hy926 cells, where the increase was 9-fold in favour to RPMI 1640 (3.5 ± 0.2 % vs. 31.5 ± 1.3 %, $p< 0.0001$, MOI 200). More notable, an 11-fold increase was detected in MG-63 cells (4.1 ± 0.1 % vs. 43.0 ± 0.9 %, $p< 0.0001$, MOI 200). The difference in the number of green HepG2 cells between RPMI 1640 and MEM (standard medium for these cells) was also statistically significant at lower MOIs. For example at MOI 200, the increase was 1.2 –fold (64.2 ± 1.2 % to 76.4 ± 1.8 %, $p < 0.0001$).

The benefit of the culture medium change was also evident when MFI was measured. In all studied cell lines and with all applied MOIs, the MFI was significantly higher and dose dependent in the RPMI 1640 cultured cells (III/ Fig. 1B). The increase in MFI was approximately 5-fold in all studied cells at MOI 1000 and there was still a good dose-dependent response compared to DMEM cultured cells.

These results confirm that DMEM interferes with the baculovirus transduction. Baculovirus-mediated gene transfer was previously enhanced by omitting $NaHCO_3$ from the medium (Shen et al., 2007). Earlier studies also revealed that Mg^{2+}, Ca^{2+}, K^+ and Na^+ did not influence transduction (Hsu et al., 2004; Shen et al., 2007). In general, DMEM contains more Ca^{2+} cations and CO_3^- anions, but less PO_4^- and NO_3^- anions than the RPMI 1640. It has also higher concentrations of glucose, amino acids and vitamins (see Table 15). However, the comparison of formulas of the tested mediums did not give any straightforward explanation for the observed results.

Table 15. Comparison of the optimized culture medium (RPMI 1640) with DMEM.

Component:	In DMEM (compared to RPMI 1640)
-Ca^{2+}	2.6 x more
-CO^{3-}	1.9 x more
-PO_4^{2-}	14 x less
-NO_3^-	667 x less
Glucose	2.3 x more
Amino acids	Generally every amino acid 3 x more
Vitamins	Generally every vitamin 4 x more
-Cl^-, -Mg^{2+}, -SO_4^{2-}, -K^+, -Na^+	No difference

5.8 Culture medium influences transgene expression also in other viruses (III)

In order to study the impact of the culture medium with other viruses, we used Gp64- and VSVG-pseudotyped lentiviruses and the transduction of cells cultured in DMEM or RPMI 1640. Both viruses showed higher transduction efficiencies in RPMI 1640 medium (III/ Fig. 2). Gp64-pseudotyped lentiviruses showed 2-fold more green 293T cells as compared to DMEM-cultured cells (50.4 ± 0.1 vs. 98.9 ± 0.4 % in favor of RPMI 1640 medium, $p < 0.0001$). The difference was even more clear in Hela cells, being 5.8-fold in favor of RPMI 1640 medium (15.5 ± 0.3 vs. 90.7 ± 0.4 %, $p < 0.0001$). VSVG-pseudotyped lentiviruses showed a 1.6-fold increase in 293T cells (14.6 ± 1.5 vs. 18.2 ± 1.1 % in favor of RPMI 1640 medium, $p < 0.0009$) whereas in Hela cells the increase was 1.8-fold (14.5 ± 1.0 vs. 24.3 ± 2.8 % in favor of RPMI 1640 medium, $p < 0.0001$). The difference was smaller in the VSVG-virus transduced cells (III/ Fig. 2B). It is known that VSVG pseudotyping improves transduction efficiency by allowing faster kinetics of the baculovirus endosomal release than Gp64 and this may explain these results (Kaikkonen et al., 2006).

The benefit of the optimized culture medium was also evident in MFI quantification (Figure 15, unpublished results). The increase was about 4.5-fold in 293T (1457 ± 174 vs. 6547 ± 164 in favor of RPMI 1640 medium, $p < 0.0022$) and 6.3-fold in Hela cells (2410 ± 94 vs. 15090 ± 439 in favor of RPMI 1640 medium, $p < 0.0013$) when tested with the Gp64-pseudotyped lentiviruses. However, with the VSVG-pseudotyped viruses, the difference was not statistically significant in 293T cells (1026 ± 71 in DMEM vs. 1145 ± 91 in RPMI 1640, $p > 0.3290$). Interestingly, with Hela cells, the optimal medium led to decline in MFI (1963 ± 50 vs. 1388 ± 23) in favor of DMEM medium ($p < 0.0001$). The reason for this remains to be studied. One possibility is, however, the known cytotoxicity of VSV-G. Indeed, earlier reports have shown that too a high quantity of VSV-G may cause cytotoxicity (Burns et al., 1993).

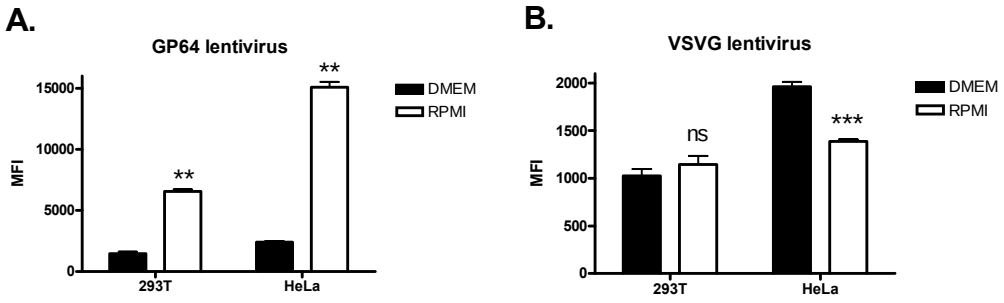


Figure 15. Transduction of 293T and Hela cells grown either in DMEM or RPMI 1640 medium with lentivirus. Mean fluorescence intensity of GP64 (A) or VSV-G (B) pseudotyped lentiviruses was determined by flow cytometry (FACSCanto™ II, BD Biosciences) (p-values: * = < 0.05, ** = < 0.01, *** = < 0.001).

Our preliminary results indicated that the medium effect was not restricted to baculoviruses or lentiviruses, but also had an impact on the adeno-associated virus (AAV) and Ad –mediated gene delivery.

5.9 $NaHCO_3$ content does not explain the medium effect (III)

Cell culture mediums contain normally $NaHCO_3$ to maintain neutral pH under CO_2 conditions. It has been claimed that $NaHCO_3$ may inhibit baculovirus transduction (Shen et al., 2007). However, no exact mechanisms for the observed results have been reported. In an attempt to study further the role of $NaHCO_3$, we adjusted the amount of $NaHCO_3$ in the RPMI 1640 medium to make it identical to that of DMEM (from 2.0 g/l up to 3.7 g/l). All solutions were adjusted to pH 7.4. The elevated level of $NaHCO_3$ in RPMI 1640 resulted in a significant drop in marker gene expression (total FI) only in Ea.hy926 cells (2.3-fold, from 1 851 000 ± 123 900 to 792 000 ± 28 350, $p < 0.001$) (III/ Fig. 3B). The effect was not significant in HepG2 and MG-63 cells. Nonetheless, the $NaHCO_3$ -adjusted RPMI 1640 still outperformed DMEM. Furthermore, HepG2 cells in RPMI 1640 resulted in a higher transduction efficiency and MFI than in MEM (III/ Fig. 1B), even though RPMI 1640 contains almost two times more HCO_3^- -ions (2 g/l) than MEM (1.2 g/l). All these data suggest that the reduced transduction efficiency in DMEM is not unambiguously attributable to the HCO_3^- -ion concentration.

5.10 Nuclear entry of nucleocapsids is improved in the optimized medium (III)

The entry of baculoviruses into vertebrate cells is a multistep process and it is thought to resemble that occurring in insect cells (van Loo et al., 2001; Matilainen et al., 2005). The first step is binding of a virion into the cell surface. The receptor is unknown, but there has been speculated about the involvement of integrins, phospholipids or heparin sulphate proteoglycans (Duisit et al., 1999; Tani et al., 2001). The attached viruses are internalized by endocytosis. The exact endocytic mechanism(s) is still unclear, but clathrin-mediated endocytosis, phagocytosis-like uptake and macropinocytosis have been implicated (Matilainen et al., 2005; Long et al., 2006; Laakkonen et al., 2009). Endocytozed virions release their nucleocapsids from endosomes into the cytoplasm by an acid-induced and Gp64-mediated fusion of the envelope and the early endosome membrane. The released nucleocapsids are transported to the nucleus and dissassembled (van Loo et al., 2001; Kukkonen et al., 2003; Salminen et al., 2005). In order to study the mechanism of the improved transduction efficiency in RPMI 1640 or MEM compared to DMEM, we analyzed the nuclear entry and intracellular localization of baculoviruses in the transduced cells in the confocal microscope.

Differences in the number of nuclear nucleocapsids were observed between RPMI 1640 and DMEM cultured cells using EGFP-tagged viruses (III/ Fig. 4). Fewer viral nucleocapsids were observed in the nucleus of DMEM cultured cells than in the cells cultured in RPMI 1640 (III/ Fig. 4A) suggesting that medium enhanced the nuclear entry of baculoviral nucleocapsids. The same phenomenon was also detected between MEM and DMEM cultured cells; hardly any viral nucleocapsids were detected in the nucleus of DMEM cells (III/ Fig.4B).

The cells in MEM and DMEM were fed with FITC-conjugated dextran in order to study endocytotic activity. The result suggested that the improved viral entry was not due to the enhanced endocytosis of nucleocapsids (Figure 16, unpublished results). All these confocal results are in accordance with the above described transduction results. All of this data emphasize that RPMI 1640 and MEM enhance transgene expression.

HIV-1 infection causes several effects on the nuclear architecture and HIV-1 PR causes amino-terminal degradation of vimentin (Shoeman et al., 1990). In addition, vimentin has also been reported to modify the shape of nucleus (Sarria et al., 1994).

Interestingly, we also observed some changes in the structure of the nuclear lamina in cells cultured in RPMI 1640 (data not shown).

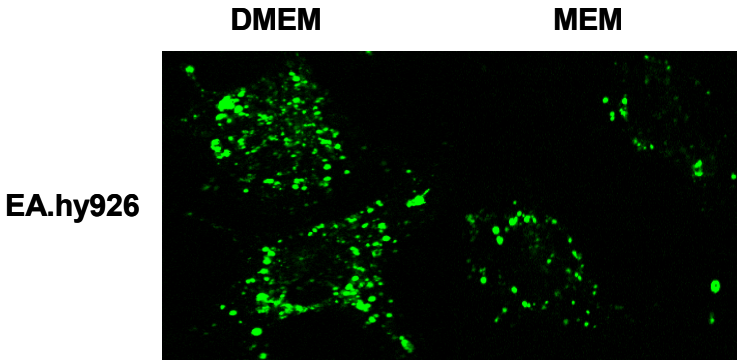


Figure 16. Endocytic activity of Ea.hy926 cells in DMEM and MEM was studied by feeding cells with FITC-conjugated dextran (green).

5.11 Intermediate filaments (vimentin) are reorganized in the optimized medium (III)

The cytoskeletal network is composed of three different structural components; IFs, MFs and MTs. The cytoskeletal network is known to play critical roles in controlling and regulating many cellular functions such as cell contraction, migration, proliferation, protein synthesis, gene expression and apoptosis as well as viral entry (Helfand et al., 2004; Radtke et al., 2006). The role of the cytoskeletal network in the baculovirus transduction is poorly undestood. In insect cells, actin filaments transport nucleocapsids from the endosome into the nucleus (Volkman, 2007). Furthermore, depolymerization of microtubules has been demonstrated to increase the number of nuclear nucleocapsids, leading to enhanced transgene expression (van Loo et al., 2001; Salminen et al., 2005). At present, the most widely distributed and highly conserved intermediate filament protein *vimentin* (Ivaska et al., 2007) has not been connected to baculovirus transduction. Vimentin is typically expressed in cells which are reported to be less permissive for baculoviruses such as leukocytes, blood vessel endothelial cells and epithelial cells as well as mesenchymal cells such as fibroblasts (Minin and Moldaver,

2008). Interestingly, earlier reports have shown that most of the intermediate filament proteins, including keratins, neurofilament triplet proteins, glial fibrillary acidic protein, desmin, vimentin, and *Drosophila* vimentin-like protein are absent in insect cells (Volkman and Zaal, 1990; Nakagawa et al., 1995). These facts led us to assume, that intermediate filaments are not essential for baculovirus infection in insect cells and infact may pose an obstacle for efficient transduction of vertebrate cells.

In an attempt to analyze the effects of the optimized medium (RPMI 1640) on the cytoskeletal network, we used confocal microscopy to study IFs (vimentin), microtubules and actin filaments in cells cultured in DMEM or RPMI 1640 (III/ Fig. 5 A-C). Reorganization of vimentin was observed in RPMI 1640 cultured cells using anti-vimentin mouse monoclonal antibody (III/ Fig. 5A). In RPMI 1640 medium, cells showed a loose, slight and re-organized vimentin network. In DMEM, the vimentin network was dense and strong when MG-63 and Ea.hy926 cells were observed. MTs (III/ Fig. 5B) and MFs (III/ Fig. 5C) were not altered in the cells. The data is in line with the transduction data and reinforce the belief that vimentin is one of the major obstacles in baculovirus transduction, and the optimized culture medium should be used to loosen the vimentin network. Several viruses, such as African swine fever virus (Carvalho et al., 1988), vaccinia virus (Ferreira et al., 1994), retrovirus (Snasel et al., 2000; Naghavi and Goff, 2007), Ad (Belin and Boulanger, 1987), bluetongue virus (Bhattacharya et al., 2007), frog virus 3 (Chen et al., 1986) and rotavirus (Weclewicz et al., 1994), have also been reported to affect IFs in order that they can replicate successfully.

5.12 Phosphorylation status of vimentin is altered in the optimized medium (III)

Phosphorylation has been shown to be an important regulator of IF dynamics, modulating the organization and subcellular distribution of IF proteins (Omary et al., 2006). For example, phosphorylation causes remarkable redistribution and disassembly of vimentin in variety of cells (Inagaki et al., 1987).

The phosphorylation status of vimentin in Ea.hy926 and MG-63 cells was changed when culturing them in the optimized medium (RPMI 1640) (III/ Fig. 6). This was in line with the confocal microscopy results (III/Fig. 5). Phosphorylation of cAMP-

dependent kinase (PKA)-specific sites (Ser-38, Ser-72) has been shown to cause a decelerated assembly kinetics of vimentin subunits *in vivo* (Eriksson et al., 2004). We also observed changes in the phosphorylation levels of these two serine sites (III/ Fig. 6B). Ser-38 and Ser-72 are important in determining the assembly state of vimentin and in regulating vimentin structure (Pallari and Eriksson, 2006). African swine fever virus was shown to evoke the CaM kinase II dependent phosphorylation of vimentin on serine 82 which led to a reorganization of vimentin. Interestingly, the same kind of changes was detected when RPMI 1640 was used as the cell culture medium (III/ Fig. 6C). Vaccinia virus has also been shown to increase phosphorylation of vimentin and desmin during infection, thereby promoting a marked reorganization of vimentin (Ferreira et al., 1994). The vimentin network was diffuse in non-infected cells, emanating from the perinuclear region through the cytoplasm towards the cell membrane in line with that observed in DMEM cultured cells in the present study (III/ Fig. 5A). However, during the early stage of vaccinia virus infection, the vimentin network was shown to be rearranged near the nucleus, which represents the situation in the RPMI 1640 cultured cells in our study (III/ Fig. 5A).

5.13 Construction of Baculovirus-Epstein-Barr Hybrid virus (BEBV) (Article IV)

Baculovirus allows transient gene expression in vertebrate cells and therefore it is not applicable for the experiments where long-term transgene expression is needed. In an attempt to overcome this issue, baculovirus-adeno-associated virus (AAV) hybrid vectors containing an expression cassette flanked by the AAV inverted terminal repeats, have been developed (Palombo et al., 1998; Wang and Wang, 2005; Zeng et al., 2007). However, these approaches rely on the gene integration and this can give rise to safety concerns. Clearly, a system which would circumvent the possibility of genomic integration would be highly desirable.

The BEBV vector consists of three different expression units: Unit I is for the gene expression under an insect active promoter (e.g. polyhedrin promoter; Polh) whereas Unit II and Unit III are designed for the expression under mammalian promoter (e.g. CAG- or EF1α-promoter) (Figure 17, from the article IV). In this study, Unit I was optional but could be used for modifying the baculovirus phenotype (Raty et al., 2004; Kaikkonen et al., 2006) or to express a desired transgene in insect cells e.g. a marker

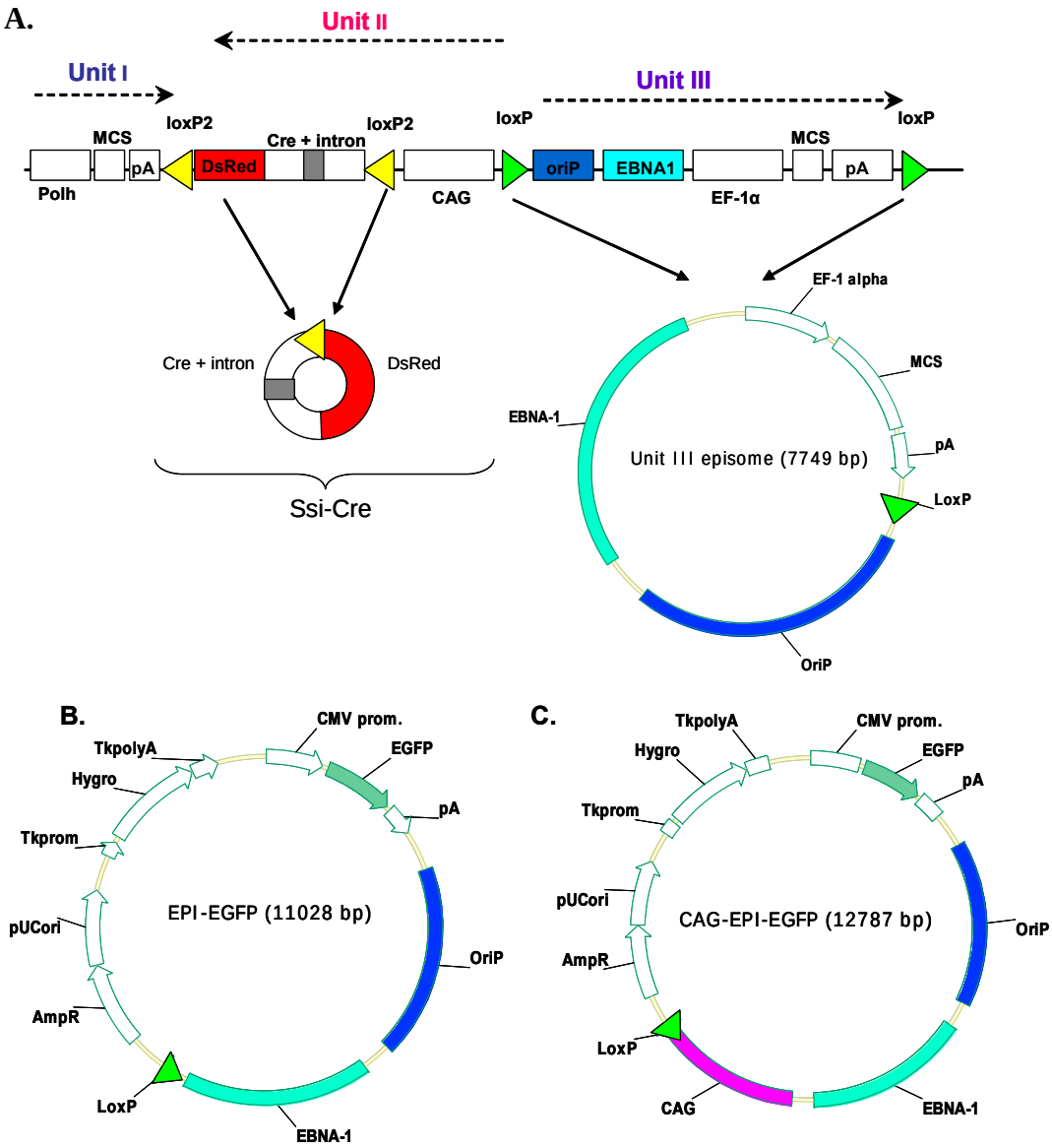


Figure 17. Schematic illustration of the constructed recombinant baculoviral vectors. A) BEBVs contain three expression units (I-III): Unit I expression cassette is active in insect cells, Unit II for Cre expression and Unit III for transgene expression (form episome after Cre expression). The new versions from Unit III were created: B) 2nd generation Unit III (shown as a episome). C) 3rd generation Unit III (shown as a episome). Control episomes were constructed without oriP and EBNA-1.

gene for successful virus production. Unit II was used to express the earlier developed SSi-Cre cassette (see Article I) in order to implement cre-mediated episome formation. Unit III was reserved for a prolonged transgene expression by an episome formation. Several BEBV generations were created. The first generation BEBV contained pEAK12-based episome (Unit III), whereas the 2nd and 3rd generation BEBVs contained pCEP4-based episome. The major difference between the 2nd and 3rd generation vectors was that in the latter the EBNA-1 expression was further enhanced by mammalian active CAG-promoter (IV/Fig. 1). The main differences between pEAK12 (EdgeBiosystems) and pCEP4 (Invitrogen) vectors are shown in table 16.

Table 16. Comparison of pCEP4 with pEAK12.

	pCEP4	**pEAK12**
Promoter	CMV	EF1α
Poly(A)	SV40 poly(A)	hGH poly(A)
Resistance gene for selection of stable cells	Hygromycin	Puromycin
Replication ori	pUCori	SV40ori

The cloning and construction procedures of BEBV vectors were troublesome and several hurdles had to be overcome. One of the greatest problems was the Cre toxicity in mammalian cells and leaky expression in *E.coli*, which were successfully solved by creating the Ssi-Cre cassette (Article I). In addition, an optimized and rapid system for the creation of baculovirus-Epstein-Barr hybrid vectors was established: the desired transgene was cloned into the Unit III containing plasmid (replicon) by using a gateway cloning system and the replicon was then transferred into a Bac-to-Bac compatible transfer plasmid (IV/ Figure 2). In summary, we succeeded in developing a novel vector, where all the necessary elements for episomal maintenance of transgene were delivered by one baculovirus.

5.14 Cre recombination target sites in the BEBV are functional (IV)

The functionality of BEBV system was tested in the 294-cre *E.coli* strain (Buchholz et al., 1996a). This strain expresses constitutively Cre-recombinase and thus provides a simple test for the recombination competence of *loxP*-constructs. Cre expression caused excision of the Unit III (episome) and the self-inactivation of the *cre/dsRed* fusion gene (Unit II) in BEBV vectors (IV/Fig. 3). This data show also that both cre-recombination pairs (the modified loxP sites in the Unit II and wild type loxP sites in the Unit III) are functional and recognized correctly by the cre-recombinase.

