Institute of Biochemistry
University of Fribourg, Switzerland

Nuclear Receptors as vehicles for Gene Transfer

MAURIZIO CEPPI
Lugano (Ticino), Switzerland

Thèse de doctorat
présentée à la Faculté des Sciences de
l'Université de Fribourg pour l'obtention du
grade de *Doctor rerum naturalium*

Dissertation Nr. 1328

Mécanographie, Fribourg, Avril 2001

Acceptée par la Faculté des Sciences de l' Université de Fribourg (Suisse) sur la proposition de Prof. Sandro Rusconi (Université de Fribourg), Dr. Elke Walter (ETH Zürich), Dr. Matt Cotten (Vienna University Biocenter) et Prof. Franzpeter Emmenegger (Université de Fribourg).

Fribourg, le 23 Novembre 2000

Le directeur de thèse
Prof. Sandro Rusconi

Le Doyen
Prof. Alexandre Zelewsky
A. v. Zelewsky

Acknowledgments

Firstly, I would like to thank my supervisor Sandro Rusconi for having welcomed me into his laboratory. I greatly enjoyed the freedom he gave me to realize some of my ideas. I will be forever thankful for having had the oppurtinity to meet some "big shots" of the gene therapy field including Jon Wolff, Phil Felgner, Jean-Paul Behr, Inder Verma and many others.

I offer much thanks to all former and current members of the "Ruscolab" for their contribution in having made the lab a friendly and pleasant environment in which to work. In particular, the contribution of Giovanni (the wolf) was essential for having cooked so often the great menu "pasta e tonno" on Tuesday (we all miss Patrick's specialities!). Special thanks go to my "labmates" Eric (vive la France!) and Patricia (her help was often fundamental) and my "ex-labmate" Stefano (boom ... boom ... pump up the volume), who had to tolerate my mood fluctuations often associated to loud music (viva la salsa cubana!) in room 2.104. I would also like to thank Alan for having improved the written English of my thesis (the work will be compensated by many pints of Guiness).

My deep gratitude goes to my collegues in Bern; Ivo (OM ... it's a pity that he left the SMGD project so early!), Dr.Wehrli, Alessio and Alex. Thanks to the fabulous molecules they created, I could actually do a big part of my work!

Special thanks to Merdol for his support at the confocal microscopy. I will never forget the many hours spent together at the microscope. Without his help, I had probably never found "the plasmids in the nucleus".

I would also thank Karola Rittner (Transgene), Martin Woodle and Kas Subramanian (GTI-Novartis) for their interest in my work and for the stimulating discussions.

My deep appreciation goes to my experts, Matt Cotten and Elke Walter, who kindly accepted to evaluate my thesis.

Un grazie di cuore a mia mamma ed a mio papà che mi hanno sempre permesso di fare quello che veramente volevo. Senza il loro rispetto della mia libertà non avrei mai raggiunto questo grande traguardo. Seguirò il vostro esempio, carissimi mami e papi ...

Mille grazie anche a Nathalie che mi ha sempre incoraggiato a non "mollare". Ti sarò sempre grato di avermi circondato del necessario affetto e della necessaria comprensione in questo "eterno" anno conclusivo di dottorato ...

"Dottor DNA"

INDEX:

Acknowledgments 3

1. SUMMARY **7**

2. INTRODUCTION **8**

2.1. HUMAN GENE TRANSFER **8**

- **2.1.1.** "GT 2000": human gene therapy between life and death
- **2.1.2.** The need of efficient gene delivery systems
- **2.1.3.** Viral vs. nonviral vectors

2.2. NONVIRAL VECTORS FOR GENE THERAPY **14**

- **2.2.1.** Brief description of current nonviral vectors **14**
    - **2.2.1.1.** Naked DNA
    - **2.2.1.2.** Gene gun
    - **2.2.1.3.** Lipoplex
    - **2.2.1.4.** Polyplex
    - **2.2.1.5.** Lipopolyplex
    - **2.2.1.6.** Receptor-targeted gene transfer (RTGT)
    - **2.2.1.7.** Hybrid viral / nonviral vectors
    - **2.2.1.8.** DNA nanospheres
- **2.2.2.** Barriers that a vector must overcome **18**
    - **2.2.2.1.** Extracellular barriers
    - **2.2.2.2.** Intracellular barriers
- **2.2.3.** Nuclear transport of exogenous DNA **19**
    - **2.2.3.1.** Nucleocytoplasmic transport
    - **2.2.3.2.** The DNA nuclear import is inefficient
    - **2.2.3.3.** NLS-mediated gene transfer
    - **2.2.3.4.** Viral strategies for DNA nuclear transport

2.3. STEROID MEDIATED GENE DELIVERY (SMGD) **22**
    2.3.1. Steroid Hormone Receptors
    2.3.2. The principle of SMGD: Nuclear Receptors as vehicles for DNA
    2.3.3. The initial purposes of SMGD
    2.3.4. Modelling the SMGD with the Glucocorticoid Receptor (GR)
    2.3.5. The "first generation" of steroid derivatives
    2.3.6. First encouraging results
    2.3.7. SMGD's "winning horse": Dr.Wherli!

2.4. DESCRIPTION AND SIGNIFICANCE OF THE PERSONAL CONTRIBUTIONS **28**
    2.4.1. The covalent attachment of steroids to DNA
    2.4.2. Three important experimental "revolutions"
    2.4.3. The proof-of-principle
    2.4.4. Detection of plasmid DNA in the nucleus of intact cells
    2.4.5. How could SMGD influence human gene therapy?

3. PAPERS **41**
3.1. PAPER 1: "SELECTIVELY ENHANCED TRANSFER OF STEROID-DECORATED TRANSGENES IN NUCLEAR RECEPTOR-POSITIVE CELLS".................. **41**

3.2. PAPER 2: "VECTORS FOR GENE DELIVERY" **80**

4. APPENDIX **110**
4.1. SUMMARY OF PROPERTIES OF SELECTED STEROID DERIVATIVES **111**
4.2. SMGD PATENT COVER **112**
4.3. CURRICULUM VITAE **113**
4.4. DECLARATION D'ORIGINALITÉ **116**

« The most beautiful thing we can experience is the mysterious. It is the source of all true art and science. »

Albert Einstein

1. SUMMARY

Human gene therapy can be defined as the directed transfer of exogenous genes or other nucleotide sequences into somatic cells, for the purpose of preventing, correcting or healing various diseases. This novel approach to the therapy of human disorders is based on the realization that many previous medical treatments have usually been directed at the consequences of causative defects or at disease symptoms rather than at the underlying causes. The field of gene therapy has been criticized for promising too much and providing too little during its first 10 years of existence. However, several recent successes achieved in human clinical trials are demonstrating that the concept of gene therapy is finally succeeding.

The basic challenge in gene therapy is to develop approaches for delivering genetic material to the appropriate cells of the patient in a way that is specific, efficient and safe. This problem of "drug delivery," where the gene is a drug, is particularly challenging for genes which are large and complex and require targeting to the nuclei of cells. If genes are appropriately delivered they can persist for the life of the cell and potentially lead to a cure.

The enabling technology of gene therapy is based on strategies for delivering genes. To do this, special gene delivery vehicles – also called *vectors* – have been developed. Vectors generally fall into two categories: viral and nonviral. Viral vectors are generally replication defective viruses with part or even all of the viral coding sequence replaced by that of therapeutic genes. Currently, the majority of the vectors for human gene therapy treatments is of viral origin. However, humans have an immune system able to fight off viruses, and attempts to deliver genes via viral vectors have been confronted by these host response. This important drawback of viral systems make synthetic vectors an attractive alternative, especially when readministration of the therapeutic genetic material may be necessary. However, nonviral vectors also have important disadvantages, including their low efficacy and their transient gene expression.

In the present study, a novel technique to improve both the efficiency and the specificity of nonviral vectors is described. This new gene transfer technology has been called Steroid Mediated Gene Delivery (SMGD). In particular, the possibility to exploit the shuttling action of nuclear receptors to facilitate the nuclear uptake of DNA has been explored. The SMGD has been modelled with the well characterized glucocorticoid receptor system. The rationale was to decorate transgenes with steroids. To this purpose, special bifunctional steroid derivatives all composed by a steroid molecule covalently linked to DNA interacting compound have been synthesized. The final aim of this work was to test the feasibility of the SMGD using the steroid derivative DR9NP, which has been ultimately selected from the initial collection of synthetic conjugates. This study clearly demonstrates that the transfer of steroid-decorated transgenes is selectively enhanced in presence of the glucocorticoid receptor. This work is the first that shows that nuclear receptors can be exploited as gene transfer vehicles. The SMGD approach can now be extended to other ligands that interact with nuclearly-shuttling intracellular receptors and offers thereby an additional selective advantage to the gene transfer-based treatment of somatic tissues that express specific steroid receptors.

2. INTRODUCTION

2.1. HUMAN GENE TRANSFER

2.1.1. "GT 2000": human gene transfer between life and death[1]

In the last twelve months, the gene therapy field has been marked by two events that have led the scientific community into contrasting emotions. In fact, we could experience both the first patient died of a gene transfer treatment[2] and the first two patients successfully cured with gene therapy[3]. Why did Jesse Gelsinger die? And why did it take apparently so long to obtain a success in a clinical trial? Based on the reactions and comments of the scientific community, I will try to answer these important questions. I will also mention some recent achievements that clearly demonstrate that after a decade of frustrations, the concept of human gene therapy may be finally succeeding.