5.15 The dual Cre/loxP system is silent during virus production (IV)

In order to study the possible detrimental activation of the Cre-expression cassette (unit II) and the formation of EBV-replicons during virus production, the virus preparations were analyzed by PCR. The results showed that EBV-replicons were not formed during virus production, indicating that the CAG promoter is not functional in insect cells (IV/Fig. 6). This feature enables the combination of both a cre-expression unit (unit II) and the cre-excisible replicon (unit III) in the same baculoviral backbone.

This approach is not possible in adenoviral vectors, because the promoters may become activated in mammalian cells during virus production. Indeed, a similar adenoviral system uses a dual virus approach; one virus contains a cre-expression cassette and the second contains cre-excisible proreplicon (Tan et al., 1999; Leblois et al., 2000). Excision of the episome occurs in target cells only if both adenoviral vectors transduce the same cell. This is very challenging setup especially *in vivo*, because high viral doses need to be used in order to generate the episomes. An attempt to solve this dual vector problem has been made by using a tetracycline-inducible cre-expression cassette (Krougliak et al., 2001) Finally, there is some evidence that EBNA-1 expression interferes with DNA replication of Ads causing serious problems in the virus production (Tan et al., 1999).

5.16 Episomes are formed in the BEBV transduced cells (IV)

In an ideal system, cre-expression is activated in the BEBV transduced target cells causing excision of a replicon from the baculoviral genome followed by inactivation of cre-expression. In order to demonstrate the formation of replicons in target cells, we amplified the episome with PCR from the transduced cells (IV/Fig. 7). The primers were designed to amplify only the formed EBV episomes. The amplified specific PCR products (EVO_{EGFP} : 369 bp or $KEVO_{EGFP}$: 322 bp) revealed the expected formation of the EBV replicons.

The corresponding system developed by Lo and co-workers was based on a FLP/Frt system, in which one baculoviral construct contained (Lo et al., 2009) FLP recombinase expression cassette and the other virus contained Frt-flanked pro-replicon. In their system, EGFP expression confirmed episome formation in transduced cells. EGFP was not driven by any promoter in the baculovirus genome and only after recombination and episome formation EGFP find the position downstream of the mammalian promoter. In addition, they recognized that sodium butyrate significantly augmented the excision of replicon

5.17 Transgene expression ceases by the time along with the disappearance of replicons (IV)

Transgene expression disappeared rapidly within two weeks after transduction in HepG2 cells (IV/Fig. 4B) and demonstrated short prolongation only in transfected Cho and 293T cells (IV/Fig. 4A). In order to determine whether replicons persist in the target cells even though transgene expression disappeared, we amplified replicons by the same primers used to prove their formation (see above) (IV/Fig. 8). The results indicated that the transgene expression declined because the replicons were lost not because of silencing of the transgene cassette by host factors. The earlier reports have shown that methylation of episomal vectors, especially in embryonic stem cells, can also cause a rapid loss of transgene expression and so limit transgene expression (Kameda et al., 2006).

5.18 Overproduction of EBNA-1 prolongs transgene expression (IV)

The 1st generation BEBV was unable to prolong transgene expression (IV/Fig. 4) in mammalian cells. The 2nd generation vector (allowing *hygromycin* –selection) prolonged transgene expression in transfected 293T cells, but sustained expression was achieved only under selection pressure (Figure 18, unpublished results). The results were the same with the recombinant 2nd generation viruses; without selection, not extension of transgene expression was observed (IV/Fig. 9) and the results were comparable to the 1st generation vector. Earlier reports have shown that widely used episomal backbones (e.g pCEP4) contain *EBNA-1* under a weak internal promoter and are as such not capable of prolonging transgene expression without selection pressure (Kreppel and Kochanek, 2004; Shan et al., 2006; Lo et al., 2009) or they require the use of cell lines constitutively expressing high amount of EBNA-1 (Kreppel and Kochanek, 2004; Howden et al., 2006). Thus, an adequate quantity of EBNA-1- protein seems to be essential in order to obtain a prolonged transgene expression and can be achieved by placing *EBNA-1* gene under a strong viral promoter such as CMV (Shan et al., 2006; Lo et al., 2009). Even though the optimization of EBNA-1 expression can prolong the expression period, the size of episome is also important. Lo and co-workers showed that the small-size replicon excised from the baculovirus genome was superior when compared to a baculovirus genome carrying the *oriP*/EBNA-1 cassette itself (Lo et al., 2009). Furthermore, the selection pressure (Neo) further prolonged and enhanced the transgene expression.

In order to enhance EBNA-1 expression, we cloned *EBNA-1*-gene under a strong CAG-promoter. In line with earlier reports, we detected significantly prolonged (> 45 days) transgene expression compared the control vector (21 days) without CAG-promoter (IV/Fig. 10). Our results support the importance of sufficient EBNA-1 expression for the episome maintenance.

In conclusion, novel baculoviruses were constructed in publications (I-IV) resulting in the altered profile of gene expression in vertebrate cells. In addition, new elements for baculoviruses and novel insights were obtained concerning the nuclear entry of baculoviruses.

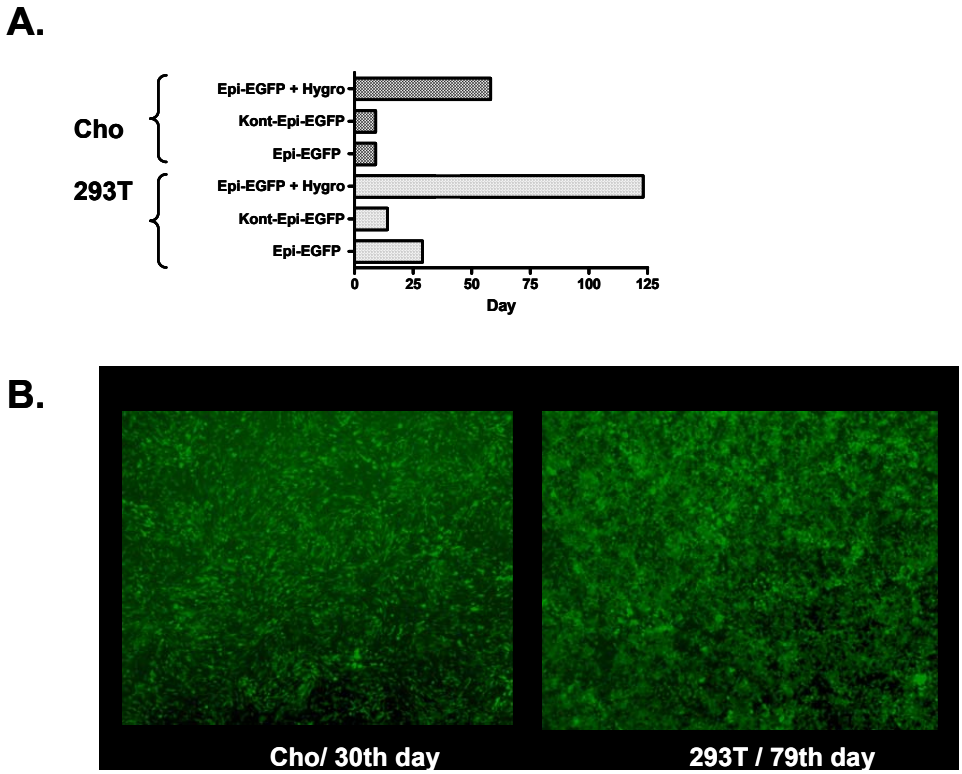


Figure 18. Hygromycin selection pressure allows long-term transgene expression. pEpiEGFP and pkontEpiEGFP constructs were tested in Cho and 293T cells. A) Time-course of EGFP production. B) Hygromycin selection led to sustained expression in both cell lines. All cells still produced EGFP at 30 days and 79 days time points (the latest time points measured) in Cho and 293T cells, respectively.

6 SUMMARY AND CONCLUSIONS

On the basis of the presented thesis, the following conclusions can be made:

1. A novel SSi-Cre can be used for the generation of Cre/*loxP* constructs and non-toxic expression of Cre recombinase in vertebrate cells. The Ssi-Cre system is compatible with double *lox*P-targeting strategies.

2. Baculovirus-mediated gene expression is boosted by the Woodchuck hepatitis virus post-transcriptional element (WPRE). A significant increase in the percentage of transduced cells and transgene expression was detected in several cell lines by a baculovirus equipped with the WPRE.

3. Culture medium has a drastic effect on the baculovirus-mediated gene delivery. Enhanced gene delivery in optimized medium was also observed with Ads and lentiviruses. The optimized medium seems to induce vimentin reorganization associating with enhanced baculovirus nuclear entry.

4. A novel all-in-one Baculovirus-Epstein-Barr hybrid virus was developed for a safe long-term episomal transgene expression, which takes advantage of the SSi-Cre cassette to liberate episomally replicating plasmids from the baculovirus genome. Three different BEBV versions were created and tested. The version having the *EBNA-1*-gene under CAG-promoter significantly prolonged the duration of transgene expression.

In conclusion, the present results widen the range of target cells which can be efficiently transduced with baculoviruses and provide new insights into important cellular factors covering baculovirus-mediate gene delivery.

7 REFERENCES

Abdelhamed, A.M., Kelley, C.M., Miller, T.G., Furman, P.A., Isom, H.C. (2002). Rebound of hepatitis B virus replication in HepG2 cells after cessation of antiviral treatment. J. Virol. *76*, 8148-8160.

Abe, T., Takahashi, H., Hamazaki, H., Miyano-Kurosaki, N., Matsuura, Y., Takaku, H. (2003). Baculovirus induces an innate immune response and confers protection from lethal influenza virus infection in mice. J. Immunol. *171*, 1133-1139.

Adams, A. (1987). Replication of latent Epstein-Barr virus genomes in Raji cells. J. Virol. *61*, 1743-1746.

Airenne, K.J., Hiltunen, M.O., Turunen, M.P., Turunen, A.M., Laitinen, O.H., Kulomaa, M.S., Yla-Herttuala, S. (2000). Baculovirus-mediated periadventitial gene transfer to rabbit carotid artery. Gene Ther. *7*, 1499-1504.

Airenne, K.J., Mahonen, A.J., Laitinen, O.H., Yla-Herttuala, S. (2009). Baculovirus-mediated Gene Transfer: An Emerging Universal Concept. In: Gene and Cell Therapy, N.S. Templeton, ed. (New York, NY: Marcel Dekker, Inc.), pp. 263-291.

Aiuti, A., Slavin, S., Aker, M., Ficara, F., Deola, S., Mortellaro, A., Morecki, S., Andolfi, G., Tabucchi, A., Carlucci, F., Marinello, E., Cattaneo, F., Vai, S., Servida, P., Miniero, R., Roncarolo, M.G., Bordignon, C. (2002). Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. Science *296*, 2410-2413.

Akopian, A., He, J., Boocock, M.R., Stark, W.M. (2003). Chimeric recombinases with designed DNA sequence recognition. Proc. Natl. Acad. Sci. U. S. A *100*, 8688-8691.

Akopian, A., Marshall, S.W. (2005). Site-specific DNA recombinases as instruments for genomic surgery. Adv. Genet. *55*, 1-23.

Alba, R., Bosch, A., Chillon, M. (2005). Gutless adenovirus: last-generation adenovirus for gene therapy. Gene Ther. *12 Suppl 1*, S18-S27.

Allen, G.C., Spiker, S., Thompson, W.F. (2000). Use of matrix attachment regions (MARs) to minimize transgene silencing. Plant Mol. Biol. *43*, 361-376.

Ambinder, R.F., Mullen, M.A., Chang, Y.N., Hayward, G.S., Hayward, S.D. (1991). Functional domains of Epstein-Barr virus nuclear antigen EBNA-1. J. Virol. *65*, 1466-1478.

Anderson, J.L., Hope, T.J. (2005). Intracellular trafficking of retroviral vectors: obstacles and advances. Gene Ther. *12*, 1667-1678.

Ando, T., Fujiyuki, T., Kawashima, T., Morioka, M., Kubo, T., Fujiwara, H. (2007). In vivo gene transfer into the honeybee using a nucleopolyhedrovirus vector. Biochem. Biophys. Res. Commun. *352*, 335-340.

Aoki, H., Sakoda, Y., Jukuroki, K., Takada, A., Kida, H., Fukusho, A. (1999a). Induction of antibodies in mice by a recombinant baculovirus expressing pseudorabies virus glycoprotein B in mammalian cells. Vet. Microbiol. *68*, 197-207.

Aoki, K., Barker, C., Danthinne, X., Imperiale, M.J., Nabel, G.J. (1999b). Efficient generation of recombinant adenoviral vectors by Cre-lox recombination in vitro. Mol. Med. *5*, 224-231.

Aranda, M., Kanellopoulou, C., Christ, N., Peitz, M., Rajewsky, K., Droge, P. (2001). Altered directionality in the Cre-LoxP site-specific recombination pathway. J. Mol. Biol. *311*, 453-459.

Arcangeletti, M.C., Pinardi, F., Missorini, S., De, C.F., Conti, G., Portincasa, P., Scherrer, K., Chezzi, C. (1997). Modification of cytoskeleton and prosome networks in relation to protein synthesis in influenza A virus-infected LLC-MK2 cells. Virus Res. *51*, 19-34.

Argyros, O., Wong, S.P., Niceta, M., Waddington, S.N., Howe, S.J., Coutelle, C., Miller, A.D., Harbottle, R.P. (2008). Persistent episomal transgene expression in liver following delivery of a scaffold/matrix attachment region containing non-viral vector. Gene Ther. *15*, 1593-1605.

Artelt, P., Grannemann, R., Stocking, C., Friel, J., Bartsch, J., Hauser, H. (1991). The prokaryotic neomycin-resistance-encoding gene acts as a transcriptional silencer in eukaryotic cells. Gene *99*, 249-254.

Ashman, C.R., Davidson, R.L. (1985). High spontaneous mutation frequency of BPV shuttle vector. Somat. Cell Mol. Genet. *11*, 499-504.

Atchison, R.W., Casto, B.C., Hammon., W.M, . (1965). Adenovirus-associated defective virus particles. Science *149*, 754-756.

Attal, J., Theron, M.C., Houdebine, L.M. (1999). The optimal use of IRES (internal ribosome entry site) in expression vectors. Genet. Anal. *15*, 161-165.

Ayres, M.D., Howard, S.C., Kuzio, J., Lopez-Ferber, M., Possee, R.D. (1994). The complete DNA sequence of Autographa californica nuclear polyhedrosis virus. Virology *202*, 586-605.

Azzouz, M., Martin-Rendon, E., Barber, R.D., Mitrophanous, K.A., Carter, E.E., Rohll, J.B., Kingsman, S.M., Kingsman, A.J., Mazarakis, N.D. (2002). Multicistronic lentiviral vector-mediated striatal gene transfer of aromatic L-amino acid decarboxylase, tyrosine hydroxylase, and GTP cyclohydrolase I induces sustained transgene expression, dopamine production, and functional improvement in a rat model of Parkinson's disease. J. Neurosci. *22*, 10302-10312.

Bank, A., Dorazio, R., Leboulch, P. (2005). A phase I/II clinical trial of beta-globin gene therapy for beta-thalassemia. Ann. N. Y. Acad. Sci. *1054*, 308-316.

Barsoum, J., Brown, R., McKee, M., Boyce, F.M. (1997). Efficient transduction of mammalian cells by a recombinant baculovirus having the vesicular stomatitis virus G glycoprotein. Hum. Gene Ther. *8*, 2011-2018.

Baum, C., Schambach, A., Bohne, J., Galla, M. (2006). Retrovirus vectors: toward the plentivirus? Mol. Ther. *13*, 1050-1063.

Bedikian, A.Y., Del, V.M. (2008). Allovectin-7 therapy in metastatic melanoma. Expert. Opin. Biol. Ther. *8*, 839-844.

Belichenko, I., Morishima, N., Separovic, D. (2001). Caspase-resistant vimentin suppresses apoptosis after photodynamic treatment with a silicon phthalocyanine in Jurkat cells. Arch. Biochem. Biophys. *390*, 57-63.

Belin, M.T., Boulanger, P. (1987). Processing of vimentin occurs during the early stages of adenovirus infection. J. Virol. *61*, 2559-2566.
Berger, E.A., Murphy, P.M., Farber, J.M. (1999). Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. Annu. Rev. Immunol. *17*, 657-700.
Berger, S.L. (2000). Gene regulation. Local or global? Nature *408*, 412-3, 415.
Bernstein, B.E., Meissner, A., Lander, E.S. (2007). The mammalian epigenome. Cell *128*, 669-681.
Bessis, N., GarciaCozar, F.J., Boissier, M.C. (2004). Immune responses to gene therapy vectors: influence on vector function and effector mechanisms. Gene Ther. *11 Suppl 1*, S10-S17.
Bhattacharya, B., Noad, R.J., Roy, P. (2007). Interaction between Bluetongue virus outer capsid protein VP2 and vimentin is necessary for virus egress. Virol. J. *4*, 7.
Black, B.C., Brennan, L.A., Dierks, P.M., Gard, I.E. (1997). Commercialization of Baculoviral Insecticides. In: The Baculoviruses, L.K. Miller, ed. (: Plenum Press, New York), pp. 341-387.
Black, J., Vos, J.M. (2002). Establishment of an oriP/EBNA1-based episomal vector transcribing human genomic beta-globin in cultured murine fibroblasts. Gene Ther. *9*, 1447-1454.
Blaese, R.M., Culver, K.W., Miller, A.D., Carter, C.S., Fleisher, T., Clerici, M., Shearer, G., Chang, L., Chiang, Y., Tolstoshev, P., Greenblatt, J.J., Rosenberg, S.A., Klein, H., Berger, M., Mullen, C.A., Ramsey, W.J., Muul, L., Morgan, R.A., Anderson, W.F. (1995). T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years. Science *270*, 475-480.
Blissard, G.W. (1996). Baculovirus--insect cell interactions. Cytotechnology *20*, 73-93.
Bode, J., Benham, C., Knopp, A., Mielke, C. (2000a). Transcriptional augmentation: modulation of gene expression by scaffold/matrix-attached regions (S/MAR elements). Crit Rev. Eukaryot. Gene Expr. *10*, 73-90.
Bode, J., Schlake, T., Iber, M., Schubeler, D., Seibler, J., Snezhkov, E., Nikolaev, L. (2000b). The transgeneticist's toolbox: novel methods for the targeted modification of eukaryotic genomes. Biol. Chem. *381*, 801-813.
Boulaire, J., Zhao, Y., Wang, S. (2009). Gene expression profiling to define host response to baculoviral transduction in the brain. J. Neurochem. *109*, 1203-1214.
Boulos, S., Meloni, B.P., Arthur, P.G., Bojarski, C., Knuckey, N.W. (2006). Assessment of CMV, RSV and SYN1 promoters and the woodchuck post-transcriptional regulatory element in adenovirus vectors for transgene expression in cortical neuronal cultures. Brain Res. *1102*, 27-38.
Boussif, O., Lezoualc'h, F., Zanta, M.A., Mergny, M.D., Scherman, D., Demeneix, B., Behr, J.P. (1995). A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proc. Natl. Acad. Sci. U. S. A *92*, 7297-7301.
Boyce, F.M., Bucher, N.L. (1996). Baculovirus-mediated gene transfer into mammalian cells. Proc. Natl. Acad. Sci. U. S. A *93*, 2348-2352.
Brun, S., Faucon-Biguet, N., Mallet, J. (2003). Optimization of transgene expression at the posttranscriptional level in neural cells: implications for gene therapy. Mol. Ther. *7*, 782-789.
Buchholz, F., Angrand, P.O., Stewart, A.F. (1996a). A simple assay to determine the functionality of Cre or FLP recombination targets in genomic manipulation constructs. Nucleic Acids Res *24*, 3118-3119.
Buchholz, F., Ringrose, L., Angrand, P.O., Rossi, F., Stewart, A.F. (1996b). Different thermostabilities of FLP and Cre recombinases: implications for applied site-specific recombination. Nucleic Acids Res. *24*, 4256-4262.
Buchholz, F., Stewart, A.F. (2001). Alteration of Cre recombinase site specificity by substrate-linked protein evolution. Nat. Biotechnol. *19*, 1047-1052.
Buchman, A.R., Berg, P. (1988). Comparison of intron-dependent and intron-independent gene expression. Mol. Cell Biol. *8*, 4395-4405.
Buerger, A., Rozhitskaya, O., Sherwood, M.C., Dorfman, A.L., Bisping, E., Abel, E.D., Pu, W.T., Izumo, S., Jay, P.Y. (2006). Dilated cardiomyopathy resulting from high-level myocardial expression of Cre-recombinase. J. Card Fail. *12*, 392-398.
Bukrinskaya, A., Brichacek, B., Mann, A., Stevenson, M. (1998). Establishment of a functional human immunodeficiency virus type 1 (HIV-1) reverse transcription complex involves the cytoskeleton. J. Exp. Med. *188*, 2113-2125.
Bunting, M., Bernstein, K.E., Greer, J.M., Capecchi, M.R., Thomas, K.R. (1999). Targeting genes for self-excision in the germ line. Genes Dev. *13*, 1524-1528.
Burges, H.D., Croizier G., Huger, J. (1980). A review of safety tests on baculoviruses. Entomaphaga *25*, 329-340.
Burns, J.C., Friedmann, T., Driever, W., Burrascano, M., Yee, J.K. (1993). Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. Proc. Natl. Acad. Sci U. S. A *90*, 8033-8037.
Bushey, A.M., Dorman, E.R., Corces, V.G. (2008). Chromatin insulators: regulatory mechanisms and epigenetic inheritance. Mol. Cell *32*, 1-9.
Bushman, F., Lewinski, M., Ciuffi, A., Barr, S., Leipzig, J., Hannenhalli, S., Hoffmann, C. (2005). Genome-wide analysis of retroviral DNA integration. Nat. Rev. Microbiol. *3*, 848-858.
Byun, Y., Chen, F., Chang, R., Trivedi, M., Green, K.J., Cryns, V.L. (2001). Caspase cleavage of vimentin disrupts intermediate filaments and promotes apoptosis. Cell Death. Differ. *8*, 443-450.
Campbell, E.M., Nunez, R., Hope, T.J. (2004). Disruption of the actin cytoskeleton can complement the ability of Nef to enhance human immunodeficiency virus type 1 infectivity. J. Virol. *78*, 5745-5755.
Carbonell, L.F., Klowden, M.J., Miller, L.K. (1985). Baculovirus-mediated expression of bacterial genes in dipteran and mammalian cells. J. Virol. *56*, 153-160.

Carbonell, L.F., Miller, L.K. (1987a). Baculovirus Interaction with Nontarget Organisms: a Virus-Borne Reporter Gene Is Not Expressed in Two Cell Lines. Applied and Environmental Microbiology *53***,** 1412-1417.
Carbonell, L.F., Miller, L.K. (1987b). Genetic Engineering of Viral Pesticides: Expression of foreing genes in nonpermissive cells. Molecular Strategies for Crop Protection 235-242.
Carter, B.J. (2005). Adeno-associated virus vectors in clinical trials. Hum. Gene Ther. *16***,** 541-550.
Carter, B.J., Burstein, H., Peluso, R.W. (2009). Adeno-Associated Virus and AAV Vectors for Gene Delivery. In: Gene and Cell Therapy, N.S. Templeton, ed. (Boca Raton: CRC Press), pp. 115-157.
Cartier, N. (2001). Gene therapy strategies for X-linked adrenoleukodystrophy. Curr. Opin. Mol. Ther. *3***,** 357-361.
Cartier, N., Aubourg, P. (2008). Hematopoietic stem cell gene therapy in Hurler syndrome, globoid cell leukodystrophy, metachromatic leukodystrophy and X-adrenoleukodystrophy. Curr. Opin. Mol. Ther. *10***,** 471-478.
Carvalho, Z.G., De Matos, A.P., Rodrigues-Pousada, C. (1988). Association of African swine fever virus with the cytoskeleton. Virus Res. *11***,** 175-192.
Cavazzana-Calvo, M., Fischer, A. (2007). Gene therapy for severe combined immunodeficiency: are we there yet? J. Clin. Invest *117***,** 1456-1465.
Cavazzana-Calvo, M., Hacein-Bey, S., de Saint, B.G., Gross, F., Yvon, E., Nusbaum, P., Selz, F., Hue, C., Certain, S., Casanova, J.L., Bousso, P., Deist, F.L., Fischer, A. (2000). Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science *288***,** 669-672.
Chapman, M.S., Rossmann, M.G. (1993). Structure, sequence, and function correlations among parvoviruses. Virology *194***,** 491-508.
Chattopadhyay, S., Pavithra, L. (2007). MARs and MARBPs: key modulators of gene regulation and disease manifestation. Subcell. Biochem. *41***,** 213-230.
Chen, C.Y., Shyu, A.B. (1995). AU-rich elements: characterization and importance in mRNA degradation. Trends Biochem. Sci. *20***,** 465-470.
Chen, H.C., Chang, Y.H., Chuang, C.K., Lin, C.Y., Sung, L.Y., Wang, Y.H., Hu, Y.C. (2009). The repair of osteochondral defects using baculovirus-mediated gene transfer with de-differentiated chondrocytes in bioreactor culture. Biomaterials *30***,** 674-681.
Chen, M., Goorha, R., Murti, K.G. (1986). Interaction of frog virus 3 with the cytomatrix. IV. Phosphorylation of vimentin precedes the reorganization of intermediate filaments around the virus assembly sites. J. Gen. Virol. *67 (Pt 5)***,** 915-922.
Chen, Y., Rice, P.A. (2003). New insight into site-specific recombination from Flp recombinase-DNA structures. Annu. Rev. Biophys. Biomol. Struct. *32***,** 135-159.
Cheng, T., Xu, C.Y., Wang, Y.B., Chen, M., Wu, T., Zhang, J., Xia, N.S. (2004). A rapid and efficient method to express target genes in mammalian cells by baculovirus. World J. Gastroenterol. *10***,** 1612-1618.
Cheshenko, N., Krougliak, N., Eisensmith, R.C., Krougliak, V.A. (2001). A novel system for the production of fully deleted adenovirus vectors that does not require helper adenovirus. Gene Ther. *8***,** 846-854.
Chisholm, G.E., Henner, D.J. (1988). Multiple early transcripts and splicing of the Autographa californica nuclear polyhedrosis virus IE-1 gene. J. Virol. *62***,** 3193-3200.
Choi, T., Huang, M., Gorman, C., Jaenisch, R. (1991). A generic intron increases gene expression in transgenic mice. Mol. Cell Biol. *11***,** 3070-3074.
Chou, Y.H., Khuon, S., Herrmann, H., Goldman, R.D. (2003). Nestin promotes the phosphorylation-dependent disassembly of vimentin intermediate filaments during mitosis. Mol. Biol. Cell *14***,** 1468-1478.
Chuang, C.K., Sung, L.Y., Hwang, S.M., Lo, W.H., Chen, H.C., Hu, Y.C. (2007). Baculovirus as a new gene delivery vector for stem cell engineering and bone tissue engineering. Gene Ther. *14***,** 1417-1424.
Chuang, C.K., Wong, T.H., Hwang, S.M., Chang, Y.H., Chen, G.Y., Chiu, Y.C., Huang, S.F., Hu, Y.C. (2009). Baculovirus transduction of mesenchymal stem cells: in vitro responses and in vivo immune responses after cell transplantation. Mol. Ther. *17***,** 889-896.
Ciuffi, A., Diamond, T.L., Hwang, Y., Marshall, H.M., Bushman, F.D. (2006). Modulating target site selection during human immunodeficiency virus DNA integration in vitro with an engineered tethering factor. Hum. Gene Ther. *17***,** 960-967.
Clarke, E.J., Allan, V. (2002). Intermediate filaments: vimentin moves in. Curr. Biol. *12***,** R596-R598.
Cohen, J.I. (2000). Epstein-Barr virus infection. N. Engl. J. Med. *343***,** 481-492.
Collins, C.H., Yokobayashi, Y., Umeno, D., Arnold, F.H. (2003). Engineering proteins that bind, move, make and break DNA. Curr. Opin. Biotechnol. *14***,** 371-378.
Colucci-Guyon, E., Portier, M.M., Dunia, I., Paulin, D., Pournin, S., Babinet, C. (1994). Mice lacking vimentin develop and reproduce without an obvious phenotype. Cell *79***,** 679-694.
Condreay, J.P., Witherspoon, S.M., Clay, W.C., Kost, T.A. (1999). Transient and stable gene expression in mammalian cells transduced with a recombinant baculovirus vector. Proc. Natl. Acad. Sci. U. S. A *96***,** 127-132.
Coppoolse, E.R., de Vroomen, M.J., Roelofs, D., Smit, J., van, G.F., Hersmus, B.J., Nijkamp, H.J., van Haaren, M.J. (2003). Cre recombinase expression can result in phenotypic aberrations in plants. Plant Mol. Biol. *51***,** 263-279.
Cordo, S.M., Candurra, N.A. (2003). Intermediate filament integrity is required for Junin virus replication. Virus Res. *97***,** 47-55.
Cornalia, E. (1856). Monografia del bombice del gelso (Bombyx mori Linneo). Memorie dell' I. R. Instituo Lombardo di Scienze, Lettere ed Arti *6***,** 3-387.
Cudmore, S., Cossart, P., Griffiths, G., Way, M. (1995). Actin-based motility of vaccinia virus. Nature *378***,** 636-638.
Cudmore, S., Reckmann, I., Way, M. (1997). Viral manipulations of the actin cytoskeleton. Trends Microbiol. *5***,** 142-148.

Cui, F.D., Kishida, T., Ohashi, S., Asada, H., Yasutomi, K., Satoh, E., Kubo, T., Fushiki, S., Imanishi, J., Mazda, O. (2001). Highly efficient gene transfer into murine liver achieved by intravenous administration of naked Epstein-Barr virus (EBV)-based plasmid vectors. Gene Ther. *8*, 1508-1513.

Dai, X., Willis, L.G., Huijskens, I., Palli, S.R., Theilmann, D.A. (2004). The acidic activation domains of the baculovirus transactivators IE1 and IE0 are functional for transcriptional activation in both insect and mammalian cells. J. Gen. Virol. *85*, 573-582.

Dai, Y., Roman, M., Naviaux, R.K., Verma, I.M. (1992). Gene therapy via primary myoblasts: long-term expression of factor IX protein following transplantation in vivo. Proc. Natl. Acad. Sci. U. S. A *89*, 10892-10895.

Davis, M.G., Huang, E.S. (1988). Transfer and expression of plasmids containing human cytomegalovirus immediate-early gene 1 promoter-enhancer sequences in eukaryotic and prokaryotic cells. Biotechnol. Appl. Biochem. *10*, 6-12.

De Geest, B., Van Linthout, S., Lox, M., Collen, D., Holvoet, P. (2000). Sustained expression of human apolipoprotein A-I after adenoviral gene transfer in C57BL/6 mice: role of apolipoprotein A-I promoter, apolipoprotein A-I introns, and human apolipoprotein E enhancer. Hum. Gene Ther. *11*, 101-112.

de, A., I, O'Hagan, R.C., Gartner, F., Malynn, B., Davidson, L., Rickert, R., Rajewsky, K., DePinho, R.A., Alt, F.W. (2001). Analysis of C-MYC function in normal cells via conditional gene-targeted mutation. Immunity. *14*, 45-55.

Deglon, N., Tseng, J.L., Bensadoun, J.C., Zurn, A.D., Arsenijevic, Y., Pereira de, A.L., Zufferey, R., Trono, D., Aebischer, P. (2000). Self-inactivating lentiviral vectors with enhanced transgene expression as potential gene transfer system in Parkinson's disease. Hum. Gene Ther. *11*, 179-190.

Dejana, E. (2006). The transcellular railway: insights into leukocyte diapedesis. Nat. Cell Biol. *8*, 105-107.

Delaney, W.E., Edwards, R., Colledge, D., Shaw, T., Torresi, J., Miller, T.G., Isom, H.C., Bock, C.T., Manns, M.P., Trautwein, C., Locarnini, S. (2001). Cross-resistance testing of antihepadnaviral compounds using novel recombinant baculoviruses which encode drug-resistant strains of hepatitis B virus. Antimicrob. Agents Chemother. *45*, 1705-1713.

Delaney, W.E., Isom, H.C. (1998). Hepatitis B virus replication in human HepG2 cells mediated by hepatitis B virus recombinant baculovirus. Hepatology *28*, 1134-1146.

Delaney, W.E., Miller, T.G., Isom, H.C. (1999). Use of the hepatitis B virus recombinant baculovirus-HepG2 system to study the effects of (-)-beta-2',3'-dideoxy-3'-thiacytidine on replication of hepatitis B virus and accumulation of covalently closed circular DNA. Antimicrob. Agents Chemother. *43*, 2017-2026.

Deng, Z., Atanasiu, C., Burg, J.S., Broccoli, D., Lieberman, P.M. (2003). Telomere repeat binding factors TRF1, TRF2, and hRAP1 modulate replication of Epstein-Barr virus OriP. J. Virol. *77*, 11992-12001.

Ding, W., Zhang, L.N., Yeaman, C., Engelhardt, J.F. (2006). rAAV2 traffics through both the late and the recycling endosomes in a dose-dependent fashion. Mol. Ther. *13*, 671-682.

Dobson, J. (2006). Gene therapy progress and prospects: magnetic nanoparticle-based gene delivery. Gene Ther. *13*, 283-287.

Doedens, J., Maynell, L.A., Klymkowsky, M.W., Kirkegaard, K. (1994). Secretory pathway function, but not cytoskeletal integrity, is required in poliovirus infection. Arch. Virol. Suppl *9*, 159-172.

Dohner, K., Sodeik, B. (2005). The role of the cytoskeleton during viral infection. Curr. Top. Microbiol. Immunol. *285*, 67-108.

Donello, J.E., Loeb, J.E., Hope, T.J. (1998). Woodchuck hepatitis virus contains a tripartite posttranscriptional regulatory element. J. Virol. *72*, 5085-5092.

Doostdar, H., Duthie, S.J., Burke, M.D., Melvin, W.T., Grant, M.H. (1988). The influence of culture medium composition on drug metabolising enzyme activities of the human liver derived Hep G2 cell line. FEBS Lett. *241*, 15-18.

Dorigo, O., Gil, J.S., Gallaher, S.D., Tan, B.T., Castro, M.G., Lowenstein, P.R., Calos, M.P., Berk, A.J. (2004). Development of a novel helper-dependent adenovirus-Epstein-Barr virus hybrid system for the stable transformation of mammalian cells. J. Virol. *78*, 6556-6566.

Douglas, J.T. (2007). Adenoviral vectors for gene therapy. Mol. Biotechnol. *36*, 71-80.

Dropulic, B., Hermankova, M., Pitha, P.M. (1996). A conditionally replicating HIV-1 vector interferes with wild-type HIV-1 replication and spread. Proc. Natl. Acad. Sci. U. S. A *93*, 11103-11108.

Dropulic, B., June, C.H. (2006). Gene-based immunotherapy for human immunodeficiency virus infection and acquired immunodeficiency syndrome. Hum. Gene Ther. *17*, 577-588.

Duan, D., Yue, Y., Yan, Z., McCray, P.B., Jr., Engelhardt, J.F. (1998). Polarity influences the efficiency of recombinant adenoassociated virus infection in differentiated airway epithelia. Hum. Gene Ther. *9*, 2761-2776.

Duisit, G., Saleun, S., Douthe, S., Barsoum, J., Chadeuf, G., Moullier, P. (1999). Baculovirus vector requires electrostatic interactions including heparan sulfate for efficient gene transfer in mammalian cells. J. Gene Med. *1*, 93-102.

Dull, T., Zufferey, R., Kelly, M., Mandel, R.J., Nguyen, M., Trono, D., Naldini, L. (1998). A third-generation lentivirus vector with a conditional packaging system. J. Virol. *72*, 8463-8471.

Duzgunes, N. (2008). Sitimagene ceradenovec, a gene therapeutic for the treatment of glioma. Curr. Opin. Mol. Ther. *10*, 187-195.

Dwarakanath, R.S., Clark, C.L., McElroy, A.K., Spector, D.H. (2001). The use of recombinant baculoviruses for sustained expression of human cytomegalovirus immediate early proteins in fibroblasts. Virology *284*, 297-307.

Earp, L.J., Delos, S.E., Park, H.E., White, J.M. (2005). The many mechanisms of viral membrane fusion proteins. Curr. Top. Microbiol. Immunol. *285*, 25-66.

Ehrhardt, A., Peng, P.D., Xu, H., Meuse, L., Kay, M.A. (2003). Optimization of cis-acting elements for gene expression from nonviral vectors in vivo. Hum. Gene Ther. *14*, 215-225.

Emery, D.W., Yannaki, E., Tubb, J., Stamatoyannopoulos, G. (2000). A chromatin insulator protects retrovirus vectors from chromosomal position effects. Proc. Natl. Acad. Sci. U. S. A *97*, 9150-9155.

Eriksson, J.E., He, T., Trejo-Skalli, A.V., Harmala-Brasken, A.S., Hellman, J., Chou, Y.H., Goldman, R.D. (2004). Specific in vivo phosphorylation sites determine the assembly dynamics of vimentin intermediate filaments. J. Cell Sci. *117*, 919-932.

Esue, O., Carson, A.A., Tseng, Y., Wirtz, D. (2006). A direct interaction between actin and vimentin filaments mediated by the tail domain of vimentin. J. Biol. Chem. *281*, 30393-30399.

Facciabene, A., Aurisicchio, L., La, M.N. (2004). Baculovirus vectors elicit antigen-specific immune responses in mice. J. Virol. *78*, 8663-8672.

Faigle, W., Colucci-Guyon, E., Louvard, D., Amigorena, S., Galli, T. (2000). Vimentin filaments in fibroblasts are a reservoir for SNAP23, a component of the membrane fusion machinery. Mol. Biol. Cell *11*, 3485-3494.

Farhood, H., Serbina, N., Huang, L. (1995). The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. Biochim. Biophys. Acta *1235*, 289-295.

Federici, B.A. (1997). Baculovirus Pathogenesis. In: The Baculoviruses, L.K. Miller, ed. (: Plenum Press, New York), pp. 33-59.

Fedorova, L., Fedorov, A. (2003). Introns in gene evolution. Genetica *118*, 123-131.

Feil, R., Brocard, J., Mascrez, B., LeMeur, M., Metzger, D., Chambon, P. (1996). Ligand-activated site-specific recombination in mice. Proc. Natl. Acad. Sci. U. S. A *93*, 10887-10890.

Felder, J.M., Sutton, R.E. (2009). Lentiviral Vectors. In: Gene and Cell Therapy, N.S. Templeton, ed. (Boca Raton: CRC Press), pp. 207-243.

Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M., Danielsen, M. (1987). Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. U. S. A *84*, 7413-7417.

Ferreira, L.R., Moussatche, N., Moura, N., V (1994). Rearrangement of intermediate filament network of BHK-21 cells infected with vaccinia virus. Arch. Virol. *138*, 273-285.

Fipaldini, C., Bellei, B., La Monica, N. (1999). Expression of hepatitis C virus cDNA in human hepatoma cell line mediated by a hybrid baculovirus-HCV vector. Virology *255*, 302-311.

Frappier, L., O'Donnell, M. (1992). EBNA1 distorts oriP, the Epstein-Barr virus latent replication origin. J. Virol. *66*, 1786-1790.

Freed, E.O., Mouland, A.J. (2006). The cell biology of HIV-1 and other retroviruses. Retrovirology. *3*, 77.

Funk, C.J., Braunagel, S.C., Rohrmann, G.F. (1997). Baculovirus Structure. In: The Baculoviruses, L.K. Miller, ed. (: Plenum Press, New York), pp. 7-32.

Gahn, T.A., Schildkraut, C.L. (1989). The Epstein-Barr virus origin of plasmid replication, oriP, contains both the initiation and termination sites of DNA replication. Cell *58*, 527-535.

Gallaher, S.D., Gil, J.S., Dorigo, O., Berk, A.J. (2009). Robust in vivo transduction of a genetically stable Epstein-Barr virus episome to hepatocytes in mice by a hybrid viral vector. J. Virol. *83*, 3249-3257.

Galou, M., Gao, J., Humbert, J., Mericskay, M., Li, Z., Paulin, D., Vicart, P. (1997). The importance of intermediate filaments in the adaptation of tissues to mechanical stress: evidence from gene knockout studies. Biol. Cell *89*, 85-97.

Gao, R., McCormick, C.J., Arthur, M.J., Ruddell, R., Oakley, F., Smart, D.E., Murphy, F.R., Harris, M.P., Mann, D.A. (2002). High efficiency gene transfer into cultured primary rat and human hepatic stellate cells using baculovirus vectors. Liver *22*, 15-22.

Gao, X., Kim, K.S., Liu, D. (2007). Nonviral gene delivery: what we know and what is next. AAPS. J. *9*, E92-104.

Garcia-Barreno, B., Jorcano, J.L., Aukenbauer, T., Lopez-Galindez, C., Melero, J.A. (1988). Participation of cytoskeletal intermediate filaments in the infectious cycle of human respiratory syncytial virus (RSV). Virus Res. *9*, 307-321.

Garcia-Otin, A.L., Guillou, F. (2006). Mammalian genome targeting using site-specific recombinases. Front Biosci. *11*, 1108-1136.

Garg, A., Barnes, P.F., Porgador, A., Roy, S., Wu, S., Nanda, J.S., Griffith, D.E., Girard, W.M., Rawal, N., Shetty, S., Vankayalapati, R. (2006). Vimentin expressed on Mycobacterium tuberculosis-infected human monocytes is involved in binding to the NKp46 receptor. J. Immunol. *177*, 6192-6198.

Ge, J., Huang, Y., Hu, X., Zhong, J. (2007). A surface-modified baculovirus vector with improved gene delivery to B-lymphocytic cells. J. Biotechnol. *129*, 367-372.

Ghosh, K., Van Duyne, G.D. (2002). Cre-loxP biochemistry. Methods *28*, 374-383.

Gilbertson, L. (2003). Cre-lox recombination: Cre-ative tools for plant biotechnology. Trends Biotechnol. *21*, 550-555.

Girod, P.A., Nguyen, D.Q., Calabrese, D., Puttini, S., Grandjean, M., Martinet, D., Regamey, A., Saugy, D., Beckmann, J.S., Bucher, P., Mermod, N. (2007). Genome-wide prediction of matrix attachment regions that increase gene expression in mammalian cells. Nat. Methods *4*, 747-753.

Glaser, S., Anastassiadis, K., Stewart, A.F. (2005). Current issues in mouse genome engineering. Nat. Genet. *37*, 1187-1193.

Glazko, G.V., Koonin, E.V., Rogozin, I.B., Shabalina, S.A. (2003). A significant fraction of conserved noncoding DNA in human and mouse consists of predicted matrix attachment regions. Trends Genet. *19*, 119-124.

Goehringer, C., Rutschow, D., Bauer, R., Schinkel, S., Weichenhan, D., Bekeredjian, R., Straub, V., Kleinschmidt, J.A., Katus, H.A., Muller, O.J. (2009). Prevention of cardiomyopathy in delta-sarcoglycan knockout mice after systemic transfer of targeted adeno-associated viral vectors. Cardiovasc. Res. *82*, 404-410.

Goff, S.P. (2001). Retroviridae: The Retroviruses and Their replication. In: Fields Virology, D.M. Knipe, P.M. Howley, eds. (Philadelphia: Lippincott Williams & Wilkins), pp. 1871-1939.

Goncalves, M.A. (2005). Adeno-associated virus: from defective virus to effective vector. Virol. J. *2*, 43.

Gonzales, M., Weksler, B., Tsuruta, D., Goldman, R.D., Yoon, K.J., Hopkinson, S.B., Flitney, F.W., Jones, J.C. (2001). Structure and function of a vimentin-associated matrix adhesion in endothelial cells. Mol. Biol. Cell *12***,** 85-100.

Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W., Bujard, H. (1995). Transcriptional activation by tetracyclines in mammalian cells. Science *268***,** 1766-1769.

Goussard, S., Grillot-Courvalin, C., Courvalin, P. (2003). Eukaryotic promoters can direct protein synthesis in Gram-negative bacteria. J. Mol. Microbiol. Biotechnol. *6***,** 211-218.

Graham, F.L., Prevec, L. (1995). Methods for construction of adenovirus vectors. Mol. Biotechnol. *3***,** 207-220.

Granados, R.R. (1978a). Replication phenomena of insect viruses *in vivo* and *in vitro*. In: Safety Aspects of Baculoviruses as Biological Insecticides, H.G. Miltenburger, ed. (Bonn: Bundesministerium fur Forschung und Technologie), pp. 163-184.

Granados, R.R., Lawler K.A. (1981). In vivo pathway of *Autographa californica* baculovirus invasion and infection. Virology *108***,** 297-308.

Granados, R.R., Williams, K.A. (1986). In vivo infection and replication of baculoviruses. In: The biology of baculoviruses, R.R. Granados, B.A. Federici, eds. (: CRC Press, Boca Raton), pp. 89-108.