In September 1999, a relatively healthy 18-year-old man with an inherited enzyme deficiency died 4 days after doctors - under the guide of James Wilson[4] - injected a genetically altered virus into his liver. Since nobody did fully understand what went wrong, no final conclusion has been made. However, some important considerations have emerged. It seems that a central factor in the tragic events that led to Gelsinger's death was the high-dose of injected viral particles. Wilson and his colleagues had given Gelsinger 38 trillions adenoviral particles, the highest dose in that 18-patients trial, hoping to get enough functioning transgene into his liver. However, only 1% of the transferred genes reached the target cells and none of the patients in the trial showed significant gene expression. Furthermore, the viral particles invaded not only the intended target, but many other organs. This triggered an activation of innate immunity followed by a systemic inflammatory response. This reaction brought Gelsinger first into a coma and later to death.

Why were not the high-dose effects foreseeable, and why were so few genes transferred? Wilson does not know why the adenoviral vectors worked so badly in humans, since animal trials had indicated much higher transduction rates. In regard to the high toxicity of the viral particles, Wilson suggested that Gelsinger's immune response was an anomaly. Wilson's collaborators found indeed that Gelsinger's bone marrow was severely depleted of erythroid precursor blood cells, suggesting the presence of an undetected genetic condition or a viral infection, either of which might have triggered the harsh immune response. Most gene therapists that analyzed the case agreed that Gelsinger's reaction was unusual. At that point, his death was considered only an accident. However, a subsequent investigation perfomed by the Food and Drug Administration resulted in the detection of important "deviations" from the protocol in Wilson's conduct of the clinical trial. It is still unknown whether those

---

[1] "GT 2000" is an abbreviation for Gene Therapy in year 2000

[2] For a recent commentary see: Eliot Marshall, Gene Therapy death prompts review of adenovirus vector, *Science* **286**, 2244-45 (1999)

[3] Cavazzano-Calvo *et al.*, Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease, *Science* **288**, 669-672 (2000)

[4] Institute of Human Gene Therapy, University of Pennsylvania

"deviations" had any influence on Gelsinger's death. However, everybody agrees that the presence of such irregularities is unaccetable and could finally lead to irreversible damages to the whole gene therapy field.

The most encouraging and long expected good news came only six months later. In fact, in April 2000 a French research team - headed by Alain Fischer[5] - reported the successfull treatment of two infants suffering from severe combined immunodeficiency (SCID). Mutations in several different genes of immune cells can result in SCID. The first gene therapy trials treated patients suffering from a type of SCID caused by a deficiency in the enzyme adenosine deaminase (ADA)[6]. In these trials, the investigators inserted a normal copy of the ADA gene (carried in a retroviral vector) into mature T-lymphocytes and later into bone marrow stem cells. In addition, the patients were always administered with PEG-ADA, a polyethylene glycol-conjugated ADA enzyme preparation that reduces the levels of the toxic molecule deoxyadenosine in ADA-deficient patients. The majority of the patients treated in these first trials have not been significantly helped. An amelioration of the symptoms occured in the first patient (Ashanti de Silva) even treated with gene therapy. However, for ethical and safety reasons the investigators never stopped administreting PEG-ADA. Therefore, the contribution of the gene therapy treatment alone could never be monitored. In Fischer's clinical trial, the two treated patients had a X-linked form of SCID (SCID-X) caused by a mutation in the gene encoding the γc subunit, a component of certain cytokine receptors. Fisher and collaborators took hematopoietic stem cells from the infants bone marrow and incubated the cells *ex vivo* with a retroviral vector carrying the γc gene. The transduced stem cells were then transfused back into the SCID-X1 patients. Clinically, the two patients improved considerably and were able to leave the hospital and go home only 3 months after the treatment!

Why are the results of Fisher and collaborators more encouraging than those from the earlier experiments that treated ADA-deficient SCID patients?[7] Unlike the earlier gene therapy trials, the SCID-X1 patients did not have to be administered with PEG-ADA. The concomitant administration of PEG-ADA is believed to lessen the potential growth advantage of ADA gene-corrected cells[8]. Still unpublished results indicate indeed that ADA-deficient SCID patients can be cured in absence of PEG-ADA[9]. Therefore, the decision to never stop the administration of PEG-ADA in earlier gene therapy trials, even if ethically correct could have played a major role in the failure of the treatments.

In addition to the success achieved with gene therapy in the treatment of SCID, recent publications reported significant progress in the treatment of hemophilia[10], cardiovascular diseases[11] and critical limb ischemia[12]. Further publications described the almost incredible efficacy of novel or improved gene therapy tools, such as ribozymes[13], chimeraplasts[14] and "gutless" adenoviral vectors[15]. These new tools will probably enable gene therapists to develop safer and more specific clinical treatments. Major new technolgies had failures and disappontments. But gene therapy, like every major innovation, will succeed with time.