Granados, R.R. (1978b). Early events in the infection of *Hiliothis zea* midgut cells by a baculovirus. Virology *90***,** 170-174.

Gray, N.K., Wickens, M. (1998). Control of translation initiation in animals. Annu. Rev. Cell Dev. Biol. *14***,** 399-458.

Greber, U.F. (2002). Signalling in viral entry. Cell Mol. Life Sci. *59***,** 608-626.

Greber, U.F., Way, M. (2006). A superhighway to virus infection. Cell *124***,** 741-754.

Greene, W.C., Peterlin, B.M. (2002). Charting HIV's remarkable voyage through the cell: Basic science as a passport to future therapy. Nat. Med. *8***,** 673-680.

Grignani, F., Kinsella, T., Mencarelli, A., Valtieri, M., Riganelli, D., Grignani, F., Lanfrancone, L., Peschle, C., Nolan, G.P., Pelicci, P.G. (1998). High-efficiency gene transfer and selection of human hematopoietic progenitor cells with a hybrid EBV/retroviral vector expressing the green fluorescence protein. Cancer Res. *58***,** 14-19.

Grimes, B.R., Monaco, Z.L. (2005). Artificial and engineered chromosomes: developments and prospects for gene therapy. Chromosoma *114***,** 230-241.

Grimes, B.R., Schindelhauer, D., McGill, N.I., Ross, A., Ebersole, T.A., Cooke, H.J. (2001). Stable gene expression from a mammalian artificial chromosome. EMBO Rep. *2***,** 910-914.

Grindley, N.D., Whiteson, K.L., Rice, P.A. (2006). Mechanisms of site-specific recombination. Annu. Rev. Biochem. *75***,** 567-605.

Grossman, Z., Mendelson, E., Brok-Simoni, F., Mileguir, F., Leitner, Y., Rechavi, G., Ramot, B. (1992). Detection of adeno-associated virus type 2 in human peripheral blood cells. J. Gen. Virol. *73 (Pt 4)***,** 961-966.

Grosveld, F., Antoniou, M., van Assendelft, G.B., de, B.E., Hurst, J., Kollias, G., MacFarlane, F., Wrighton, N. (1987). The regulation of expression of human beta-globin genes. Prog. Clin. Biol. Res. *251***,** 133-144.

Gruenberg, J., van der Goot, F.G. (2006). Mechanisms of pathogen entry through the endosomal compartments. Nat. Rev. Mol. Cell Biol. *7***,** 495-504.

Gruenheid, S., Finlay, B.B. (2003). Microbial pathogenesis and cytoskeletal function. Nature *422***,** 775-781.

Grunert, S., St, J.D. (1996). RNA localization and the development of asymmetry during Drosophila oogenesis. Curr. Opin. Genet. Dev. *6***,** 395-402.

Guo, J., Xin, H. (2006). Chinese gene therapy. Splicing out the West? Science *314***,** 1232-1235.

Habel, M.E., Drouin, M., Jung, D. (2004). Maintenance of Epstein-Barr virus-derived episomal vectors in the murine Sp2/0 myeloma cell line is dependent upon exogenous expression of human EBP2. Biochem. Cell Biol. *82***,** 375-380.

Hacein-Bey-Abina, S., Garrigue, A., Wang, G.P., Soulier, J., Lim, A., Morillon, E., Clappier, E., Caccavelli, L., Delabesse, E., Beldjord, K., Asnafi, V., Macintyre, E., Dal, C.L., Radford, I., Brousse, N., Sigaux, F., Moshous, D., Hauer, J., Borkhardt, A., Belohradsky, B.H., Wintergerst, U., Velez, M.C., Leiva, L., Sorensen, R., Wulffraat, N., Blanche, S., Bushman, F.D., Fischer, A., Cavazzana-Calvo, M. (2008). Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. J. Clin. Invest *118***,** 3132-3142.

Hacein-Bey-Abina, S., von, K.C., Schmidt, M., Le, D.F., Wulffraat, N., McIntyre, E., Radford, I., Villeval, J.L., Fraser, C.C., Cavazzana-Calvo, M., Fischer, A. (2003a). A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. N. Engl. J. Med. *348***,** 255-256.

Hacein-Bey-Abina, S., von, K.C., Schmidt, M., McCormack, M.P., Wulffraat, N., Leboulch, P., Lim, A., Osborne, C.S., Pawliuk, R., Morillon, E., Sorensen, R., Forster, A., Fraser, P., Cohen, J.I., de Saint, B.G., Alexander, I., Wintergerst, U., Frebourg, T., Aurias, A., Stoppa-Lyonnet, D., Romana, S., Radford-Weiss, I., Gross, F., Valensi, F., Delabesse, E., Macintyre, E., Sigaux, F., Soulier, J., Leiva, L.E., Wissler, M., Prinz, C., Rabbitts, T.H., Le, D.F., Fischer, A., Cavazzana-Calvo, M. (2003b). LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science *302***,** 415-419.

Haeseleer, F., Imanishi, Y., Saperstein, D.A., Palczewski, K. (2001). Gene transfer mediated by recombinant baculovirus into mouse eye. Invest Ophthalmol. Vis. Sci. *42***,** 3294-3300.

Hager, S., Frame, F.M., Collins, A.T., Burns, J.E., Maitland, N.J. (2008). An internal polyadenylation signal substantially increases expression levels of lentivirus-delivered transgenes but has the potential to reduce viral titer in a promoter-dependent manner. Hum. Gene Ther. *19***,** 840-850.

Hagstrom, J.N., Couto, L.B., Scallan, C., Burton, M., McCleland, M.L., Fields, P.A., Arruda, V.R., Herzog, R.W., High, K.A. (2000). Improved muscle-derived expression of human coagulation factor IX from a skeletal actin/CMV hybrid enhancer/promoter. Blood *95***,** 2536-2542.

Hamilton, D.L., Abremski, K. (1984). Site-specific recombination by the bacteriophage P1 lox-Cre system. Cre-mediated synapsis of two lox sites. J. Mol. Biol. *178***,** 481-486.

Harper, D.M., Franco, E.L., Wheeler, C., Ferris, D.G., Jenkins, D., Schuind, A., Zahaf, T., Innis, B., Naud, P., De Carvalho, N.S., Roteli-Martins, C.M., Teixeira, J., Blatter, M.M., Korn, A.P., Quint, W., Dubin, G. (2004). Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial. Lancet *364***,** 1757-1765.

Harrington, J.J., Van, B.G., Mays, R.W., Gustashaw, K., Willard, H.F. (1997). Formation of de novo centromeres and construction of first-generation human artificial microchromosomes. Nat. Genet. *15***,** 345-355.

Harris, K.F., Christensen, J.B., Imperiale, M.J. (1996). BK virus large T antigen: interactions with the retinoblastoma family of tumor suppressor proteins and effects on cellular growth control. J. Virol. *70***,** 2378-2386.

Heidmann, D., Lehner, C.F. (2001). Reduction of Cre recombinase toxicity in proliferating Drosophila cells by estrogen-dependent activity regulation. Dev. Genes Evol. *211***,** 458-465.

Heipertz, R.A., Jr., Miller, T.G., Kelley, C.M., Delaney, W.E., Locarnini, S.A., Isom, H.C. (2007). In vitro study of the effects of precore and lamivudine-resistant mutations on hepatitis B virus replication. J. Virol. *81***,** 3068-3076.

Helfand, B.T., Chang, L., Goldman, R.D. (2004). Intermediate filaments are dynamic and motile elements of cellular architecture. J. Cell Sci. *117***,** 133-141.

Hermening, S., Kugler, S., Bahr, M., Isenmann, S. (2006). Improved high-capacity adenoviral vectors for high-level neuron-restricted gene transfer to the CNS. J. Virol. Methods *136***,** 30-37.

Herrmann, H., Fouquet, B., Franke, W.W. (1989). Expression of intermediate filament proteins during development of Xenopus laevis. I. cDNA clones encoding different forms of vimentin. Development *105***,** 279-298.

Higashi, A.Y., Ikawa, T., Muramatsu, M., Economides, A.N., Niwa, A., Okuda, T., Murphy, A.J., Rojas, J., Heike, T., Nakahata, T., Kawamoto, H., Kita, T., Yanagita, M. (2009). Direct hematological toxicity and illegitimate chromosomal recombination caused by the systemic activation of CreERT2. J. Immunol. *182***,** 5633-5640.

Higashimoto, T., Urbinati, F., Perumbeti, A., Jiang, G., Zarzuela, A., Chang, L.J., Kohn, D.B., Malik, P. (2007). The woodchuck hepatitis virus post-transcriptional regulatory element reduces readthrough transcription from retroviral vectors. Gene Ther. *14***,** 1298-1304.

Hirokawa, N. (1998). Kinesin and dynein superfamily proteins and the mechanism of organelle transport. Science *279***,** 519-526.

Hirokawa, N., Noda, Y. (2008). Intracellular transport and kinesin superfamily proteins, KIFs: structure, function, and dynamics. Physiol Rev. *88***,** 1089-1118.

Hirosue, S., Senn, K., Clement, N., Nonnenmacher, M., Gigout, L., Linden, R.M., Weber, T. (2007). Effect of inhibition of dynein function and microtubule-altering drugs on AAV2 transduction. Virology *367***,** 10-18.

Ho, Y.C., Chung, Y.C., Hwang, S.M., Wang, K.C., Hu, Y.C. (2005). Transgene expression and differentiation of baculovirus-transduced human mesenchymal stem cells. J. Gene Med. *7***,** 860-868.

Ho, Y.C., Lee, H.P., Hwang, S.M., Lo, W.H., Chen, H.C., Chung, C.K., Hu, Y.C. (2006). Baculovirus transduction of human mesenchymal stem cell-derived progenitor cells: variation of transgene expression with cellular differentiation states. Gene Ther. *13***,** 1471-1479.

Hoare, J., Waddington, S., Thomas, H.C., Coutelle, C., McGarvey, M.J. (2005). Complement inhibition rescued mice allowing observation of transgene expression following intraportal delivery of baculovirus in mice. J. Gene Med. *7***,** 325-333.

Hoess, R.H., Ziese, M., Sternberg, N. (1982). P1 site-specific recombination: nucleotide sequence of the recombining sites. Proc. Natl. Acad. Sci. U. S. A *79***,** 3398-3402.

Hofmann, C., Sandig, V., Jennings, G., Rudolph, M., Schlag, P., Strauss, M. (1995). Efficient gene transfer into human hepatocytes by baculovirus vectors. Proc. Natl. Acad. Sci. U. S. A *92***,** 10099-10103.

Hofmann, C., Strauss, M. (1998). Baculovirus-mediated gene transfer in the presence of human serum or blood facilitated by inhibition of the complement system. Gene Ther. *5***,** 531-536.

Hofmann, C., Wolfgang, L., Strauss, M. (1998). The Baculovirus vector system for gene delivery into hepatocytes. Gene Therapy and Molecular Biology *1***,** 231-239.

Holleran, E.A., Karki, S., Holzbaur, E.L. (1998). The role of the dynactin complex in intracellular motility. Int. Rev. Cytol. *182***,** 69-109.

Homan, S.M., Mercurio, A.M., LaFlamme, S.E. (1998). Endothelial cells assemble two distinct alpha6beta4-containing vimentin-associated structures: roles for ligand binding and the beta4 cytoplasmic tail. J. Cell Sci. *111 (Pt 18)***,** 2717-2728.

Hook, P., Vallee, R.B. (2006). The dynein family at a glance. J. Cell Sci. *119***,** 4369-4371.

Hoopes, R.R., Jr., Rohrmann, G.F. (1991). In vitro transcription of baculovirus immediate early genes: accurate mRNA initiation by nuclear extracts from both insect and human cells. Proc. Natl. Acad. Sci U. S. A *88***,** 4513-4517.

Horwitz, M.S. (2001). Adenoviruses. In: Fields Virology, D.M. Knipe, P.M. Howley, eds. (Philadelphia: Lippincott Williams & Wilkins), pp. 2301-2326.

Howden, S.E., Wardan, H., Voullaire, L., McLenachan, S., Williamson, R., Ioannou, P., Vadolas, J. (2006). Chromatin-binding regions of EBNA1 protein facilitate the enhanced transfection of Epstein-Barr virus-based vectors. Hum. Gene Ther. *17***,** 833-844.

Howe, S.J., Mansour, M.R., Schwarzwaelder, K., Bartholomae, C., Hubank, M., Kempski, H., Brugman, M.H., Pike-Overzet, K., Chatters, S.J., de, R.D., Gilmour, K.C., Adams, S., Thornhill, S.I., Parsley, K.L., Staal, F.J., Gale, R.E., Linch, D.C., Bayford, J., Brown, L., Quaye, M., Kinnon, C., Ancliff, P., Webb, D.K., Schmidt, M., von, K.C., Gaspar, H.B., Thrasher,

A.J. (2008). Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. J. Clin. Invest *118*, 3143-3150.
Hsu, C.S., Ho, Y.C., Wang, K.C., Hu, Y.C. (2004). Investigation of optimal transduction conditions for baculovirus-mediated gene delivery into mammalian cells. Biotechnol. Bioeng. *88*, 42-51.
Hu, Y.C., Yao, K., Wu, T.Y. (2008). Baculovirus as an expression and/or delivery vehicle for vaccine antigens. Expert. Rev. Vaccines. *7*, 363-371.
Huang, M.T., Gorman, C.M. (1990). The simian virus 40 small-t intron, present in many common expression vectors, leads to aberrant splicing. Mol. Cell Biol. *10*, 1805-1810.
Huang, W., Tian, X.L., Wu, Y.L., Zhong, J., Yu, L.F., Hu, S.P., Li, B. (2008). Suppression of gastric cancer growth by baculovirus vector-mediated transfer of normal epithelial cell specific-1 gene. World J. Gastroenterol. *14*, 5810-5815.
Huard, J., Lochmuller, H., Acsadi, G., Jani, A., Massie, B., Karpati, G. (1995). The route of administration is a major determinant of the transduction efficiency of rat tissues by adenoviral recombinants. Gene Ther. *2*, 107-115.
Huebner, R.J., Rowe, W.P., Schatten, W.E., Smith, R.R., Thomas, L.B. (1956). Studies on the use of viruses in the treatment of carcinoma of the cervix. Cancer *9*, 1211-1218.
Hughes, T.A. (2006). Regulation of gene expression by alternative untranslated regions. Trends Genet. *22*, 119-122.
Hughes, V. (2007). Therapy on trial. Nat. Med. *13*, 1008-1009.
Hunt, L., Batard, P., Jordan, M., Wurm, F.M. (2002). Fluorescent proteins in animal cells for process development: optimization of sodium butyrate treatment as an example. Biotechnol. Bioeng. *77*, 528-537.
Hüser, A., Rudolph, M., Hofmann, C. (2001). Incorporation of decay-accelerating factor into the baculovirus envelope generates complement-resistant gene transfer vectors. Nat. Biotechnol. *19*, 451-455.
Hynes, R.O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. Cell *69*, 11-25.
Inagaki, M., Nishi, Y., Nishizawa, K., Matsuyama, M., Sato, C. (1987). Site-specific phosphorylation induces disassembly of vimentin filaments in vitro. Nature *328*, 649-652.
Irvine, D.V., Shaw, M.L., Choo, K.H., Saffery, R. (2005). Engineering chromosomes for delivery of therapeutic genes. Trends Biotechnol. *23*, 575-583.
Ito, S., Gotoh, E., Ozawa, S., Yanagi, K. (2002). Epstein-Barr virus nuclear antigen-1 is highly colocalized with interphase chromatin and its newly replicated regions in particular. J. Gen. Virol. *83*, 2377-2383.
Ivaska, J., Pallari, H.M., Nevo, J., Eriksson, J.E. (2007). Novel functions of vimentin in cell adhesion, migration, and signaling. Exp. Cell Res. *313*, 2050-2062.
Ivaska, J., Vuoriluoto, K., Huovinen, T., Izawa, I., Inagaki, M., Parker, P.J. (2005). PKCepsilon-mediated phosphorylation of vimentin controls integrin recycling and motility. EMBO J. *24*, 3834-3845.
Iwai, M., Harada, Y., Tanaka, S., Muramatsu, A., Mori, T., Kashima, K., Imanishi, J., Mazda, O. (2002). Polyethylenimine-mediated suicide gene transfer induces a therapeutic effect for hepatocellular carcinoma in vivo by using an Epstein-Barr virus-based plasmid vector. Biochem. Biophys. Res. Commun. *291*, 48-54.
Iyengar, S., Hildreth, J.E., Schwartz, D.H. (1998). Actin-dependent receptor colocalization required for human immunodeficiency virus entry into host cells. J. Virol. *72*, 5251-5255.
Izumo, T., Ohtsuru, A., Tokunaga, Y., Namba, H., Kaneda, Y., Nagata, I., Yamashita, S. (2007). Epstein-Barr virus-based vector improves the tumor cell killing effect of pituitary tumor in HVJ-liposome-mediated transcriptional targeting suicide gene therapy. Int. J. Oncol. *31*, 379-387.
Jackson, R.J., Standart, N. (1990). Do the poly(A) tail and 3' untranslated region control mRNA translation? Cell *62*, 15-24.
Jasmund, I., Schwientek, S., Acikgoz, A., Langsch, A., Machens, H.G., Bader, A. (2007). The influence of medium composition and matrix on long-term cultivation of primary porcine and human hepatocytes. Biomol. Eng *24*, 59-69.
Jenke, A.C., Stehle, I.M., Herrmann, F., Eisenberger, T., Baiker, A., Bode, J., Fackelmayer, F.O., Lipps, H.J. (2004). Nuclear scaffold/matrix attached region modules linked to a transcription unit are sufficient for replication and maintenance of a mammalian episome. Proc. Natl. Acad. Sci. U. S. A *101*, 11322-11327.
Jia, H., Kling, J. (2006). China offers alternative gateway for experimental drugs. Nat. Biotechnol. *24*, 117-118.
Jo, D., Nashabi, A., Doxsee, C., Lin, Q., Unutmaz, D., Chen, J., Ruley, H.E. (2001). Epigenetic regulation of gene structure and function with a cell-permeable Cre recombinase. Nat. Biotechnol. *19*, 929-933.
Jolly, C., Mitar, I., Sattentau, Q.J. (2007). Requirement for an intact T-cell actin and tubulin cytoskeleton for efficient assembly and spread of human immunodeficiency virus type 1. J. Virol. *81*, 5547-5560.
Jones, P.L., Veenstra, G.J., Wade, P.A., Vermaak, D., Kass, S.U., Landsberger, N., Strouboulis, J., Wolffe, A.P. (1998). Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. Nat. Genet. *19*, 187-191.
Jones, R.J., Smith, L.J., Dawson, C.W., Haigh, T., Blake, N.W., Young, L.S. (2003). Epstein-Barr virus nuclear antigen 1 (EBNA1) induced cytotoxicity in epithelial cells is associated with EBNA1 degradation and processing. Virology *313*, 663-676.
Kaczmarczyk, S.J., Green, J.E. (2001). A single vector containing modified cre recombinase and LOX recombination sequences for inducible tissue-specific amplification of gene expression. Nucleic Acids Res. *29*, E56.
Kaikkonen, M.U., Raty, J.K., Airenne, K.J., Wirth, T., Heikura, T., Yla-Herttuala, S. (2006). Truncated vesicular stomatitis virus G protein improves baculovirus transduction efficiency in vitro and in vivo. Gene Ther. *13*, 304-312.
Kaikkonen, M.U., Viholainen, J.I., Narvanen, A., Yla-Herttuala, S., Airenne, K.J. (2008). Targeting and purification of metabolically biotinylated baculovirus. Hum. Gene Ther. *19*, 589-600.
Kaiser, J. (2007). Clinical trials. Gene transfer an unlikely contributor to patient's death. Science *318*, 1535.