---

[5] INSERM, Hôpital Necker-Enfants Malades, Paris

[6] (1) R.M. Blaese *et al.*, T lymphocyte-directed gene therapy for ADA-SCID: initial trial results after 4 years.*Science* **270**, 475-480 (1995); (2) C.Bordignon *et al.*, Gene therapy in peripheral blood lymphocytes and bone marrow for ADA- immunodeficient patients, *Science* **270**, 470-475 (1995)

[7] For a recent commentary see: W. French Anderson, The best of times, the worst of times, *Science* **288**, 627-629 (2000)

[8] D.B. Kohn *et al.*, T lymphocytes with a normal ADA gene accumulate after transplantation of transduced autologous umbilical cord blood CD34+ cells in ADA-deficient SCID neonates, *Nature Medicine* **4**, 775-780 (1998)

[9] Claudio Bordignon, ESGT annnual meeting, October 2000, Stockholm

2.1.2. The need of efficient gene delivery systems

Conceptually, gene therapy involves the identification of a disease to cure and its related potential genetic target. The subsequent steps are the identification of the appropriate cell types to treat and the development of a suitable way to get enough of the therapeutic genetic material specifically into these cells. In practice, considerable obstacles have emerged, mostly caused by the inefficacy of current gene delivery systems. All of the current gene transfer systems - also called *vectors* - have some limitation. The "perfect" vector does not exist yet and will probably never exist (see Figure 1). The choice of the vector is often dictated by the aim of the treatment. If the expression of a gene is required for only a short time (e.g., expression of a toxic protein in cancer cells), then the adenoviral vectors are ideal. If a sustained expression is needed (such as in most genetic diseases), then integrating vectors such as retroviruses or adeno-associated viruses (AAV) are suitable candidates. If a vaccination against a particular antigen is required, then no particular vector is required and the injection of naked plasmid DNA may be sufficient.

An "ideal" vector may have properties of both viral and synthetic gene transfer systems, and it should include:

- the ability to transfer genetic material with high efficiency
- the capacity to target the desired type of cells (specificity)
- the ability to integrate in a site-specific location in the host chromosome, or to be successfully maintained as a stable episome (persistence)
- the ability to target non-dividing cells (somatic cells)
- no components that elicit an immune response
- an unlimited packaging size
- convenience, reproducibility and biosafety compliance of production
- high concentration, allowing many cells to be infected

---

[10] M.A. Kay, Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector, *Nature Genetics* **24**, 257 (2000)

[11] M. Isner *et al.*, Therapeutic angiogenesis for heart failure, *Nature Medicine* **5**, 491-492 (1999)

[12] I. Baumgartner, Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia, *Circulation* **97,** 1114-1123 (1998)

[13] A.S. Lewin, Ribozyme rescue of photoreceptor cells in a transgenic rat model of autosomal dominant retinitis pigmentosa, *Nature Medicine* **4,** 967-970 (1998)

[14] R.J. Bartlett, In vivo targeted ripair of a point mutation in the canine dystrophin gene by a chimeric RNA/DNA oligonucleotide, *Nature Biotechnology* **18**, 615-622 (2000)

[15] Morral *et al.*, Administration of helper-dependent adenoviral vector and sequential delivery of different vector serotype for long-term liver directed gene transfer in baboons, *PNAS*, **22**, 12816-21 (1999)

- an inducible transcriptional unit for regulated gene expression.

Although such a vector isn't available, all of these properties exist, individually, in disparate delivery systems. The features of some of the most common gene transfer vectors are listed in Table 1.

Table 1 does not include the most innovative viral vectors. Among these, particularly interesting is the possibility to combine the advantages of two independent viruses in one single vector. As an example, hybrid viral vectors that combine the great gene transfer efficiency of adenoviruses and the integration capacity of retroviruses have been proposed[16]. Similarly, an hybrid HSV-AAV vector, that combines the large packaging capacity of HSV and the integration ability of AAV has also been proposed[17]. Other revolutionary viral systems are the so-called "gutless" (or helper-dependent) adenoviral vectors. These adenoviruses are devoided of all viral coding sequences and are therefore uncapable to express any viral protein. A recent report described their efficacy after liver-directed gene transfer in baboons[18]. The authors reported long-term expression (longer than a year!) and reduced immunogenicity and toxicity. By looking at the rapid evolution of adenoviral vectors, we can envisage similar improvements for similar gene transfer systems.

The new generation of viral vectors is probably still at its primitive stage. However, these innovations will probably enable vectors to stay competitive for certain *in vivo* treatments. In fact, new therapeutical procedures such as *stem cell therapy* are rapidly emerging. If the expectations related to stem cells will become reality, soon their *ex vivo* cultivation and their reimplantation will enable the regeneration of various tissues. In presence of a genetic disorder, the corresponding correction could be easily obtained with conventional (inefficient!) gene transfection systems and subsequent selection of pre-characterized recombinant cell clones. The most optimists even envisage the reconstruction of entire organs starting from a population of explanted bone marrow cells. Stem cells are such powerful tools that risk to render obsolete most of the efforts undertaken to obtain high efficiency gene delivery vectors. Those latter, would be required only for treatments that do not aim the use of stem cells, such as acute treatments (e.g., organ transplantations, infections) and correction of gain-of-function disorders (e.g., cancers, auto-immune disorders).

2.1.3. Viral vs. nonviral vectors

Vectors for gene therapy applications generally fall into two categories: viral and nonviral. They primarily differ in their assembling process. In fact, viral vectors are produced in cells, whereas nonviral vectors are assembled in a test tube. Viral vectors are generally replication defective viruses with part or even all of the viral coding sequence replaced by that of therapeutic genes. The major use of viral vectors for human gene therapy applications is mostly due to their superior gene transfer efficiency (80-99% of targeted cells express the transgene), if compared to that of nonviral vectors (0.5-20%). Other features that make them the vectors of choice for clinical applications are their ability to integrate in the host genome (retroviral and AAV vectors) and their capacity to persistently express the transgene (lentiviruses, AAV and HSV). However, humans have an immune system able to fight off viruses, and the attempts to deliver genes in viral vectors have been confronted by these host responses. Other problems of viral vectors include insufficient pharmaceutical quantities (low viral titers), toxicity, and the potential emergence of replication-competent viruses. The features of the major viral vectors and a direct comparison with nonviral vectors are reported in Table 1 (see 2.1.2.). Further important aspects of gene transfer vectors are extensively discussed in Paper 2 (see 3.2.).

---

[16] Feng *et al*, Stable in vivo gene transduction via a novel adenoviral/retroviral chimeric vector, *Nature Biotechnology*, **15**, 866-870 (1997)

[17] Fraefel *et al.*, Gene transfer into hepatocytes mediated by helper virus-free HSV/AAV hybrid vectors, *Mol Med*, **3**, 813-825, (1997)

[18] Morral *et al.*, Administration of helper-dependent adenoviral vector and sequential delivery of different vector serotype for long-term liver directed gene transfer in baboons, *PNAS*, **22**, 12816-21 (1999)

VECTOR	**Retroviral**	**Adenoviral**	**AAV**	**HSV**	**Non-viral**
Genome type and size	ssRNA 9 kb	dsDNA 36 kb	ssDNA 5 kb	dsDNA 150 kb	dsDNA from plasmids to BACs
Maximum insert size	7 - 7.5 kb	30 kb	3.5 - 4 kb	15 -150 kb	no limitation
Concentration (partcles/ml)	low titer 10^6	high titer 10^{10}	high titer 10^{11}	high titer 10^{10}	no limitation
Integration	yes	no	yes	no	"no"
Duration of expression in vivo	"short" (long in lentiviruses)	"short" (long in non-replicative viruses)	long	long (mostly in neurons)	short
Immunological problems	few	yes	few	yes	"no"
Safety problems	insertional mutagenesis	inflammatory response, toxicity	insertional mutagenesis, low toxicity	cytotoxicity	no
Targeting of non-dividing cells	only lentiviruses	yes	yes	yes	very inefficient
Gene transfer efficiency	high	high	high	high	low

TABLE 1. Comparison of properties of various vector systems. Abbreviations: AAV, adeno-associated virus; HSV, herpes simplex virus; ssRNA, single stranded RNA; dsDNA, double stranded DNA; ssDNA, single stranded DNA; kb, kilobases; BAC, bacterial artificial chromosome.

2.2. NONVIRAL VECTORS FOR GENE THERAPY

2.2.1. Brief desciption of current nonviral vectors

The drawbacks of viral vectors (see 2.1.3.) make synthetic vectors an attractive alternative. Followings are the advantages of nonviral vectors:

- nonimmunogenicity
- low acute toxicity
- assembled for defined components
- convenience of production on a large scale.

However, nonviral vectors also have important disadvantages, including their low gene transfer efficiency and their transient gene expression. Many types of nonviral vectors have been proposed. In the current chapter I will briefly summarize the most common nonviral delivery systems.

2.2.1.1. Naked DNA

Ten years ago Wolff and co-workers made a key discovery that led to an increased interest in nonviral gene transfer technologies[19]. These investigators were the first to show that muscle tissues could absorb intramuscular injected naked plasmids, leading to expression of the encoded protein persisiting for periods of weeks to several months. The original gene transfer efficiencies were quite satisfactory (5% of targeted cells expressing the transgene). However, higher expression levels were recently achieved by delivering naked DNA intravascularly under high pressure[20] or intratumourally via electrically enhanced transfer[21]. Injection of naked DNA is indicated for all the treatments that do not need persistent transgene expression[22]. Therapeutic expression levels were indeed obtained after intratumoral injection. The most encouraging clinical results (among the first achieved in human gene therapy!) were obtained in the treatment of limb ischemia after intramuscular injection of plasmid encoding VEGF[23]. Because of the semplicity and the relative low costs of the approach, injections of DNA encoding antigens from infectious organisms are currently investigated for immunization purposes. DNA-based vaccinations have indeed a great potential in the prevention of infectious diseases (influenza, HIV, malaria, hepatitis B) and cancer.