Kakoki, M., Tsai, Y.S., Kim, H.S., Hatada, S., Ciavatta, D.J., Takahashi, N., Arnold, L.W., Maeda, N., Smithies, O. (2004). Altering the expression in mice of genes by modifying their 3' regions. Dev. Cell *6*, 597-606.
Kameda, T., Smuga-Otto, K., Thomson, J.A. (2006). A severe de novo methylation of episomal vectors by human ES cells. Biochem. Biophys. Res. Commun. *349*, 1269-1277.
Kaneda, Y., Saeki, Y., Nakabayashi, M., Zhou, W.Z., Kaneda, M.W., Morishita, R. (2000). Enhancement of transgene expression by cotransfection of oriP plasmid with EBNA-1 expression vector. Hum. Gene Ther. *11*, 471-479.
Kaneko, H., Suzuki, H., Abe, T., Miyano-Kurosaki, N., Takaku, H. (2006). Inhibition of HIV-1 replication by vesicular stomatitis virus envelope glycoprotein pseudotyped baculovirus vector-transduced ribozyme in mammalian cells. Biochem. Biophys. Res. Commun. *349*, 1220-1227.
Kang, M.S., Soni, V., Bronson, R., Kieff, E. (2008). Epstein-Barr virus nuclear antigen 1 does not cause lymphoma in C57BL/6J mice. J. Virol. *82*, 4180-4183.
Kann, M., Sodeik, B., Vlachou, A., Gerlich, W.H., Helenius, A. (1999). Phosphorylation-dependent binding of hepatitis B virus core particles to the nuclear pore complex. J. Cell Biol. *145*, 45-55.
Karczewski, M.K., Strebel, K. (1996). Cytoskeleton association and virion incorporation of the human immunodeficiency virus type 1 Vif protein. J. Virol. *70*, 494-507.
Karmali, P.P., Chaudhuri, A. (2007). Cationic liposomes as non-viral carriers of gene medicines: resolved issues, open questions, and future promises. Med. Res. Rev. *27*, 696-722.
Keddie, B.A., Aponte, G.W., Volkman, L.E. (1989). The pathway of infection of Autographa californica nuclear polyhedrosis virus in an insect host. Science *243*, 1728-1730.
Kelkar, S.A., Pfister, K.K., Crystal, R.G., Leopold, P.L. (2004). Cytoplasmic dynein mediates adenovirus binding to microtubules. J. Virol. *78*, 10122-10132.
Kenoutis, C., Efrose, R.C., Swevers, L., Lavdas, A.A., Gaitanou, M., Matsas, R., Iatrou, K. (2006). Baculovirus-mediated gene delivery into Mammalian cells does not alter their transcriptional and differentiating potential but is accompanied by early viral gene expression. J. Virol. *80*, 4135-4146.
Kichler, A. (2004). Gene transfer with modified polyethylenimines. J. Gene Med. *6 Suppl 1*, S3-10.
Kim, J.M., Kim, J.S., Park, D.H., Kang, H.S., Yoon, J., Baek, K., Yoon, Y. (2004). Improved recombinant gene expression in CHO cells using matrix attachment regions. J. Biotechnol. *107*, 95-105.
Kim, Y.K., Park, I.K., Jiang, H.L., Choi, J.Y., Je, Y.H., Jin, H., Kim, H.W., Cho, M.H., Cho, C.S. (2006). Regulation of transduction efficiency by pegylation of baculovirus vector in vitro and in vivo. J. Biotechnol. *125*, 104-109.
Kingsman, S.M., Mitrophanous, K., Olsen, J.C. (2005). Potential oncogene activity of the woodchuck hepatitis post-transcriptional regulatory element (WPRE). Gene Ther. *12*, 3-4.
Kinnunen, K., Kalesnykas, G., Mahonen, A.J., Laidinen, S., Holma, L., Heikura, T., Airenne, K., Uusitalo, H., Yla-Herttuala, S. (2009). Baculovirus is an efficient vector for the transduction of the eye: comparison of baculovirus- and adenovirus-mediated intravitreal vascular endothelial growth factor D gene transfer in the rabbit eye. J. Gene Med. *11*, 382-389.
Kircheis, R., Wightman, L., Schreiber, A., Robitza, B., Rossler, V., Kursa, M., Wagner, E. (2001). Polyethylenimine/DNA complexes shielded by transferrin target gene expression to tumors after systemic application. Gene Ther. *8*, 28-40.
Kishida, T., Asada, H., Satoh, E., Tanaka, S., Shinya, M., Hirai, H., Iwai, M., Tahara, H., Imanishi, J., Mazda, O. (2001). In vivo electroporation-mediated transfer of interleukin-12 and interleukin-18 genes induces significant antitumor effects against melanoma in mice. Gene Ther. *8*, 1234-1240.
Kitajima, M., Hamazaki, H., Miyano-Kurosaki, N., Takaku, H. (2006). Characterization of baculovirus Autographa californica multiple nuclear polyhedrosis virus infection in mammalian cells. Biochem. Biophys. Res. Commun. *343*, 378-384.
Klein, R., Ruttkowski, B., Knapp, E., Salmons, B., Gunzburg, W.H., Hohenadl, C. (2006). WPRE-mediated enhancement of gene expression is promoter and cell line specific. Gene *372*, 153-161.
Klein, R.L., Hamby, M.E., Gong, Y., Hirko, A.C., Wang, S., Hughes, J.A., King, M.A., Meyer, E.M. (2002). Dose and promoter effects of adeno-associated viral vector for green fluorescent protein expression in the rat brain. Exp. Neurol. *176*, 66-74.
Kohn, D.B. (2007). Lentiviral vectors ready for prime-time. Nat. Biotechnol. *25*, 65-66.
Kolb, A.F. (2002). Genome engineering using site-specific recombinases. Cloning Stem Cells *4*, 65-80.
Komano, J., Miyauchi, K., Matsuda, Z., Yamamoto, N. (2004). Inhibiting the Arp2/3 complex limits infection of both intracellular mature vaccinia virus and primate lentiviruses. Mol. Biol. Cell *15*, 5197-5207.
Koresawa, Y., Miyagawa, S., Ikawa, M., Matsunami, K., Yamada, M., Shirakura, R., Okabe, M. (2000). Synthesis of a new Cre recombinase gene based on optimal codon usage for mammalian systems. J. Biochem. *127*, 367-372.
Kost, T.A., Condreay, J.P. (2002). Recombinant baculoviruses as mammalian cell gene-delivery vectors. Trends Biotechnol. *20*, 173-180.
Kozak, M. (1984). Point mutations close to the AUG initiator codon affect the efficiency of translation of rat preproinsulin in vivo. Nature *308*, 241-246.
Kozak, M. (1987a). An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res. *15*, 8125-8148.
Kozak, M. (1987b). At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. J. Mol. Biol. *196*, 947-950.
Kozak, M. (1999). Initiation of translation in prokaryotes and eukaryotes. Gene *234*, 187-208.
Kozak, M. (2005). Regulation of translation via mRNA structure in prokaryotes and eukaryotes. Gene *361*, 13-37.
Kozarsky, K.F., Wilson, J.M. (1993). Gene therapy: adenovirus vectors. Curr. Opin. Genet. Dev. *3*, 499-503.

Kreis, S., Schonfeld, H.J., Melchior, C., Steiner, B., Kieffer, N. (2005). The intermediate filament protein vimentin binds specifically to a recombinant integrin alpha2/beta1 cytoplasmic tail complex and co-localizes with native alpha2/beta1 in endothelial cell focal adhesions. Exp. Cell Res. *305*, 110-121.

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

Kronschnabl, M., Marschall, M., Stamminger, T. (2002). Efficient and tightly regulated expression systems for the human cytomegalovirus major transactivator protein IE2p86 in permissive cells. Virus Res. *83*, 89-102.

Krougliak, V.A., Krougliak, N., Eisensmith, R.C. (2001). Stabilization of transgenes delivered by recombinant adenovirus vectors through extrachromosomal replication. J. Gene Med. *3*, 51-58.

Kuhn, R., Schwenk, F., Aguet, M., Rajewsky, K. (1995). Inducible gene targeting in mice. Science *269*, 1427-1429.

Kukkonen, S.P., Airenne, K.J., Marjomaki, V., Laitinen, O.H., Lehtolainen, P., Kankaanpaa, P., Mahonen, A.J., Raty, J.K., Nordlund, H.R., Oker-Blom, C., Kulomaa, M.S., Yla-Herttuala, S. (2003). Baculovirus capsid display: a novel tool for transduction imaging. Mol. Ther. *8*, 853-862.

Kumar, N., Robidoux, J., Daniel, K.W., Guzman, G., Floering, L.M., Collins, S. (2007). Requirement of vimentin filament assembly for beta3-adrenergic receptor activation of ERK MAP kinase and lipolysis. J. Biol. Chem. *282*, 9244-9250.

Laakkonen, J.P., Kaikkonen, M.U., Ronkainen, P.H., Ihalainen, T.O., Niskanen, E.A., Hakkinen, M., Salminen, M., Kulomaa, M.S., Yla-Herttuala, S., Airenne, K.J., Vihinen-Ranta, M. (2008). Baculovirus-mediated immediate-early gene expression and nuclear reorganization in human cells. Cell Microbiol. *10*, 667-681.

Laakkonen, J.P., Makela, A.R., Kakkonen, E., Turkki, P., Kukkonen, S., Peranen, J., Yla-Herttuala, S., Airenne, K.J., Oker-Blom, C., Vihinen-Ranta, M., Marjomaki, V. (2009). Clathrin-independent entry of baculovirus triggers uptake of E. coli in non-phagocytic human cells. PLoS. ONE. *4*, e5093.

Laitinen, O.H., Airenne, K.J., Hytonen, V.P., Peltomaa, E., Mahonen, A.J., Wirth, T., Lind, M.M., Makela, K.A., Toivanen, P.I., Schenkwein, D., Heikura, T., Nordlund, H.R., Kulomaa, M.S., Yla-Herttuala, S. (2005). A multipurpose vector system for the screening of libraries in bacteria, insect and mammalian cells and expression in vivo. Nucleic Acids Res. *33*, e42.

Laitinen, O.H., Hytonen, V.P., Ahlroth, M.K., Pentikainen, O.T., Gallagher, C., Nordlund, H.R., Ovod, V., Marttila, A.T., Porkka, E., Heino, S., Johnson, M.S., Airenne, K.J., Kulomaa, M.S. (2002). Chicken avidin-related proteins show altered biotin-binding and physico-chemical properties as compared with avidin. Biochem. J. *363*, 609-617.

Lakadamyali, M., Rust, M.J., Babcock, H.P., Zhuang, X. (2003). Visualizing infection of individual influenza viruses. Proc. Natl. Acad. Sci. U. S. A *100*, 9280-9285.

Lakso, M., Sauer, B., Mosinger, B., Jr., Lee, E.J., Manning, R.W., Yu, S.H., Mulder, K.L., Westphal, H. (1992). Targeted oncogene activation by site-specific recombination in transgenic mice. Proc. Natl. Acad. Sci. U. S. A *89*, 6232-6236.

Lancaster, W.D., Olson, C. (1982). Animal papillomaviruses. Microbiol. Rev. *46*, 191-207.

Larin, Z., Mejia, J.E. (2002). Advances in human artificial chromosome technology. Trends Genet. *18*, 313-319.

Lazarou, J., Pomeranz, B.H., Corey, P.N. (1998). Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies. JAMA *279*, 1200-1205.

Leblois, H., Roche, C., Di, F.N., Orsini, C., Yeh, P., Perricaudet, M. (2000). Stable transduction of actively dividing cells via a novel adenoviral/episomal vector. Mol. Ther. *1*, 314-322.

Lee, C.G., Vieira, W.D., Pastan, I., Gottesman, M.M. (2001a). An episomally maintained MDR1 gene for gene therapy. Hum. Gene Ther. *12*, 945-953.

Lee, D.F., Chen, C.C., Hsu, T.A., Juang, J.L. (2000). A baculovirus superinfection system: efficient vehicle for gene transfer into Drosophila S2 cells. J. Virol. *74*, 11873-11880.

Lee, H.P., Chen, Y.L., Shen, H.C., Lo, W.H., Hu, Y.C. (2007a). Baculovirus transduction of rat articular chondrocytes: roles of cell cycle. J. Gene Med. *9*, 33-43.

Lee, H.P., Ho, Y.C., Hwang, S.M., Sung, L.Y., Shen, H.C., Liu, H.J., Hu, Y.C. (2007b). Variation of baculovirus-harbored transgene transcription among mesenchymal stem cell-derived progenitors leads to varied expression. Biotechnol. Bioeng. *97*, 649-655.

Lee, J.Y., Ristow, M., Lin, X., White, M.F., Magnuson, M.A., Hennighausen, L. (2006). RIP-Cre revisited, evidence for impairments of pancreatic beta-cell function. J. Biol. Chem. *281*, 2649-2653.

Lee, Y.J., Galoforo, S.S., Battle, P., Lee, H., Corry, P.M., Jessup, J.M. (2001b). Replicating adenoviral vector-mediated transfer of a heat-inducible double suicide gene for gene therapy. Cancer Gene Ther. *8*, 397-404.

Lefkowitz, E.J., Pattnaik, A.K., Ball, L.A. (1990). Complementation of a vesicular stomatitis virus glycoprotein G mutant with wild-type protein expressed from either a bovine papilloma virus or a vaccinia virus vector system. Virology *178*, 373-383.

Lehtolainen, P., Tyynela, K., Kannasto, J., Airenne, K.J., Yla-Herttuala, S. (2002). Baculoviruses exhibit restricted cell type specificity in rat brain: a comparison of baculovirus- and adenovirus-mediated intracerebral gene transfer in vivo. Gene Ther. *9*, 1693-1699.

Lehtolainen, P., Wirth, T., Taskinen, A.K., Lehenkari, P., Leppanen, O., Lappalainen, M., Pulkkanen, K., Marttila, A., Marjomaki, V., Airenne, K.J., Horton, M., Kulomaa, M.S., Yla-Herttuala, S. (2003). Targeting of biotinylated compounds to its target tissue using a low-density lipoprotein receptor-avidin fusion protein. Gene Ther. *10*, 2090-2097.

Leight, E.R., Sugden, B. (2001). Establishment of an oriP replicon is dependent upon an infrequent, epigenetic event. Mol. Cell Biol. *21*, 4149-4161.

Leisy, D.J., Lewis, T.D., Leong, J.A., Rohrmann, G.F. (2003). Transduction of cultured fish cells with recombinant baculoviruses. J. Gen. Virol. *84*, 1173-1178.

Leopold, P.L., Crystal, R.G. (2007). Intracellular trafficking of adenovirus: many means to many ends. Adv. Drug Deliv. Rev. *59*, 810-821.

Leppanen, P., Kholova, I., Mahonen, A.J., Airenne, K., Koota, S., Mansukoski, H., Narvainen, J., Wirzenius, M., Alhonen, L., Janne, J., Alitalo, K., Yla-Herttuala, S. (2006). Short and long-term effects of hVEGF-A(165) in Cre-activated transgenic mice. PLoS. ONE. *1*, e13.

Lesch, H.P., Turpeinen, S., Niskanen, E.A., Mahonen, A.J., Airenne, K.J., Yla-Herttuala, S. (2008). Generation of lentivirus vectors using recombinant baculoviruses. Gene Ther. *15*, 1280-1286.

Levine, B.L., Humeau, L.M., Boyer, J., MacGregor, R.R., Rebello, T., Lu, X., Binder, G.K., Slepushkin, V., Lemiale, F., Mascola, J.R., Bushman, F.D., Dropulic, B., June, C.H. (2006). Gene transfer in humans using a conditionally replicating lentiviral vector. Proc. Natl. Acad. Sci. U. S. A *103*, 17372-17377.

Lewis, P.F., Emerman, M. (1994). Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. J. Virol. *68*, 510-516.

Li, E., Stupack, D., Bokoch, G.M., Nemerow, G.R. (1998). Adenovirus endocytosis requires actin cytoskeleton reorganization mediated by Rho family GTPases. J. Virol. *72*, 8806-8812.

Li, Q., Peterson, K.R., Fang, X., Stamatoyannopoulos, G. (2002). Locus control regions. Blood *100*, 3077-3086.

Li, Y., Wang, X., Guo, H., Wang, S. (2004). Axonal transport of recombinant baculovirus vectors. Mol. Ther. *10*, 1121-1129.

Li, Y., Yang, Y., Wang, S. (2005). Neuronal gene transfer by baculovirus-derived vectors accommodating a neurone-specific promoter. Exp. Physiol *90*, 39-44.

Li, Z., Sharma, R.V., Duan, D., Davisson, R.L. (2003). Adenovirus-mediated gene transfer to adult mouse cardiomyocytes is selectively influenced by culture medium. J. Gene Med. *5*, 765-772.

Liang, C.Y., Wang, H.Z., Li, T.X., Hu, Z.H., Chen, X.W. (2004). High efficiency gene transfer into mammalian kidney cells using baculovirus vectors. Arch. Virol. *149*, 51-60.

Lindahl, T., Adams, A., Bjursell, G., Bornkamm, G.W., Kaschka-Dierich, C., Jehn, U. (1976). Covalently closed circular duplex DNA of Epstein-Barr virus in a human lymphoid cell line. J. Mol. Biol. *102*, 511-530.

Lipshutz, G.S., Titre, D., Brindle, M., Bisconte, A.R., Contag, C.H., Gaensler, K.M. (2003). Comparison of gene expression after intraperitoneal delivery of AAV2 or AAV5 in utero. Mol. Ther. *8*, 90-98.

Litzinger, D.C., Huang, L. (1992). Phosphatidylethanolamine liposomes: drug delivery, gene transfer and immunodiagnostic applications. Biochim. Biophys. Acta *1113*, 201-227.

Liu, B.H., Yang, Y., Paton, J.F., Li, F., Boulaire, J., Kasparov, S., Wang, S. (2006a). GAL4-NF-kappaB fusion protein augments transgene expression from neuronal promoters in the rat brain. Mol. Ther. *14*, 872-882.

Liu, X., Li, K., Song, J., Liang, C., Wang, X., Chen, X. (2006b). Efficient and stable gene expression in rabbit intervertebral disc cells transduced with a recombinant baculovirus vector. Spine *31*, 732-735.

Lo, W.H., Hwang, S.M., Chuang, C.K., Chen, C.Y., Hu, Y.C. (2009). Development of a Hybrid Baculoviral Vector for Sustained Transgene Expression. Mol. Ther. *17*, 658-666.

Loeb, J.E., Cordier, W.S., Harris, M.E., Weitzman, M.D., Hope, T.J. (1999). Enhanced expression of transgenes from adeno-associated virus vectors with the woodchuck hepatitis virus posttranscriptional regulatory element: implications for gene therapy. Hum. Gene Ther. *10*, 2295-2305.

Long, G., Pan, X., Kormelink, R., Vlak, J.M. (2006). Functional entry of baculovirus into insect and mammalian cells is dependent on clathrin-mediated endocytosis. J. Virol. *80*, 8830-8833.

Loonstra, A., Vooijs, M., Beverloo, H.B., Allak, B.A., van, D.E., Kanaar, R., Berns, A., Jonkers, J. (2001). Growth inhibition and DNA damage induced by Cre recombinase in mammalian cells. Proc. Natl. Acad. Sci. U. S. A *98*, 9209-9214.

Lu, L., Ho, Y., Kwang, J. (2006). Suppression of porcine arterivirus replication by baculovirus-delivered shRNA targeting nucleoprotein. Biochem. Biophys. Res. Commun. *340*, 1178-1183.

Lu, W., Zheng, S., Li, X.F., Huang, J.J., Zheng, X., Li, Z. (2004a). Intra-tumor injection of H101, a recombinant adenovirus, in combination with chemotherapy in patients with advanced cancers: a pilot phase II clinical trial. World J. Gastroenterol. *10*, 3634-3638.

Lu, X., Yu, Q., Binder, G.K., Chen, Z., Slepushkina, T., Rossi, J., Dropulic, B. (2004b). Antisense-mediated inhibition of human immunodeficiency virus (HIV) replication by use of an HIV type 1-based vector results in severely attenuated mutants incapable of developing resistance. J. Virol. *78*, 7079-7088.

Luby-Phelps, K. (2000). Cytoarchitecture and physical properties of cytoplasm: volume, viscosity, diffusion, intracellular surface area. Int. Rev. Cytol. *192*, 189-221.

Lucifora, J., Durantel, D., Belloni, L., Barraud, L., Villet, S., Vincent, I.E., Margeridon-Thermet, S., Hantz, O., Kay, A., Levrero, M., Zoulim, F. (2008). Initiation of hepatitis B virus genome replication and production of infectious virus following delivery in HepG2 cells by novel recombinant baculovirus vector. J. Gen. Virol. *89*, 1819-1828.

Lufino, M.M., Edser, P.A., Wade-Martins, R. (2008). Advances in high-capacity extrachromosomal vector technology: episomal maintenance, vector delivery, and transgene expression. Mol. Ther. *16*, 1525-1538.

Lufino, M.M., Manservigi, R., Wade-Martins, R. (2007). An S/MAR-based infectious episomal genomic DNA expression vector provides long-term regulated functional complementation of LDLR deficiency. Nucleic Acids Res. *35*, e98.

Lukacs, G.L., Haggie, P., Seksek, O., Lechardeur, D., Freedman, N., Verkman, A.S. (2000). Size-dependent DNA mobility in cytoplasm and nucleus. J. Biol. Chem. *275*, 1625-1629.

Lupton, S., Levine, A.J. (1985). Mapping genetic elements of Epstein-Barr virus that facilitate extrachromosomal persistence of Epstein-Barr virus-derived plasmids in human cells. Mol. Cell Biol. *5*, 2533-2542.

Luz-Madrigal, A., Clapp, C., Aranda, J., Vaca, L. (2007). In vivo transcriptional targeting into the retinal vasculature using recombinant baculovirus carrying the human flt-1 promoter. Virol. J. *4*, 88.
Ma, L., Tamarina, N., Wang, Y., Kuznetsov, A., Patel, N., Kending, C., Hering, B.J., Philipson, L.H. (2000). Baculovirus-mediated gene transfer into pancreatic islet cells. Diabetes *49*, 1986-1991.
Mackey, D., Sugden, B. (1999). Applications of oriP plasmids and their mode of replication. Methods Enzymol. *306*, 308-328.
Macnab, S., Whitehouse, A. (2009). Progress and prospects: human artificial chromosomes. Gene Ther. *16*, 1180-1188.
Magin, T.M., Reichelt, J., Hatzfeld, M. (2004). Emerging functions: diseases and animal models reshape our view of the cytoskeleton. Exp. Cell Res. *301*, 91-102.
Magin-Lachmann, C., Kotzamanis, G., D'Aiuto, L., Wagner, E., Huxley, C. (2003). Retrofitting BACs with G418 resistance, luciferase, and oriP and EBNA-1 - new vectors for in vitro and in vivo delivery. BMC. Biotechnol. *3*, 2.
Makela, A.R., Enback, J., Laakkonen, J.P., Vihinen-Ranta, M., Laakkonen, P., Oker-Blom, C. (2008). Tumor targeting of baculovirus displaying a lymphatic homing peptide. J. Gene Med. *10*, 1019-1031.
Makela, A.R., Matilainen, H., White, D.J., Ruoslahti, E., Oker-Blom, C. (2006). Enhanced baculovirus-mediated transduction of human cancer cells by tumor-homing peptides. J. Virol. *80*, 6603-6611.
Makinen, P.I., Koponen, J.K., Karkkainen, A.M., Malm, T.M., Pulkkinen, K.H., Koistinaho, J., Turunen, M.P., Yla-Herttuala, S. (2006). Stable RNA interference: comparison of U6 and H1 promoters in endothelial cells and in mouse brain. J. Gene Med. *8*, 433-441.
Makrides, S.C. (1996). Strategies for achieving high-level expression of genes in Escherichia coli. Microbiol. Rev. *60*, 512-538.
Makrides, S.C. (1999). Components of vectors for gene transfer and expression in mammalian cells. Protein Expr. Purif. *17*, 183-202.
Mangel, W.F., Baniecki, M.L., McGrath, W.J. (2003). Specific interactions of the adenovirus proteinase with the viral DNA, an 11-amino-acid viral peptide, and the cellular protein actin. Cell Mol. Life Sci. *60*, 2347-2355.
Manzini, S., Vargiolu, A., Stehle, I.M., Bacci, M.L., Cerrito, M.G., Giovannoni, R., Zannoni, A., Bianco, M.R., Forni, M., Donini, P., Papa, M., Lipps, H.J., Lavitrano, M. (2006). Genetically modified pigs produced with a nonviral episomal vector. Proc. Natl. Acad. Sci. U. S. A *103*, 17672-17677.
Mariati, Ho, S.C., Yap, M.G., Yang, Y. (2010). Evaluating post-transcriptional regulatory elements for enhancing transient gene expression levels in CHO K1 and HEK293 cells. Protein Expr. Purif. *69*, 9-15.
Marples, B., Scott, S.D., Hendry, J.H., Embleton, M.J., Lashford, L.S., Margison, G.P. (2000). Development of synthetic promoters for radiation-mediated gene therapy. Gene Ther. *7*, 511-517.
Marsh, M., Helenius, A. (2006). Virus entry: open sesame. Cell *124*, 729-740.
Martyn, J.C., Dong, X., Holmes-Brown, S., Pribul, P., Li, S., Drummer, H.E., Gowans, E.J. (2007). Transient and stable expression of the HCV envelope glycoproteins in cell lines and primary hepatocytes transduced with a recombinant baculovirus. Arch. Virol. *152*, 329-343.
Maruyama-Tabata, H., Harada, Y., Matsumura, T., Satoh, E., Cui, F., Iwai, M., Kita, M., Hibi, S., Imanishi, J., Sawada, T., Mazda, O. (2000). Effective suicide gene therapy in vivo by EBV-based plasmid vector coupled with polyamidoamine dendrimer. Gene Ther. *7*, 53-60.
Mastroyiannopoulos, N.P., Feldman, M.L., Uney, J.B., Mahadevan, M.S., Phylactou, L.A. (2005). Woodchuck post-transcriptional element induces nuclear export of myotonic dystrophy 3' untranslated region transcripts. EMBO Rep. *6*, 458-463.
Matilainen, H., Makela, A.R., Riikonen, R., Saloniemi, T., Korhonen, E., Hyypia, T., Heino, J., Grabherr, R., Oker-Blom, C. (2006). RGD motifs on the surface of baculovirus enhance transduction of human lung carcinoma cells. J. Biotechnol. *125*, 114-126.
Matilainen, H., Rinne, J., Gilbert, L., Marjomaki, V., Reunanen, H., Oker-Blom, C. (2005). Baculovirus entry into human hepatoma cells. J. Virol. *79*, 15452-15459.
Mattia, E., Ceridono, M., Chichiarelli, S., D'Erme, M. (1999). Interactions of Epstein-Barr virus origins of replication with nuclear matrix in the latent and in the lytic phases of viral infection. Virology *262*, 9-17.
Mazda, O. (2002). Improvement of nonviral gene therapy by Epstein-Barr virus (EBV)-based plasmid vectors. Curr. Gene Ther. *2*, 379-392.
McCaughan, K.K., Brown, C.M., Dalphin, M.E., Berry, M.J., Tate, W.P. (1995). Translational termination efficiency in mammals is influenced by the base following the stop codon. Proc. Natl. Acad. Sci. U. S. A *92*, 5431-5435.
McCormick, C.J., Brown, D., Griffin, S., Challinor, L., Rowlands, D.J., Harris, M. (2006). A link between translation of the hepatitis C virus polyprotein and polymerase function; possible consequences for hyperphosphorylation of NS5A. J. Gen. Virol. *87*, 93-102.
McDonald, D., Vodicka, M.A., Lucero, G., Svitkina, T.M., Borisy, G.G., Emerman, M., Hope, T.J. (2002). Visualization of the intracellular behavior of HIV in living cells. J. Cell Biol. *159*, 441-452.
McIntosh, A.H., Shamy, R. (1975). Effects of the nuclear polyhedrosis virus (NPV) of *Autographa californica* on a vertebrate viper cell line. Ann. N. Y. Acad. Sci *266*, 327-331.
Medina-Kauwe, L.K. (2003). Endocytosis of adenovirus and adenovirus capsid proteins. Adv. Drug Deliv. Rev. *55*, 1485-1496.
Mehier-Humbert, S., Guy, R.H. (2005). Physical methods for gene transfer: improving the kinetics of gene delivery into cells. Adv. Drug Deliv. Rev. *57*, 733-753.
Mehta, A.K., Majumdar, S.S., Alam, P., Gulati, N., Brahmachari, V. (2009). Epigenetic regulation of cytomegalovirus major immediate-early promoter activity in transgenic mice. Gene *428*, 20-24.