2.2.1.2. Gene gun

This physical method enables to shoot DNA into cells via biolistic bombardment. The gene gun uses gold or tungsten particles coated with DNA. An electric high-voltage discharge or a sudden pressure accelerate the DNA-coated particles to high velocity, enhabling efficient penetration of single cell layers *in vitro* or target organs *in*

---

[19] Wolff *et al.*, Direct gene transfer into mouse muscle in vivo, *Science* **247**, 1465-1468 (1990)

[20] Budker *et al.*, The efficient expression of intravascularly delivered DNA in rat muscle, *Gene Therapy* **5**, 272-276 (1998)

[21] Wells *et al.*, Electroporation enhanced gene delivery in mammary tumours, *Gene Therapy* **7**, 541-547 (2000)

[22] For a recent review see: Jon A. Wolff, *Naked DNA gene transfer in mammalian cells,* book chapter in *The development of human gene therapy,* Cold Spring Harbor Laboratory Press (1999)

[23] Baumgartner *et al.*, Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia, *Circulation* **97**, 1114-1123 (1998)

vivo. Although the DNA transfer is relatively efficient, the major drawback of this system is that the target tissues must be surgically exposed.

2.2.1.3. Lipoplex

Lipoplexes are complexes formed by the interaction of cationic liposomes and DNA[24]. Liposomes generally consist of a mixtures of positively charged lipids (cationic lipids, e.g.: DOTMA) and helper lipids (colipid, e.g.: DOPE). The cationic lipid-mediated transfection is also called *lipofection*. Lipoplexes are capable of delivering functional nucleic acid molecules into cultured cells with relatively high efficiency. Cationic-liposome formulations have three properties that are particularly important for efficient gene delivery. First, liposomes spontaneosly condense with DNA to form a complex in which up to 100 % of the DNA is entrapped. This high entrapment efficiency is not limited by the size of the DNA. Second, all biological surfaces, including cultured cell surfaces, carry a net negative charge. Consequently, positively charged lipid vescicles interact spontaneously with the negatively charged cell surfaces. Therefore, the liposomes-entrapped genetic material is co-delivered to the cell surface in a condensed form. Finally, cationic lipids fuse with cell membranes in a manner that allows the entrapped DNA to enter the cytoplasm and escape the degradative lysosomal pathway. Several reports described the ability of cationic lipids to deliver genes also *in vivo*. The obtained results pushed several gene therapists to test this gene transfer approach in humans. Cationic lipid-based delivery systems have indeed been evaluated in phase I and phase II clinical trials for the treatment of a variety of different types of human cancer and for the treatment of cystic fibrosis.

2.2.1.4. Polyplex

Polyplexes are complexes formed by the interaction of hydrophlic cationic polymeres and DNA[25]. The polycations-mediated transfection is also called *polyfection*. The hydrophhilic polymers are of two general types: the linear polymers, such as polylysine and spermine, and the branched chain, spherical, or globular polycations such as polyethyleneimine (PEI) or dendrimers. An active area of scientific research involves understanding and controlling the DNA condensation and packaging processes with these agents and determing the structure of the complexes. The cationic polymer-based systems have been most widely associated with the generation of receptor-mediated gene delivery systems (see 2.2.1.6).

2.2.1.5. Lipopolyplex

Lipopolyplexes are obtained by incorporating polylysine (see 2.2.1.4.) into lipoplex (see 2.2.1.3.) to form ternary complexes. In lipopolyplexes DNA is more tightly condensed preventing the complex from further aggregation and nuclease degradation. Cationic and anionic lipopolyplexes called LPDI and LPDII, respectively, have been formulated and shown to be effective in gene transfer.

---

[24] For a recent review see: Philip L. Felgner, *Advances in synthetic gene-delivery systems technology,* book chapter in *The development of human gene therapy,* Cold Spring Harbor Laboratory Press (1999)

[25] For a recent review see: A.Kichler *et al.*, *Polyethylenimines: a family of potent polymers for nucleic acid delivery*, book chapter in *Nonviral vectors for gene therapy,* Academic Press (1999)

2.2.1.6. Receptor-targeted gene transfer (RTGT)

Many cell surface receptors recognize and bind specific extracellular ligands with high affinity and in a specific mode. Ligands can be proteins, peptides, carbohydrates, vitamins and antibodies. Some of these have been incorporated into gene transfer systems for two major reasons: (a) to target specific cell types and (b) to enhance intracellular uptake after binding to target cells[26]. Ligands have been linked either directly to DNA, to lipoplexes (see 2.2.1.3) or to polyplexes (2.2.1.4). Similarly, the cell-binding domains of viral vectors have been modified to alter or broaden the target cell specificity (change the viral tropism).

RTGT systems generally contain three molecular elements: (a) the DNA, (b) a DNA-binding polycation and (c) the targeting ligand which is covalently linked to the polycation. Additional elements can be incorporated that modify the properties of the complex, promoting intracellular release, nuclear targeting or persistence of the introduced transgene (see 2.2.1.7.). Wu and co-workers linked the ligand asialoorosomucoid to the DNA condesing compound polylysine. This conjugate could deliver DNA to hepatocytes, by targeting the cell-specific asialoglycoprotein (ASGP) receptor[27]. Another ligand that has been linked to different polycations was transferrin, taking advantage of the ubiquitously expressed transferrin receptor[28]. Tranferrin-mediated gene transfer has been called *transferrinfection*. Similar observations were made with a variety of other receptor-ligand systems, demonstrating the feasibility of the RTGT approach.