Mei, W.H., Qian, G.X., Zhang, X.Q., Zhang, P., Lu, J. (2006). Sustained expression of Epstein-Barr virus episomal vector mediated factor VIII in vivo following muscle electroporation. Haemophilia. *12***,** 271-279.
Meier, O., Greber, U.F. (2004). Adenovirus endocytosis. J. Gene Med. *6 Suppl 1***,** S152-S163.
Mejia, J.E., Willmott, A., Levy, E., Earnshaw, W.C., Larin, Z. (2001). Functional complementation of a genetic deficiency with human artificial chromosomes. Am. J. Hum. Genet. *69***,** 315-326.
Mercer, J., Helenius, A. (2009). Virus entry by macropinocytosis. Nat. Cell Biol. *11***,** 510-520.
Mermall, V., Post, P.L., Mooseker, M.S. (1998). Unconventional myosins in cell movement, membrane traffic, and signal transduction. Science *279***,** 527-533.
Miller, J.C., Holmes, M.C., Wang, J., Guschin, D.Y., Lee, Y.L., Rupniewski, I., Beausejour, C.M., Waite, A.J., Wang, N.S., Kim, K.A., Gregory, P.D., Pabo, C.O., Rebar, E.J. (2007). An improved zinc-finger nuclease architecture for highly specific genome editing. Nat. Biotechnol. *25***,** 778-785.
Miller, L.K., 1997. The Baculoviruses. Plenum Press, New York.
Min, K.A., Oh, S.T., Yoon, K.H., Kim, C.K., Lee, S.K. (2003). Prolonged gene expression in primary porcine pancreatic cells using an Epstein-Barr virus-based episomal vector. Biochem. Biophys. Res. Commun. *305***,** 108-115.
Mingozzi, F., High, K.A. (2007). Immune responses to AAV in clinical trials. Curr. Gene Ther. *7***,** 316-324.
Minin, A.A., Moldaver, M.V. (2008). Intermediate vimentin filaments and their role in intracellular organelle distribution. Biochemistry (Mosc.) *73***,** 1453-1466.
Mitsialis, S.A., Young, J.F., Palese, P., Guntaka, R.V. (1981). An avian tumor virus promoter directs expression of plasmid genes in Escherichia coli. Gene *16***,** 217-225.
Mizuguchi, H., Hosono, T., Hayakawa, T. (2000). Long-term replication of Epstein-Barr virus-derived episomal vectors in the rodent cells. FEBS Lett. *472***,** 173-178.
Mlynarova, L., Nap, J.P. (2003). A self-excising Cre recombinase allows efficient recombination of multiple ectopic heterospecific lox sites in transgenic tobacco. Transgenic Res. *12***,** 45-57.
Moehle, E.A., Rock, J.M., Lee, Y.L., Jouvenot, Y., DeKelver, R.C., Gregory, P.D., Urnov, F.D., Holmes, M.C. (2007). Targeted gene addition into a specified location in the human genome using designed zinc finger nucleases. Proc. Natl. Acad. Sci. U. S. A *104***,** 3055-3060.
Mondor, I., Ugolini, S., Sattentau, Q.J. (1998). Human immunodeficiency virus type 1 attachment to HeLa CD4 cells is CD4 independent and gp120 dependent and requires cell surface heparans. J. Virol. *72***,** 3623-3634.
Mor-Vaknin, N., Punturieri, A., Sitwala, K., Markovitz, D.M. (2003). Vimentin is secreted by activated macrophages. Nat. Cell Biol. *5***,** 59-63.
Muller, L., Saydam, O., Saeki, Y., Heid, I., Fraefel, C. (2005). Gene transfer into hepatocytes mediated by herpes simplex virus-Epstein-Barr virus hybrid amplicons. J. Virol. Methods *123***,** 65-72.
Murges, D., Kremer, A., Knebel-Morsdorf, D. (1997). Baculovirus transactivator IE1 is functional in mammalian cells. J. Gen. Virol. *78 (Pt 6)***,** 1507-1510.
Murti, K.G., Goorha, R., Klymkowsky, M.W. (1988). A functional role for intermediate filaments in the formation of frog virus 3 assembly sites. Virology *162***,** 264-269.
Naghavi, M.H., Goff, S.P. (2007). Retroviral proteins that interact with the host cell cytoskeleton. Curr. Opin. Immunol. *19***,** 402-407.
Nagy, A. (2000). Cre recombinase: the universal reagent for genome tailoring. Genesis. *26***,** 99-109.
Naiche, L.A., Papaioannou, V.E. (2007). Cre activity causes widespread apoptosis and lethal anemia during embryonic development. Genesis. *45***,** 768-775.
Nakagawa, T., Chen, J., Zhang, Z., Kanai, Y., Hirokawa, N. (1995). Two distinct functions of the carboxyl-terminal tail domain of NF-M upon neurofilament assembly: cross-bridge formation and longitudinal elongation of filaments. J. Cell Biol. *129***,** 411-429.
Nakai, H., Herzog, R.W., Hagstrom, J.N., Walter, J., Kung, S.H., Yang, E.Y., Tai, S.J., Iwaki, Y., Kurtzman, G.J., Fisher, K.J., Colosi, P., Couto, L.B., High, K.A. (1998). Adeno-associated viral vector-mediated gene transfer of human blood coagulation factor IX into mouse liver. Blood *91***,** 4600-4607.
Nakai, H., Yant, S.R., Storm, T.A., Fuess, S., Meuse, L., Kay, M.A. (2001). Extrachromosomal recombinant adeno-associated virus vector genomes are primarily responsible for stable liver transduction in vivo. J. Virol. *75***,** 6969-6976.
Nakanishi, H., Mazda, O., Satoh, E., Asada, H., Morioka, H., Kishida, T., Nakao, M., Mizutani, Y., Kawauchi, A., Kita, M., Imanishi, J., Miki, T. (2003). Nonviral genetic transfer of Fas ligand induced significant growth suppression and apoptotic tumor cell death in prostate cancer in vivo. Gene Ther. *10***,** 434-442.
Nakano, M., Odaka, K., Takahashi, Y., Ishimura, M., Saito, I., Kanegae, Y. (2005). Production of viral vectors using recombinase-mediated cassette exchange. Nucleic Acids Res. *33***,** e76.
Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F.H., Verma, I.M., Trono, D. (1996). In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science *272***,** 263-267.
Nazir, S.A., Metcalf, J.P. (2005). Innate immune response to adenovirus. J. Investig. Med. *53***,** 292-304.
Nedellec, P., Vicart, P., Laurent-Winter, C., Martinat, C., Prevost, M.C., Brahic, M. (1998). Interaction of Theiler's virus with intermediate filaments of infected cells. J. Virol. *72***,** 9553-9560.
Negrete, A., Kotin, R.M. (2007). Production of recombinant adeno-associated vectors using two bioreactor configurations at different scales. J. Virol. Methods *145***,** 155-161.

Nelson, W.J., Traub, P. (1982). Intermediate (10 nm) filament proteins and the Ca2+-activated proteinase specific for vimentin and desmin in the cells from fish to man: an example of evolutionary conservation. J. Cell Sci. *57*, 25-49.

Nemerow, G.R., Stewart, P.L. (1999). Role of alpha(v) integrins in adenovirus cell entry and gene delivery. Microbiol. Mol. Biol. Rev. *63*, 725-734.

Nicholson, L.J., Philippe, M., Paine, A.J., Mann, D.A., Dolphin, C.T. (2005). RNA interference mediated in human primary cells via recombinant baculoviral vectors. Mol. Ther. *11*, 638-644.

Nieminen, M., Henttinen, T., Merinen, M., Marttila-Ichihara, F., Eriksson, J.E., Jalkanen, S. (2006). Vimentin function in lymphocyte adhesion and transcellular migration. Nat. Cell Biol. *8*, 156-162.

Nishibe, Y., Kaneko, H., Suzuki, H., Abe, T., Matsuura, Y., Takaku, H. (2008). Baculovirus-mediated interferon alleviates dimethylnitrosamine-induced liver cirrhosis symptoms in a murine model. Gene Ther. *15*, 990-997.

Nishizaki, K., Mazda, O., Dohi, Y., Kawata, T., Mizuguchi, K., Kitamura, S., Taniguchi, S. (2000). In vivo gene gun-mediated transduction into rat heart with Epstein-Barr virus-based episomal vectors. Ann. Thorac. Surg. *70*, 1332-1337.

Novina, C.D., Murray, M.F., Dykxhoorn, D.M., Beresford, P.J., Riess, J., Lee, S.K., Collman, R.G., Lieberman, J., Shankar, P., Sharp, P.A. (2002). siRNA-directed inhibition of HIV-1 infection. Nat. Med. *8*, 681-686.

O'Neal, K.R., Agah, R. (2007). Conditional targeting: inducible deletion by Cre recombinase. Methods Mol. Biol. *366*, 309-320.

O'Reilly, D.R., Miller, L.K., Luckov, V.A., 1994. Baculovirus expression vectors. A laboratory manual. Oxford University Press, New York.

Olafsdottir, G., Svansson, V., Ingvarsson, S., Marti, E., Torsteinsdottir, S. (2008). In vitro analysis of expression vectors for DNA vaccination of horses: the effect of a Kozak sequence. Acta Vet. Scand. *50*, 44.

Omary, M.B., Coulombe, P.A., McLean, W.H. (2004). Intermediate filament proteins and their associated diseases. N. Engl. J. Med. *351*, 2087-2100.

Omary, M.B., Ku, N.O., Tao, G.Z., Toivola, D.M., Liao, J. (2006). "Heads and tails" of intermediate filament phosphorylation: multiple sites and functional insights. Trends Biochem. Sci. *31*, 383-394.

Ong, S.T., Li, F., Du, J., Tan, Y.W., Wang, S. (2005). Hybrid cytomegalovirus enhancer-h1 promoter-based plasmid and baculovirus vectors mediate effective RNA interference. Hum. Gene Ther. *16*, 1404-1412.

Oppenheimer, D.I., MacNicol, A.M., Patel, N.H. (1999). Functional conservation of the wingless-engrailed interaction as shown by a widely applicable baculovirus misexpression system. Curr. Biol. *9*, 1288-1296.

Osborne, R. (2008). Ark floats gene therapy's boat, for now. Nat. Biotechnol. *26*, 1057-1059.

Ossevoort, M., Visser, B.M., van den Wollenberg, D.J., van, d., V, Offringa, R., Melief, C.J., Toes, R.E., Hoeben, R.C. (2003). Creation of immune 'stealth' genes for gene therapy through fusion with the Gly-Ala repeat of EBNA-1. Gene Ther. *10*, 2020-2028.

Otomo, T., Yamamoto, S., Morishita, R., Kaneda, Y. (2001). EBV replicon vector system enhances transgene expression in vivo: applications to cancer gene therapy. J. Gene Med. *3*, 345-352.

Ott, M.G., Schmidt, M., Schwarzwaelder, K., Stein, S., Siler, U., Koehl, U., Glimm, H., Kuhlcke, K., Schilz, A., Kunkel, H., Naundorf, S., Brinkmann, A., Deichmann, A., Fischer, M., Ball, C., Pilz, I., Dunbar, C., Du, Y., Jenkins, N.A., Copeland, N.G., Luthi, U., Hassan, M., Thrasher, A.J., Hoelzer, D., von, K.C., Seger, R., Grez, M. (2006). Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1, PRDM16 or SETBP1. Nat. Med. *12*, 401-409.

Owen-Hughes, T. (2003). Colworth memorial lecture. Pathways for remodelling chromatin. Biochem. Soc. Trans. *31*, 893-905.

Pallari, H.M., Eriksson, J.E. (2006). Intermediate filaments as signaling platforms. Sci. STKE. *2006*, e53.

Palmiter, R.D., Sandgren, E.P., Avarbock, M.R., Allen, D.D., Brinster, R.L. (1991). Heterologous introns can enhance expression of transgenes in mice. Proc. Natl. Acad. Sci U. S. A *88*, 478-482.

Palombo, F., Monciotti, A., Recchia, A., Cortese, R., Ciliberto, G., La Monica, N. (1998). Site-specific integration in mammalian cells mediated by a new hybrid baculovirus-adeno-associated virus vector. J. Virol. *72*, 5025-5034.

Papadakis, E.D., Nicklin, S.A., Baker, A.H., White, S.J. (2004). Promoters and control elements: designing expression cassettes for gene therapy. Curr. Gene Ther. *4*, 89-113.

Papapetrou, E.P., Ziros, P.G., Micheva, I.D., Zoumbos, N.C., Athanassiadou, A. (2006). Gene transfer into human hematopoietic progenitor cells with an episomal vector carrying an S/MAR element. Gene Ther. *13*, 40-51.

Park, S.W., Lee, H.K., Kim, T.G., Yoon, S.K., Paik, S.Y. (2001). Hepatocyte-specific gene expression by baculovirus pseudotyped with vesicular stomatitis virus envelope glycoprotein. Biochem. Biophys. Res. Commun. *289*, 444-450.

Paterna, J.C., Moccetti, T., Mura, A., Feldon, J., Bueler, H. (2000). Influence of promoter and WHV post-transcriptional regulatory element on AAV-mediated transgene expression in the rat brain. Gene Ther. *7*, 1304-1311.

Pavlakis, G.N., Hamer, D.H. (1983). Regulation of a metallothionein-growth hormone hybrid gene in bovine papilloma virus. Proc. Natl. Acad. Sci. U. S. A *80*, 397-401.

Peitz, M., Pfannkuche, K., Rajewsky, K., Edenhofer, F. (2002). Ability of the hydrophobic FGF and basic TAT peptides to promote cellular uptake of recombinant Cre recombinase: a tool for efficient genetic engineering of mammalian genomes. Proc. Natl. Acad. Sci. U. S. A *99*, 4489-4494.

Pelkmans, L., Fava, E., Grabner, H., Hannus, M., Habermann, B., Krausz, E., Zerial, M. (2005). Genome-wide analysis of human kinases in clathrin- and caveolae/raft-mediated endocytosis. Nature *436*, 78-86.

Pelkmans, L., Kartenbeck, J., Helenius, A. (2001). Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. Nat. Cell Biol. *3*, 473-483.

Pelletier, J., Sonenberg, N. (1987). The involvement of mRNA secondary structure in protein synthesis. Biochem. Cell Biol. *65*, 576-581.

Peng, Z. (2005). Current status of gendicine in China: recombinant human Ad-p53 agent for treatment of cancers. Hum. Gene Ther. *16*, 1016-1027.

Perlson, E., Hanz, S., Ben-Yaakov, K., Segal-Ruder, Y., Seger, R., Fainzilber, M. (2005). Vimentin-dependent spatial translocation of an activated MAP kinase in injured nerve. Neuron *45*, 715-726.

Pesole, G., Mignone, F., Gissi, C., Grillo, G., Licciulli, F., Liuni, S. (2001). Structural and functional features of eukaryotic mRNA untranslated regions. Gene *276*, 73-81.

Petitclerc, D., Attal, J., Theron, M.C., Bearzotti, M., Bolifraud, P., Kann, G., Stinnakre, M.G., Pointu, H., Puissant, C., Houdebine, L.M. (1995). The effect of various introns and transcription terminators on the efficiency of expression vectors in various cultured cell lines and in the mammary gland of transgenic mice. J. Biotechnol. *40*, 169-178.

Pfeifer, A., Brandon, E.P., Kootstra, N., Gage, F.H., Verma, I.M. (2001). Delivery of the Cre recombinase by a self-deleting lentiviral vector: efficient gene targeting in vivo. Proc. Natl. Acad. Sci. U. S. A *98*, 11450-11455.

Philippe, S., Sarkis, C., Barkats, M., Mammeri, H., Ladroue, C., Petit, C., Mallet, J., Serguera, C. (2006). Lentiviral vectors with a defective integrase allow efficient and sustained transgene expression in vitro and in vivo. Proc. Natl. Acad. Sci. U. S. A *103*, 17684-17689.

Piechaczek, C., Fetzer, C., Baiker, A., Bode, J., Lipps, H.J. (1999). A vector based on the SV40 origin of replication and chromosomal S/MARs replicates episomally in CHO cells. Nucleic Acids Res. *27*, 426-428.

Pieroni, L., Maione, D., La Monica, N. (2001). In vivo gene transfer in mouse skeletal muscle mediated by baculovirus vectors. Hum. Gene Ther. *12*, 871-881.

Ping, W., Ge, J., Li, S., Zhou, H., Wang, K., Feng, Y., Lou, Z. (2006). Baculovirus-mediated gene expression in chicken primary cells. Avian Dis. *50*, 59-63.

Ploubidou, A., Way, M. (2001). Viral transport and the cytoskeleton. Curr. Opin. Cell Biol. *13*, 97-105.

Poluri, A., van, M.M., Sutton, R.E. (2003). Genetic therapy for HIV/AIDS. Expert. Opin. Biol. Ther. *3*, 951-963.

Poranen, M.M., Daugelavicius, R., Bamford, D.H. (2002). Common principles in viral entry. Annu. Rev. Microbiol. *56*, 521-538.

Porret, A., Merillat, A.M., Guichard, S., Beermann, F., Hummler, E. (2006). Tissue-specific transgenic and knockout mice. Methods Mol. Biol. *337*, 185-205.

Proudfoot, N.J. (1986). Transcriptional interference and termination between duplicated alpha-globin gene constructs suggests a novel mechanism for gene regulation. Nature *322*, 562-565.

Proudfoot, N.J., Furger, A., Dye, M.J. (2002). Integrating mRNA processing with transcription. Cell *108*, 501-512.

Qing, K., Mah, C., Hansen, J., Zhou, S., Dwarki, V., Srivastava, A. (1999). Human fibroblast growth factor receptor 1 is a co-receptor for infection by adeno-associated virus 2. Nat. Med. *5*, 71-77.

Radtke, K., Dohner, K., Sodeik, B. (2006). Viral interactions with the cytoskeleton: a hitchhiker's guide to the cell. Cell Microbiol. *8*, 387-400.

Ramezani, A., Hawley, T.S., Hawley, R.G. (2000). Lentiviral vectors for enhanced gene expression in human hematopoietic cells. Mol. Ther. *2*, 458-469.

Ramos, L., Kopec, L.A., Sweitzer, S.M., Fornwald, J.A., Zhao, H., McAllister, P., McNulty, D.E., Trill, J.J., Kane, J.F. (2002). Rapid expression of recombinant proteins in modified CHO cells using the baculovirus system. Cytotechnology *38*, 37-41.

Raper, S.E., Chirmule, N., Lee, F.S., Wivel, N.A., Bagg, A., Gao, G.P., Wilson, J.M., Batshaw, M.L. (2003). Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. Mol. Genet. Metab *80*, 148-158.

Raty, J.K., Airenne, K.J., Marttila, A.T., Marjomaki, V., Hytonen, V.P., Lehtolainen, P., Laitinen, O.H., Mahonen, A.J., Kulomaa, M.S., Yla-Herttuala, S. (2004). Enhanced gene delivery by avidin-displaying baculovirus. Mol. Ther. *9*, 282-291.

Raty, J.K., Liimatainen, T., Huhtala, T., Kaikkonen, M.U., Airenne, K.J., Hakumaki, J.M., Narvanen, A., Yla-Herttuala, S. (2007). SPECT/CT imaging of baculovirus biodistribution in rat. Gene Ther. *14*, 930-938.

Raty, J.K., Liimatainen, T., Wirth, T., Airenne, K.J., Ihalainen, T.O., Huhtala, T., Hamerlynck, E., Vihinen-Ranta, M., Narvanen, A., Yla-Herttuala, S., Hakumaki, J.M. (2006). Magnetic resonance imaging of viral particle biodistribution in vivo. Gene Ther. *13*, 1440-1446.

Rawlins, D.R., Milman, G., Hayward, S.D., Hayward, G.S. (1985). Sequence-specific DNA binding of the Epstein-Barr virus nuclear antigen (EBNA-1) to clustered sites in the plasmid maintenance region. Cell *42*, 859-868.

Raymond, C.S., Soriano, P. (2007). High-efficiency FLP and PhiC31 site-specific recombination in mammalian cells. PLoS. ONE. *2*, e162.

Reisman, D., Yates, J., Sugden, B. (1985). A putative origin of replication of plasmids derived from Epstein-Barr virus is composed of two cis-acting components. Mol. Cell Biol. *5*, 1822-1832.

Ren, C., Zhao, M., Yang, X., Li, D., Jiang, X., Wang, L., Shan, W., Yang, H., Zhou, L., Zhou, W., Zhang, H. (2006). Establishment and applications of epstein-barr virus-based episomal vectors in human embryonic stem cells. Stem Cells *24*, 1338-1347.

Ren, C.P., Zhao, M., Shan, W.J., Yang, X.Y., Yin, Z.H., Jiang, X.J., Zhang, H.B., Yao, K.T. (2005). Establishment of human embryonic stem cell line stably expressing Epstein-Barr virus-encoded nuclear antigen 1. Acta Biochim. Biophys. Sin. (Shanghai) *37*, 68-73.

Revenu, C., Athman, R., Robine, S., Louvard, D. (2004). The co-workers of actin filaments: from cell structures to signals. Nat. Rev. Mol. Cell Biol. *5*, 635-646.

Roelvink, P.W., Lizonova, A., Lee, J.G., Li, Y., Bergelson, J.M., Finberg, R.W., Brough, D.E., Kovesdi, I., Wickham, T.J. (1998). The coxsackievirus-adenovirus receptor protein can function as a cellular attachment protein for adenovirus serotypes from subgroups A, C, D, E, and F. J. Virol. *72*, 7909-7915.

Rosenberg, S.A., Aebersold, P., Cornetta, K., Kasid, A., Morgan, R.A., Moen, R., Karson, E.M., Lotze, M.T., Yang, J.C., Topalian, S.L., . (1990). Gene transfer into humans--immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. N. Engl. J. Med. *323*, 570-578.

Ross, J. (1995). mRNA stability in mammalian cells. Microbiol. Rev. *59*, 423-450.

Runembert, I., Couette, S., Federici, P., Colucci-Guyon, E., Babinet, C., Briand, P., Friedlander, G., Terzi, F. (2004). Recovery of Na-glucose cotransport activity after renal ischemia is impaired in mice lacking vimentin. Am. J. Physiol Renal Physiol *287*, F960-F968.

Ruponen, M., Arkko, S., Urtti, A., Reinisalo, M., Ranta, V.P. (2009). Intracellular DNA release and elimination correlate poorly with transgene expression after non-viral transfection. J. Control Release *136*, 226-231.

Ruponen, M., Honkakoski, P., Ronkko, S., Pelkonen, J., Tammi, M., Urtti, A. (2003). Extracellular and intracellular barriers in non-viral gene delivery. J. Control Release *93*, 213-217.

Saeki, Y., Wataya-Kaneda, M., Tanaka, K., Kaneda, Y. (1998). Sustained transgene expression in vitro and in vivo using an Epstein-Barr virus replicon vector system combined with HVJ liposomes. Gene Ther. *5*, 1031-1037.

Safdar, A., Rodriguez, M.A., Fayad, L.E., Rodriguez, G.H., Pro, B., Wang, M., Romaguera, J.E., Goy, A.H., Hagemeister, F.B., McLaughlin, P., Bodey, G.P., Kwak, L.W., Raad, I.I., Couch, R.B. (2006). Dose-related safety and immunogenicity of baculovirus-expressed trivalent influenza vaccine: a double-blind, controlled trial in adult patients with non-Hodgkin B cell lymphoma. J. Infect. Dis. *194*, 1394-1397.

Saha, A., Wittmeyer, J., Cairns, B.R. (2006). Chromatin remodelling: the industrial revolution of DNA around histones. Nat. Rev. Mol. Cell Biol. *7*, 437-447.

Salminen, M., Airenne, K.J., Rinnankoski, R., Reimari, J., Valilehto, O., Rinne, J., Suikkanen, S., Kukkonen, S., Yla-Herttuala, S., Kulomaa, M.S., Vihinen-Ranta, M. (2005). Improvement in nuclear entry and transgene expression of baculoviruses by disintegration of microtubules in human hepatocytes. J. Virol. *79*, 2720-2728.

Salmon, P., Kindler, V., Ducrey, O., Chapuis, B., Zubler, R.H., Trono, D. (2000). High-level transgene expression in human hematopoietic progenitors and differentiated blood lineages after transduction with improved lentiviral vectors. Blood *96*, 3392-3398.

Sambrook, J., Rodgers, L., White, J., Gething, M.J. (1985). Lines of BPV-transformed murine cells that constitutively express influenza virus hemagglutinin. EMBO J. *4*, 91-103.

Sandig, V., Hofmann, C., Steinert, S., Jennings, G., Schlag, P., Strauss, M. (1996). Gene transfer into hepatocytes and human liver tissue by baculovirus vectors. Hum. Gene Ther. *7*, 1937-1945.

Santoro, S.W., Schultz, P.G. (2002). Directed evolution of the site specificity of Cre recombinase. Proc. Natl. Acad. Sci. U. S. A *99*, 4185-4190.

Sarkis, C., Serguera, C., Petres, S., Buchet, D., Ridet, J.L., Edelman, L., Mallet, J. (2000). Efficient transduction of neural cells in vitro and in vivo by a baculovirus-derived vector. Proc. Natl. Acad. Sci. U. S. A *97*, 14638-14643.

Sarria, A.J., Lieber, J.G., Nordeen, S.K., Evans, R.M. (1994). The presence or absence of a vimentin-type intermediate filament network affects the shape of the nucleus in human SW-13 cells. J. Cell Sci. *107 (Pt 6)*, 1593-1607.

Sarver, N., Gruss, P., Law, M.F., Khoury, G., Howley, P.M. (1981). Bovine papilloma virus deoxyribonucleic acid: a novel eucaryotic cloning vector. Mol. Cell Biol. *1*, 486-496.

Sauer, B. (1992). Identification of cryptic lox sites in the yeast genome by selection for Cre-mediated chromosome translocations that confer multiple drug resistance. J. Mol. Biol. *223*, 911-928.

Sauer, B. (1996). Multiplex Cre/lox recombination permits selective site-specific DNA targeting to both a natural and an engineered site in the yeast genome. Nucleic Acids Res. *24*, 4608-4613.

Sauer, B. (1998). Inducible gene targeting in mice using the Cre/lox system. Methods *14*, 381-392.

Schaffeld, M., Herrmann, H., Schultess, J., Markl, J. (2001). Vimentin and desmin of a cartilaginous fish, the shark Scyliorhinus stellaris: sequence, expression patterns and in vitro assembly. Eur. J. Cell Biol. *80*, 692-702.

Schaffer, D.V., Koerber, J.T., Lim, K.I. (2008). Molecular engineering of viral gene delivery vehicles. Annu. Rev. Biomed. Eng *10*, 169-194.

Schaft, J., shery-Padan, R., van der, H.F., Gruss, P., Stewart, A.F. (2001). Efficient FLP recombination in mouse ES cells and oocytes. Genesis. *31*, 6-10.

Schambach, A., Baum, C. (2008). Clinical application of lentiviral vectors - concepts and practice. Curr. Gene Ther. *8*, 474-482.

Schambach, A., Bohne, J., Baum, C., Hermann, F.G., Egerer, L., von, L.D., Giroglou, T. (2006). Woodchuck hepatitis virus post-transcriptional regulatory element deleted from X protein and promoter sequences enhances retroviral vector titer and expression. Gene Ther. *13*, 641-645.

Schambach, A., Galla, M., Maetzig, T., Loew, R., Baum, C. (2007). Improving transcriptional termination of self-inactivating gamma-retroviral and lentiviral vectors. Mol. Ther. *15*, 1167-1173.

Schambach, A., Wodrich, H., Hildinger, M., Bohne, J., Krausslich, H.G., Baum, C. (2000). Context dependence of different modules for posttranscriptional enhancement of gene expression from retroviral vectors. Mol. Ther. *2*, 435-445.

Schiedner, G., Morral, N., Parks, R.J., Wu, Y., Koopmans, S.C., Langston, C., Graham, F.L., Beaudet, A.L., 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*, 180-183.

Schmidt, E.E., Taylor, D.S., Prigge, J.R., Barnett, S., Capecchi, M.R. (2000). Illegitimate Cre-dependent chromosome rearrangements in transgenic mouse spermatids. Proc. Natl. Acad. Sci. U. S. A *97***,** 13702-13707.
Schmidt-Wolf, G.D., Schmidt-Wolf, I.G. (2003). Non-viral and hybrid vectors in human gene therapy: an update. Trends Mol. Med. *9***,** 67-72.
Schnepp, B.C., Clark, K.R., Klemanski, D.L., Pacak, C.A., Johnson, P.R. (2003). Genetic fate of recombinant adeno-associated virus vector genomes in muscle. J. Virol. *77***,** 3495-3504.
Schulz, T.F., Cordes, S. (2009). Is the Epstein-Barr virus EBNA-1 protein an oncogen? Proc. Natl. Acad. Sci. U. S. A *106***,** 2091-2092.
Sclimenti, C.R., Neviaser, A.S., Baba, E.J., Meuse, L., Kay, M.A., Calos, M.P. (2003). Epstein-Barr virus vectors provide prolonged robust factor IX expression in mice. Biotechnol. Prog. *19***,** 144-151.
Sclimenti, C.R., Thyagarajan, B., Calos, M.P. (2001). Directed evolution of a recombinase for improved genomic integration at a native human sequence. Nucleic Acids Res. *29***,** 5044-5051.
Seif, I., Khoury, G., Dhar, R. (1979). The genome of human papovavirus BKV. Cell *18***,** 963-977.
Seksek, O., Biwersi, J., Verkman, A.S. (1997). Translational diffusion of macromolecule-sized solutes in cytoplasm and nucleus. J. Cell Biol. *138***,** 131-142.
Shan, L., Wang, L., Yin, J., Zhong, P., Zhong, J. (2006). An OriP/EBNA-1-based baculovirus vector with prolonged and enhanced transgene expression. J. Gene Med. *8***,** 1400-1406.
Sharp, P.M., Cowe, E., Higgins, D.G., Shields, D.C., Wolfe, K.H., Wright, F. (1988). Codon usage patterns in Escherichia coli, Bacillus subtilis, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Drosophila melanogaster and Homo sapiens; a review of the considerable within-species diversity. Nucleic Acids Res. *16***,** 8207-8211.
Shaw, T., Bartholomeusz, A., Locarnini, S. (2006). HBV drug resistance: mechanisms, detection and interpretation. J. Hepatol. *44***,** 593-606.
Shen, H.C., Lee, H.P., Lo, W.H., Yang, D.G., Hu, Y.C. (2007). Baculovirus-mediated gene transfer is attenuated by sodium bicarbonate. J. Gene Med. *9***,** 470-478.
Shen, H.C., Yeh, C.N., Chen, G.Y., Huang, S.F., Chen, C.Y., Chiu, Y.C., Hu, Y.C. (2008). Sustained baculovirus-mediated expression in myogenic cells. J. Gene Med. *10***,** 1190-1197.
Shibata, T., Giaccia, A.J., Brown, J.M. (2000). Development of a hypoxia-responsive vector for tumor-specific gene therapy. Gene Ther. *7***,** 493-498.
Shimshek, D.R., Kim, J., Hubner, M.R., Spergel, D.J., Buchholz, F., Casanova, E., Stewart, A.F., Seeburg, P.H., Sprengel, R. (2002). Codon-improved Cre recombinase (iCre) expression in the mouse. Genesis. *32***,** 19-26.
Shoeman, R.L., Honer, B., Stoller, T.J., Kesselmeier, C., Miedel, M.C., Traub, P., Graves, M.C. (1990). Human immunodeficiency virus type 1 protease cleaves the intermediate filament proteins vimentin, desmin, and glial fibrillary acidic protein. Proc. Natl. Acad. Sci. U. S. A *87***,** 6336-6340.
Shoeman, R.L., Huttermann, C., Hartig, R., Traub, P. (2001). Amino-terminal polypeptides of vimentin are responsible for the changes in nuclear architecture associated with human immunodeficiency virus type 1 protease activity in tissue culture cells. Mol. Biol. Cell *12***,** 143-154.
Shoji, I., Aizaki, H., Tani, H., Ishii, K., Chiba, T., Saito, I., Miyamura, T., Matsuura, Y. (1997). Efficient gene transfer into various mammalian cells, including non-hepatic cells, by baculovirus vectors. J. Gen. Virol. *78***,** 2657-2664.
Siegel, R.W., Jain, R., Bradbury, A. (2001). Using an in vivo phagemid system to identify non-compatible loxP sequences. FEBS Lett. *505***,** 467-473.
Sihag, R.K., Inagaki, M., Yamaguchi, T., Shea, T.B., Pant, H.C. (2007). Role of phosphorylation on the structural dynamics and function of types III and IV intermediate filaments. Exp. Cell Res. *313***,** 2098-2109.
Silver, D.P., Livingston, D.M. (2001). Self-excising retroviral vectors encoding the Cre recombinase overcome Cre-mediated cellular toxicity. Mol. Cell *8***,** 233-243.
Slack, J., Arif, B.M. (2007). The baculoviruses occlusion-derived virus: virion structure and function. Adv. Virus Res. *69***,** 99-165.
Smith, G.J., III, Donello, J.E., Luck, R., Steger, G., Hope, T.J. (1998). The hepatitis B virus post-transcriptional regulatory element contains two conserved RNA stem-loops which are required for function. Nucleic Acids Res. *26***,** 4818-4827.
Smith, M.C., Thorpe, H.M. (2002). Diversity in the serine recombinases. Mol. Microbiol. *44***,** 299-307.
Snasel, J., Shoeman, R., Horejsi, M., Hruskova-Heidingsfeldova, O., Sedlacek, J., Ruml, T., Pichova, I. (2000). Cleavage of vimentin by different retroviral proteases. Arch. Biochem. Biophys. *377***,** 241-245.
Sonabend, A.M., Ulasov, I.V., Lesniak, M.S. (2006). Conditionally replicative adenoviral vectors for malignant glioma. Rev. Med. Virol. *16***,** 99-115.
Sonawane, N.D., Szoka, F.C., Jr., Verkman, A.S. (2003). Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. J. Biol. Chem. *278***,** 44826-44831.
Song, J., Liang, C., Chen, X. (2006). Transduction of avian cells with recombinant baculovirus. J. Virol. Methods *135***,** 157-162.
Sorrell, D.A., Kolb, A.F. (2005). Targeted modification of mammalian genomes. Biotechnol. Adv. *23***,** 431-469.
Stark, W.M., Boocock, M.R., Sherratt, D.J. (1992). Catalysis by site-specific recombinases. Trends Genet. *8***,** 432-439.
Starkey, J.L., Chiari, E.F., Isom, H.C. (2009). Hepatitis B virus (HBV)-specific short hairpin RNA is capable of reducing the formation of HBV covalently closed circular (CCC) DNA but has no effect on established CCC DNA in vitro. J. Gen. Virol. *90***,** 115-126.

Staufenbiel, M., Epple, P., Deppert, W. (1986). Progressive reorganization of the host cell cytoskeleton during adenovirus infection. J. Virol. *60***,** 1186-1191.

Stefanovic, S., Windsor, M., Nagata, K.I., Inagaki, M., Wileman, T. (2005). Vimentin rearrangement during African swine fever virus infection involves retrograde transport along microtubules and phosphorylation of vimentin by calcium calmodulin kinase II. J. Virol. *79***,** 11766-11775.

Steinwaerder, D.S., Lieber, A. (2000). Insulation from viral transcriptional regulatory elements improves inducible transgene expression from adenovirus vectors in vitro and in vivo. Gene Ther. *7***,** 556-567.

Stoll, S.M., Sclimenti, C.R., Baba, E.J., Meuse, L., Kay, M.A., Calos, M.P. (2001). Epstein-Barr virus/human vector provides high-level, long-term expression of alpha1-antitrypsin in mice. Mol. Ther. *4***,** 122-129.

Stone, D., Lieber, A. (2006). New serotypes of adenoviral vectors. Curr. Opin. Mol. Ther. *8***,** 423-431.

Stone, D., Liu, Y., Li, Z.Y., Tuve, S., Strauss, R., Lieber, A. (2007). Comparison of adenoviruses from species B, C, E, and F after intravenous delivery. Mol. Ther. *15***,** 2146-2153.

Strahl, B.D., Allis, C.D. (2000). The language of covalent histone modifications. Nature *403***,** 41-45.

Strauss, R., Huser, A., Ni, S., Tuve, S., Kiviat, N., Sow, P.S., Hofmann, C., Lieber, A. (2007). Baculovirus-based vaccination vectors allow for efficient induction of immune responses against plasmodium falciparum circumsporozoite protein. Mol. Ther. *15***,** 193-202.

Street, A., Macdonald, A., McCormick, C., Harris, M. (2005). Hepatitis C virus NS5A-mediated activation of phosphoinositide 3-kinase results in stabilization of cellular beta-catenin and stimulation of beta-catenin-responsive transcription. J. Virol. *79***,** 5006-5016.

Su, H., rakawa-Hoyt, J., Kan, Y.W. (2002). Adeno-associated viral vector-mediated hypoxia response element-regulated gene expression in mouse ischemic heart model. Proc. Natl. Acad. Sci. U. S. A *99***,** 9480-9485.

Sugden, B., Warren, N. (1989). A promoter of Epstein-Barr virus that can function during latent infection can be transactivated by EBNA-1, a viral protein required for viral DNA replication during latent infection. J. Virol. *63***,** 2644-2649.

Suikkanen, S., Aaltonen, T., Nevalainen, M., Valilehto, O., Lindholm, L., Vuento, M., Vihinen-Ranta, M. (2003). Exploitation of microtubule cytoskeleton and dynein during parvoviral traffic toward the nucleus. J. Virol. *77***,** 10270-10279.

Summerford, C., Bartlett, J.S., Samulski, R.J. (1999). AlphaVbeta5 integrin: a co-receptor for adeno-associated virus type 2 infection. Nat. Med. *5***,** 78-82.

Summerford, C., Samulski, R.J. (1998). Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. J. Virol. *72***,** 1438-1445.

Summers, M.D. (1971). Electron microscopic observations on granulosis virus entry, uncoating and replication processes during infection of the midgut cells of Trichoplusia ni. J. Ultrastruct. Res *35***,** 606-625.

Summers, M.D. (2006). Milestones leading to the genetic engineering of baculoviruses as expression vector systems and viral pesticides. Adv. Virus Res. *68***,** 3-73.

Sung, L.Y., Lo, W.H., Chiu, H.Y., Chen, H.C., Chung, C.K., Lee, H.P., Hu, Y.C. (2007). Modulation of chondrocyte phenotype via baculovirus-mediated growth factor expression. Biomaterials *28***,** 3437-3447.

Suomalainen, M., Nakano, M.Y., Keller, S., Boucke, K., Stidwill, R.P., Greber, U.F. (1999). Microtubule-dependent plus- and minus end-directed motilities are competing processes for nuclear targeting of adenovirus. J. Cell Biol. *144***,** 657-672.

Suzuki, H., Kaneko, H., Tamai, N., Miyano-Kurosaki, N., Hashimoto, K., Shimotohno, K., Takaku, H. (2005). Suppression of HCV RNA replication by baculovirus-mediated shRNA expression. Nucleic Acids Symp. Ser. (Oxf) 339-340.

Suzuki, H., Matsumoto, N., Suzuki, T., Chang, M.O., Takaku, H. (2009). Stable replication of the EBNA1/OriP-mediated baculovirus vector and its application to anti-HCV gene therapy. Virol. J. *6***,** 156.

Tan, B.T., Wu, L., Berk, A.J. (1999). An adenovirus-Epstein-Barr virus hybrid vector that stably transforms cultured cells with high efficiency. J. Virol. *73***,** 7582-7589.

Tan, W., Zhu, K., Segal, D.J., Barbas, C.F., III, Chow, S.A. (2004). Fusion proteins consisting of human immunodeficiency virus type 1 integrase and the designed polydactyl zinc finger protein E2C direct integration of viral DNA into specific sites. J. Virol. *78***,** 1301-1313.

Tani, H., Abe, T., Matsunaga, T.M., Moriishi, K., Matsuura, Y. (2008). Baculovirus vector for gene delivery and vaccine development. Future Virol. *3***,** 35-43.

Tani, H., Limn, C.K., Yap, C.C., Onishi, M., Nozaki, M., Nishimune, Y., Okahashi, N., Kitagawa, Y., Watanabe, R., Mochizuki, R., Moriishi, K., Matsuura, Y. (2003). In vitro and in vivo gene delivery by recombinant baculoviruses. J. Virol. *77***,** 9799-9808.

Tani, H., Nishijima, M., Ushijima, H., Miyamura, T., Matsuura, Y. (2001). Characterization of cell-surface determinants important for baculovirus infection. Virology *279***,** 343-353.

Themis, M., Waddington, S.N., Schmidt, M., von, K.C., Wang, Y., Al-Allaf, F., Gregory, L.G., Nivsarkar, M., Themis, M., Holder, M.V., Buckley, S.M., Dighe, N., Ruthe, A.T., Mistry, A., Bigger, B., Rahim, A., Nguyen, T.H., Trono, D., Thrasher, A.J., Coutelle, C. (2005). Oncogenesis following delivery of a nonprimate lentiviral gene therapy vector to fetal and neonatal mice. Mol. Ther. *12***,** 763-771.

Thomas, C.E., Ehrhardt, A., Kay, M.A. (2003). Progress and problems with the use of viral vectors for gene therapy. Nat. Rev. Genet. *4***,** 346-358.

Thyagarajan, B., Guimaraes, M.J., Groth, A.C., Calos, M.P. (2000). Mammalian genomes contain active recombinase recognition sites. Gene *244***,** 47-54.

Thyagarajan, B., Scheyhing, K., Xue, H., Fontes, A., Chesnut, J., Rao, M., Lakshmipathy, U. (2009). A single EBV-based vector for stable episomal maintenance and expression of GFP in human embryonic stem cells. Regen. Med. *4*, 239-250.

Toivola, D.M., Tao, G.Z., Habtezion, A., Liao, J., Omary, M.B. (2005). Cellular integrity plus: organelle-related and protein-targeting functions of intermediate filaments. Trends Cell Biol. *15*, 608-617.