Different *in vivo* applications of RTGT have been tested, ranging from direct injection into the target tissue to systemic delivery. Local injection of naked or complexed DNA directly into tumors resulted in significant reporter gene expression, with DNA-transferrin-PEI complexes or adenovirus-linked DNA-transferrin-polylysine complexes being 10- to 100-fold more efficient than naked DNA[29]. Systemic delivery of a reporter gene complexed to a galactose-modified polylysine resulted in prolonged expression in rat hepatocytes[30]. This observation demonstrated the ASGP receptor could be also targeted *in vivo.* The current RTGT systems are very promising, but still need to be improved. Better gene transfer efficacies could be achieved by incorporating additive biological elements that would help to overcome further extracellular and intracellular barriers (see 2.2.2.).

2.2.1.7. Hybrid viral / nonviral vectors

Some new gene transfer systems have combined the advantages of both viral and nonviral vectors. A report described the capability of adenovirus to promote the gene transfer of transferrin-polylysine conjugates[31] (see 2.2.1.6.). Adenovirus is indeed able to disrupt the endosomal membrane, facilitating the release of DNA into the cytoplasm and avoiding its lysosomal degradation. A further report descibed the construction of a virus-like gene-transfer vehicle (also called *artificial virus*), avoiding the addition of intact viral particles. In fact, Wagner and co-workers augmented the gene transfer efficiency of transferrin-polylysine-DNA complexes (see 2.2.1.6.) by adding an influenza virus hemagglutinin HA-2 N-terminal fusogenic peptide[32]. These DNA complexes contained polylysine as nucleic acid packaging module (see 2.2.1.4.), transferrin as receptor binding module (see 2.2.1.6.) and influenza peptides as endosomolytic module. Together with hybrid viral vectors (see 2.1.2.), artificial viruses have the potential to become the DNA transfer tools of choice for future gene therapists.

---

[26] For a recent review see: Cotten and Wagner, *Receptor-mediated gene delivery strategies,* book chapter in *The development of human gene therapy,* Cold Spring Harbor Laboratory Press (1999)

[27] Wu *et al.,* Receptor-mediated in vitro gene transformation by a soluble DNA carrier system, *J.Biol.Chem.* **262**, 4429-4432 (1987)

[28] Wagner *et al.*, Transferrin-polycation conjugates as carriers for DNA uptake into cells, *PNAS* **87**, 3410-3414 (1990)

[29] Kircheis *et al.*, Polycation-based DNA complexes for tumor-targeted gene delivery *in vivo*, *J.Gene Med* (1999)

[30] Perales *et al.*, Gene transfer *in vivo*: sustained expression and regulation of genes introduced into liver by receptor-targeted uptake, *PNAS* **91**, 4086-4090 (1994)

2.2.1.8. DNA nanospheres

Nanospheres are particles formed by the complexation of DNA with gelatin or chitosan[33]. Gelatin, the denatured form of collagen, is a polyampholyte that gels below 35-40°C. Chitosan is a natural and biodegradable polycationic linear polysaccharide. In addition to the benefits common to other nonviral gene delivery systems, characteritics unique to nanospheres inlcude coencapsulation of bioactive reagent and sustained release of DNA. The former raises the possibility of combining drug and gene therapy in one single vehicle, and the latter may improve the tissue bioavailability of DNA. In recent studies[34], similar general properties were found for chitosan and PEI (see 2.2.1.4.). Both formed stable complexes with plasmid DNA, having a diameter of approximatively 120nm and a positive net charge (positive zeta potential). Transfection experiments in cultured cells resulted in similar reporter activities for the two systems. However, *in vivo* PEI mediated 10-fold higher expression levels than chitosan.

The efficacy of nanospheres has been tested in further experiments. In animal models, positive gene transfer has been observed in the lung, muscle and gastrointestinal tissues. Therein, the measured reporter activities were relatively low. Therefore, nanospheres are particularly suitable for applications that do not require high gene expression levels, such as DNA vaccinations. Nanospheres-protected DNA vaccines could be more advantageous than simple injections of naked DNA (see 2.2.1.1.). In fact, free DNA is subject to extracellular degradation and tissue clearance. A local and sustained delivery of DNA should increase the fraction of DNA taken up by the target cell or tissue. Targeting could be obtained by attaching ligands externally to nanospheres. As a matter of fact, transferrin (see 2.2.1.6.) has been already successfully linked to chitosan-DNA complexes. Another type of nanosphere-based immunization that is particularly promising is the oral administration of food allergenes. In a recent report, the oral delivery of the dominant peanut allergen gene provided immunologic protection and reduced anaphylaxis in a murine model of peanut allergy[35]. Therefore, this simple immunoprohylactic strategy could become the treatment of choice for the often fatal food allergies, that are very common and currently without an effective treatment.