Tolstonog, G.V., Mothes, E., Shoeman, R.L., Traub, P. (2001). Isolation of SDS-stable complexes of the intermediate filament protein vimentin with repetitive, mobile, nuclear matrix attachment region, and mitochondrial DNA sequence elements from cultured mouse and human fibroblasts. DNA Cell Biol. *20*, 531-554.

Tolstonog, G.V., Wang, X., Shoeman, R., Traub, P. (2000). Intermediate filaments reconstituted from vimentin, desmin, and glial fibrillary acidic protein selectively bind repetitive and mobile DNA sequences from a mixture of mouse genomic DNA fragments. DNA Cell Biol. *19*, 647-677.

Tomiyasu, K., Satoh, E., Oda, Y., Nishizaki, K., Kondo, M., Imanishi, J., Mazda, O. (1998). Gene transfer in vitro and in vivo with Epstein-Barr virus-based episomal vector results in markedly high transient expression in rodent cells. Biochem. Biophys. Res. Commun. *253*, 733-738.

Trabanelli, C., Corallini, A., Gruppioni, R., Sensi, A., Bonfatti, A., Campioni, D., Merlin, M., Calza, N., Possati, L., Barbanti-Brodano, G. (1998). Chromosomal aberrations induced by BK virus T antigen in human fibroblasts. Virology *243*, 492-496.

Traub, P. (1995). Intermediate filaments and gene regulation. Physiol Chem. Phys. Med. NMR *27*, 377-400.

Treanor, J.J., Schiff, G.M., Couch, R.B., Cate, T.R., Brady, R.C., Hay, C.M., Wolff, M., She, D., Cox, M.M. (2006). Dose-related safety and immunogenicity of a trivalent baculovirus-expressed influenza-virus hemagglutinin vaccine in elderly adults. J. Infect. Dis. *193*, 1223-1228.

Treanor, J.J., Schiff, G.M., Hayden, F.G., Brady, R.C., Hay, C.M., Meyer, A.L., Holden-Wiltse, J., Liang, H., Gilbert, A., Cox, M. (2007). Safety and immunogenicity of a baculovirus-expressed hemagglutinin influenza vaccine: a randomized controlled trial. JAMA *297*, 1577-1582.

Tsai, C.T., Chuang, C.K., Hu, Y.C. (2009). Baculovirus-mediated gene transfer into mesenchymal stem cells. Methods Mol. Biol. *515*, 339-351.

Tsujie, M., Isaka, Y., Nakamura, H., Kaneda, Y., Imai, E., Hori, M. (2001). Prolonged transgene expression in glomeruli using an EBV replicon vector system combined with HVJ liposomes. Kidney Int. *59*, 1390-1396.

Tsukamoto, H., Wells, D., Brown, S., Serpente, P., Strong, P., Drew, J., Inui, K., Okada, S., Dickson, G. (1999). Enhanced expression of recombinant dystrophin following intramuscular injection of Epstein-Barr virus (EBV)-based mini-chromosome vectors in mdx mice. Gene Ther. *6*, 1331-1335.

Tsurumi, T., Fujita, M., Kudoh, A. (2005). Latent and lytic Epstein-Barr virus replication strategies. Rev. Med. Virol. *15*, 3-15.

Tsuruta, Y., Pereboeva, L., Glasgow, J.N., Rein, D.T., Kawakami, Y., Alvarez, R.D., Rocconi, R.P., Siegal, G.P., Dent, P., Fisher, P.B., Curiel, D.T. (2007). A mosaic fiber adenovirus serotype 5 vector containing reovirus sigma 1 and adenovirus serotype 3 knob fibers increases transduction in an ovarian cancer ex vivo system via a coxsackie and adenovirus receptor-independent pathway. Clin. Cancer Res. *13*, 2777-2783.

Tu, G., Kirchmaier, A.L., Liggitt, D., Liu, Y., Liu, S., Yu, W.H., Heath, T.D., Thor, A., Debs, R.J. (2000). Non-replicating Epstein-Barr virus-based plasmids extend gene expression and can improve gene therapy in vivo. J. Biol. Chem. *275*, 30408-30416.

Turner, B.M. (2000). Histone acetylation and an epigenetic code. Bioessays *22*, 836-845.

Uemura, N., Ozawa, K., Tojo, A., Takahashi, K., Okano, A., Karasuyama, H., Tani, K., Asano, S. (1992). Acquisition of interleukin-3 independence in FDC-P2 cells after transfection with the activated c-H-ras gene using a bovine papillomavirus-based plasmid vector. Blood *80*, 3198-3204.

Ungewickell, E.J., Hinrichsen, L. (2007). Endocytosis: clathrin-mediated membrane budding. Curr. Opin. Cell Biol. *19*, 417-425.

Utomo, A.R., Nikitin, A.Y., Lee, W.H. (1999). Temporal, spatial, and cell type-specific control of Cre-mediated DNA recombination in transgenic mice. Nat. Biotechnol. *17*, 1091-1096.

Valenzuela, L., Kamakaka, R.T. (2006). Chromatin insulators. Annu. Rev. Genet. *40*, 107-138.

Van Craenenbroeck, K., Vanhoenacker, P., Haegeman, G. (2000). Episomal vectors for gene expression in mammalian cells. Eur. J. Biochem. *267*, 5665-5678.

van Loo, N.D., Fortunati, E., Ehlert, E., Rabelink, M., Grosveld, F., Scholte, B.J. (2001). Baculovirus infection of nondividing mammalian cells: mechanisms of entry and nuclear transport of capsids. J. Virol. *75*, 961-970.

van Oers, M.M. (2006). Vaccines for viral and parasitic diseases produced with baculovirus vectors. Adv. Virus Res. *68*, 193-253.

Vargas, J., Jr., Gusella, G.L., Najfeld, V., Klotman, M.E., Cara, A. (2004). Novel integrase-defective lentiviral episomal vectors for gene transfer. Hum. Gene Ther. *15*, 361-372.

Vargas, J., Jr., Klotman, M.E., Cara, A. (2008). Conditionally replicating lentiviral-hybrid episomal vectors for suicide gene therapy. Antiviral Res. *80*, 288-294.

Velazquez, L., Camarena, L., Reyes, J.L., Bastarrachea, F. (1991). Mutations affecting the Shine-Dalgarno sequences of the untranslated region of the Escherichia coli gltBDF operon. J. Bacteriol. *173*, 3261-3264.

Vile, R., Miller, N., Chernajovsky, Y., Hart, I. (1994). A comparison of the properties of different retroviral vectors containing the murine tyrosinase promoter to achieve transcriptionally targeted expression of the HSVtk or IL-2 genes. Gene Ther. *1*, 307-316.

Viswanathan, P., Venkaiah, B., Kumar, M.S., Rasheedi, S., Vrati, S., Bashyam, M.D., Hasnain, S.E. (2003). The homologous region sequence (hr1) of Autographa californica multinucleocapsid polyhedrosis virus can enhance transcription from non-baculoviral promoters in mammalian cells. J. Biol. Chem. *278***,** 52564-52571.

Volkman, L.E. (2007). Baculovirus infectivity and the actin cytoskeleton. Curr. Drug Targets. *8***,** 1075-1083.

Volkman, L.E., Goldsmith, P.A. (1983). In vitro Survey of *Autographa californica* Nuclear Polyhedrosis Virus Interaction with Nontarget Vertebrate Host Cells. Applied and Environmental Microbiology *45***,** 1085-1093.

Volkman, L.E., Zaal, K.J. (1990). Autographa californica M nuclear polyhedrosis virus: microtubules and replication. Virology *175***,** 292-302.

Voziyanov, Y., Konieczka, J.H., Stewart, A.F., Jayaram, M. (2003). Stepwise manipulation of DNA specificity in Flp recombinase: progressively adapting Flp to individual and combinatorial mutations in its target site. J. Mol. Biol. *326***,** 65-76.

Wade, R.H. (2007). Microtubules: an overview. Methods Mol. Med. *137***,** 1-16.

Wade-Martins, R., Frampton, J., James, M.R. (1999). Long-term stability of large insert genomic DNA episomal shuttle vectors in human cells. Nucleic Acids Res *27***,** 1674-1682.

Wade-Martins, R., Saeki, Y., Chiocca, E.A. (2003). Infectious delivery of a 135-kb LDLR genomic locus leads to regulated complementation of low-density lipoprotein receptor deficiency in human cells. Mol. Ther. *7***,** 604-612.

Waehler, R., Russell, S.J., Curiel, D.T. (2007). Engineering targeted viral vectors for gene therapy. Nat. Rev. Genet. *8***,** 573-587.

Wagle, M., Grunewald, B., Subburaju, S., Barzaghi, C., Le, G.S., Chan, J., Jesuthasan, S. (2004). EphrinB2a in the zebrafish retinotectal system. J. Neurobiol. *59***,** 57-65.

Wagle, M., Jesuthasan, S. (2003). Baculovirus-mediated gene expression in zebrafish. Mar. Biotechnol. (NY) *5***,** 58-63.

Wagner, J.A., Nepomuceno, I.B., Messner, A.H., Moran, M.L., Batson, E.P., Dimiceli, S., Brown, B.W., Desch, J.K., Norbash, A.M., Conrad, C.K., Guggino, W.B., Flotte, T.R., Wine, J.J., Carter, B.J., Reynolds, T.C., Moss, R.B., Gardner, P. (2002). A phase II, double-blind, randomized, placebo-controlled clinical trial of tgAAVCF using maxillary sinus delivery in patients with cystic fibrosis with antrostomies. Hum. Gene Ther. *13***,** 1349-1359.

Waldenstrom, M., Schenstrom, K., Sollerbrant, K., Hansson, L. (1992). Replication of bovine papillomavirus vectors in murine cells. Gene *120***,** 175-181.

Wang, C. (2008). Hybrid baculovirus-adeno-associated virus vectors for prolonged transgene expression in human neural cells. J. Neurovirol. 1-6.

Wang, C.Y., Li, F., Yang, Y., Guo, H.Y., Wu, C.X., Wang, S. (2006). Recombinant baculovirus containing the diphtheria toxin A gene for malignant glioma therapy. Cancer Res. *66***,** 5798-5806.

Wang, C.Y., Wang, S. (2005). Adeno-associated virus inverted terminal repeats improve neuronal transgene expression mediated by baculoviral vectors in rat brain. Hum. Gene Ther. *16***,** 1219-1226.

Wang, C.Y., Wang, S. (2006). Astrocytic expression of transgene in the rat brain mediated by baculovirus vectors containing an astrocyte-specific promoter. Gene Ther. *13***,** 1447-1456.

Wang, K.C., Wu, J.C., Chung, Y.C., Ho, Y.C., Chang, M.D., Hu, Y.C. (2005a). Baculovirus as a highly efficient gene delivery vector for the expression of hepatitis delta virus antigens in mammalian cells. Biotechnol. Bioeng. *89***,** 464-473.

Wang, L., Menon, S., Bolin, S.R., Bello, L.J. (2003). A hepadnavirus regulatory element enhances expression of a type 2 bovine viral diarrhea virus E2 protein from a bovine herpesvirus 1 vector. J. Virol. *77***,** 8775-8782.

Wang, L., Shan, L., Lo, K.W., Yin, J., Zhang, Y., Sun, R., Zhong, J. (2008). Inhibition of nasopharyngeal carcinoma growth by RTA-expressing baculovirus vectors containing oriP. J. Gene Med. *10***,** 1124-1133.

Wang, P., Hammer, D.A., Granados, R.R. (1997). Binding and fusion of Autographa californica nucleopolyhedrovirus to cultured insect cells. J. Gen. Virol. *78***,** 3081-3089.

Wang, S., Vos, J.M. (1996). A hybrid herpesvirus infectious vector based on Epstein-Barr virus and herpes simplex virus type 1 for gene transfer into human cells in vitro and in vivo. J. Virol. *70***,** 8422-8430.

Wang, X., Wang, C., Zeng, J., Xu, X., Hwang, P.Y., Yee, W.C., Ng, Y.K., Wang, S. (2005b). Gene transfer to dorsal root ganglia by intrathecal injection: effects on regeneration of peripheral nerves. Mol. Ther. *12***,** 314-320.

Weclewicz, K., Kristensson, K., Svensson, L. (1994). Rotavirus causes selective vimentin reorganization in monkey kidney CV-1 cells. J. Gen. Virol. *75 (Pt 11)***,** 3267-3271.

Weissenhorn, W., Hinz, A., Gaudin, Y. (2007). Virus membrane fusion. FEBS Lett. *581***,** 2150-2155.

Werner, M., Kraunus, J., Baum, C., Brocker, T. (2004). B-cell-specific transgene expression using a self-inactivating retroviral vector with human CD19 promoter and viral post-transcriptional regulatory element. Gene Ther. *11***,** 992-1000.

White, R.E., Wade-Martins, R., James, M.R. (2002). Infectious delivery of 120-kilobase genomic DNA by an epstein-barr virus amplicon vector. Mol. Ther. *5***,** 427-435.

Whittaker, G.R. (2003). Virus nuclear import. Adv. Drug Deliv. Rev. *55***,** 733-747.

Wickham, T.J. (2000). Targeting adenovirus. Gene Ther. *7***,** 110-114.

Wickham, T.J., Davis, T., Granados, R.R., Shuler, M.L., Wood, H.A. (1992a). Screening of insect cell lines for the production of recombinant proteins and infectious virus in the baculovirus expression system. Biotechnol. Prog. *8***,** 391-396.

Wickham, T.J., Filardo, E.J., Cheresh, D.A., Nemerow, G.R. (1994). Integrin alpha v beta 5 selectively promotes adenovirus mediated cell membrane permeabilization. J. Cell Biol. *127***,** 257-264.

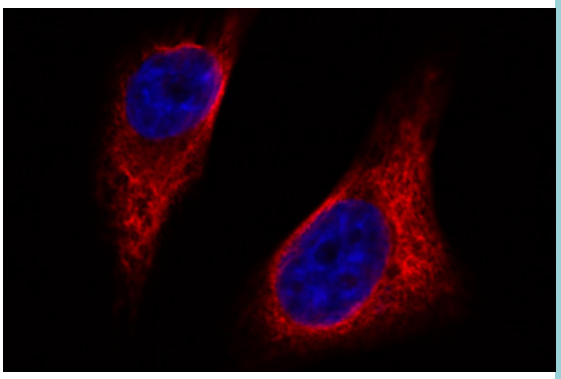
Wickham, T.J., Shuler, M.L., Hammer, D.A., Granados, R.R., Wood, H.A. (1992b). Equilibrium and kinetic analysis of Autographa californica nuclear polyhedrosis virus attachment to different insect cell lines. J. Gen. Virol. *73 (Pt 12)***,** 3185-3194.

Williams, D.A. (2008). NIH recombinant DNA Advisory Committee continues to ponder adverse event associated with AAV gene therapy trial. Mol. Ther. *16*, 427-428.
Williams, D.A. (2009). Recombinant DNA advisory committee updates recommendations on gene transfer for x-linked severe combined immunodeficiency. Mol. Ther. *17*, 751-752.
Wilson, J.B., Bell, J.L., Levine, A.J. (1996). Expression of Epstein-Barr virus nuclear antigen-1 induces B cell neoplasia in transgenic mice. EMBO J. *15*, 3117-3126.
Wirth, D., Gama-Norton, L., Riemer, P., Sandhu, U., Schucht, R., Hauser, H. (2007). Road to precision: recombinase-based targeting technologies for genome engineering. Curr. Opin. Biotechnol. *18*, 411-419.
Wolff, J.A., Rozema, D.B. (2008). Breaking the bonds: non-viral vectors become chemically dynamic. Mol. Ther. *16*, 8-15.
Wu, G.Y., Wu, C.H. (1987). Receptor-mediated in vitro gene transformation by a soluble DNA carrier system. J. Biol. Chem. *262*, 4429-4432.
Wu, Z., Asokan, A., Samulski, R.J. (2006). Adeno-associated virus serotypes: vector toolkit for human gene therapy. Mol. Ther. *14*, 316-327.
Xu, Z.L., Mizuguchi, H., Ishii-Watabe, A., Uchida, E., Mayumi, T., Hayakawa, T. (2001). Optimization of transcriptional regulatory elements for constructing plasmid vectors. Gene *272*, 149-156.
Xu, Z.L., Mizuguchi, H., Ishii-Watabe, A., Uchida, E., Mayumi, T., Hayakawa, T. (2002). Strength evaluation of transcriptional regulatory elements for transgene expression by adenovirus vector. J. Control Release *81*, 155-163.
Xu, Z.L., Mizuguchi, H., Mayumi, T., Hayakawa, T. (2003). Woodchuck hepatitis virus post-transcriptional regulation element enhances transgene expression from adenovirus vectors. Biochim. Biophys. Acta *1621*, 266-271.
Xuan, Z., Zhao, F., Wang, J., Chen, G., Zhang, M.Q. (2005). Genome-wide promoter extraction and analysis in human, mouse, and rat. Genome Biol. *6*, R72.
Yang, X., Wang, J., Liu, C., Grizzle, W.E., Yu, S., Zhang, S., Barnes, S., Koopman, W.J., Mountz, J.D., Kimberly, R.P., Zhang, H.G. (2005). Cleavage of p53-vimentin complex enhances tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis of rheumatoid arthritis synovial fibroblasts. Am. J. Pathol. *167*, 705-719.
Yang, Y., Lo, S.L., Yang, J., Yang, J., Goh, S.S., Wu, C., Feng, S.S., Wang, S. (2009). Polyethylenimine coating to produce serum-resistant baculoviral vectors for in vivo gene delivery. Biomaterials *30*, 5767-5774.
Yap, C.C., Ishii, K., Aoki, Y., Aizaki, H., Tani, H., Shimizu, H., Ueno, Y., Miyamura, T., Matsuura, Y. (1997). A hybrid baculovirus-T7 RNA polymerase system for recovery of an infectious virus from cDNA. Virology *231*, 192-200.
Yates, J., Warren, N., Reisman, D., Sugden, B. (1984). A cis-acting element from the Epstein-Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells. Proc. Natl. Acad. Sci U. S. A *81*, 3806-3810.
Yates, J.L., Camiolo, S.M., Bashaw, J.M. (2000). The minimal replicator of Epstein-Barr virus oriP. J. Virol. *74*, 4512-4522.
Yates, J.L., Guan, N. (1991). Epstein-Barr virus-derived plasmids replicate only once per cell cycle and are not amplified after entry into cells. J. Virol. *65*, 483-488.
Yates, J.L., Warren, N., Sugden, B. (1985). Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells. Nature *313*, 812-815.
Ye, Z.Q., Qiu, P., Burkholder, J.K., Turner, J., Culp, J., Roberts, T., Shahidi, N.T., Yang, N.S. (1998). Cytokine transgene expression and promoter usage in primary CD34+ cells using particle-mediated gene delivery. Hum. Gene Ther. *9*, 2197-2205.
Yla-Herttuala, S., Luoma, J., Viita, H., Hiltunen, T., Sisto, T., Nikkari, T. (1995). Transfer of 15-lipoxygenase gene into rabbit iliac arteries results in the appearance of oxidation-specific lipid-protein adducts characteristic of oxidized low density lipoprotein. J. Clin. Invest *95*, 2692-2698.
Young, J.A.T. (2001). Virus Entry and Uncoating. In: Fields Virology, D.M. Knipe, P.M. Howley, eds. (Philadelphia: Lippincott Williams & Wilkins), pp. 87-103.
Yu, S.F., von, R.T., Kantoff, P.W., Garber, C., Seiberg, M., Ruther, U., Anderson, W.F., Wagner, E.F., Gilboa, E. (1986). Self-inactivating retroviral vectors designed for transfer of whole genes into mammalian cells. Proc. Natl. Acad. Sci. U. S. A *83*, 3194-3198.
Zanta-Boussif, M.A., Charrier, S., Brice-Ouzet, A., Martin, S., Opolon, P., Thrasher, A.J., Hope, T.J., Galy, A. (2009). Validation of a mutated PRE sequence allowing high and sustained transgene expression while abrogating WHV-X protein synthesis: application to the gene therapy of WAS. Gene Ther. *16*, 605-619.
Zeng, J., Du, J., Zhao, Y., Palanisamy, N., Wang, S. (2007). Baculoviral vector-mediated transient and stable transgene expression in human embryonic stem cells. Stem Cells *25*, 1055-1061.
Zhang, J., Wilson, A., Alber, S., Ma, Z., Tang, Z.L., Satoh, E., Mazda, O., Watkins, S., Huang, L., Pitt, B., Li, S. (2003). Prolonged gene expression in mouse lung endothelial cells following transfection with Epstein-Barr virus-based episomal plasmid. Gene Ther. *10*, 822-826.
Zhang, M.H., Lee, J.S., Kim, H.J., Jin, D.I., Kim, J.I., Lee, K.J., Seo, J.S. (2006). HSP90 protects apoptotic cleavage of vimentin in geldanamycin-induced apoptosis. Mol. Cell Biochem. *281*, 111-121.
Zhu, Y., Cullen, J.M., Aldrich, C.E., Saputelli, J., Miller, D., Seeger, C., Mason, W.S., Jilbert, A.R. (2004). Adenovirus-based gene therapy during clevudine treatment of woodchucks chronically infected with woodchuck hepatitis virus. Virology *327*, 26-40.
Zhu, Y., QI, Y., Liu, Z., Xiao, G. (1998). Baculovirus-mediated gene tranfer into mammalian cells. Science in China *41*, 473-478.

Zolotukhin, S., Potter, M., Hauswirth, W.W., Guy, J., Muzyczka, N. (1996). A "humanized" green fluorescent protein cDNA adapted for high-level expression in mammalian cells. J. Virol. *70*, 4646-4654.

Zufferey, R., Donello, J.E., Trono, D., Hope, T.J. (1999). Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. J. Virol. *73*, 2886-2892.

Zuo, J., Niu, Q.W., Moller, S.G., Chua, N.H. (2001). Chemical-regulated, site-specific DNA excision in transgenic plants. Nat. Biotechnol. *19*, 157-161.



Anssi J. Mähönen

The Challenge of Enhanced and Long-term Baculovirus-mediated Gene Expression in Vertebrate Cells

The aim of this study was to enhance and extend the transient gene expression properties of baculoviruses. In addition, the effect of cytoskeleton on the baculoviral transduction of mammalian cells was studied. As a result, novel vectors were generated resulting in increased and prolonged gene expression in vertebrate cells. In addition, this thesis provides new insights concerning the nuclear entry of baculoviruses.

UNIVERSITY OF
EASTERN FINLAND

Publications of the University of Eastern Finland
Dissertations in Health Sciences

ISBN 978-952-61-0021-0