---

[31] Curiel *et al.*, Adenovirus enhancement of trasferrin-polylysine-mediated gene delivery, *PNAS*, **88**, 8850-8854 (1991)

[32] Wagner *et al.*, Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle, *PNAS* **89**, 7934-7938 (1992)

[33] For a recent review see: Kam W. Leong., *Biopolymer-DNA nanospheres*, book chapter in *Nonviral vectors for gene therapy,* Academic Press (1999)

[34] Holly Guan, *Chitosans as gene delivery systems,* Monte Verità Workshop on *Gene and oligonucleotide delivery of therapeutic and vaccines,* 18-23 April 1999

2.2.2. Barriers that a vector must overcome

The *in vivo* applications of nonviral vectors remain limited mainly because of their low gene transfer efficiency. The obstacles to efficient gene delivery and expression can be described in terms of "barriers". In the current chapter I will focus on the currently identified extracellular and intracellular barriers.

2.2.2.1. Extracellular barriers

The extracellular barriers are the obstacles that the delivered genetic material encounters before it reaches its target cell. They are particularly important for *in vivo* gene transfer procedures. The main barriers are:

- opsonins
- phagocytic cells
- extracellular matrix
- degradative enzymes
- other anatomical barriers

Opsonins are serum proteins (complement system, neutralizing antibodies) that stick to the vector, inactivating the transgene and its carrier. *Phagocytes* are cells capable of engulfing and actively digesting the invading particles. *Extracellular matrices* are polymerized proteins and carbohydrates that are present between cells protecting plasma membranes. These matrices are particularly difficult to overcome for large DNA vehicles. The extracellular fluid is rich in *DNases* that can rapidly digest unprotected DNA. Finally, examples of *anatomical barriers* are the skin and the blood-brain barrier.

2.2.2.2. Intracellular barriers

The intracellular barriers are the obstacles that a delivered transgene encounters once it reaches its target cell. They are important for both *in vitro* (cultured cells) and *in vivo* gene transfer procedures. By overcoming these barriers, the vectors will finally enable the transgene to enter the nucleus in order to be transcribed. The main intracellular barriers are:

- the plasma membrane
- the cytoplasm
- the nuclear envelope.

Current nonviral vectors are generally designed to allow the genetic material to cross the *plasma membrane* with high efficiency. However only a small portion of the delivered nucleic acids undergoes nuclear translocation.[36] *Cytosolic obstacles* to nuclear accumulation are the entrapment of DNA in the endolysosomal compartment, the degradation of DNA by nucleases and the DNA sequestration by still uncharacterized cellular structures. However, the major intracellular barrier for successful gene transfer is represented by the *nuclear envelope* (see 2.2.3.).

---

[35] Roy *et al.*, Oral gene delivery with chitosan-DNA nanoparticles generates immunologic protection in a murine model of peanut allergy, *Nature Medicine* **5** (4), 1999

[36] Zabner *et al.*, Cellular and molecular barriers to gene transfer by a cationic lipid, *J Biol Chem* **270,** 18997-9007 (1995)

2.2.3. Nuclear transport of exogenous DNA

2.2.3.1. Nucleocytoplasmic transport

There is a very dense traffic across the nuclear membrane. Every minute several millions RNA and protein molecules are transported into the nucleus and vice versa. This intense traffic is regulated by special transport factors that are called importins and exportins. These factors are continously shuttling between the cytoplasm and the nucleus, passing through the Nuclear Pore Complex (NPC), a large protein structure embedded in the nuclear envelope[37]. The NPC contains a central channel with a diameter of 9 nm that allows free diffusion of small proteins (<50 kD). Larger particles or molecules (up to 25 nm) can be transported only in presence of appropriate signatures, such as the Nuclear Localization Signals (NLS). One of the first NLS was identified in the SV40 large-antigen and has the sequence PKKKRKV. Such NLS is first recognized by importins in the cytoplasm. The formed complex is then transported into the nucleus via an energy-dependent reaction. Because nondividing cells are poorly transfectable by nonviral vectors and transfection particles generally have a diameter larger than 25 nm, it was postulated that nuclear uptake occurs preferentially in cells that are entering mitosis, consequently to breakdown of the nuclear envelope[38].

2.2.3.2. The DNA nuclear import is inefficient

Already in 1980 the nuclear import step has been recognized to be a major barrier for gene expression. In a pioneering experiment, Capecchi and co-workers reported that 50-100 % of thymidine kinase (TK)-deficient mouse fibroblast cells expressed TK, after having injected a TK encoding plasmid into their nuclei. In contrast, cytoplasmic injection of over 1000 cells did not result in any detectable TK activity[39]. Further reports confirmed this result in other cell lines. However, in 1995 the laboratory of Jon Wolff surprisingly reported that cytoplasmically injected plasmid DNA can enter postmitotic and thereby intact nuclei[40]. In the same report there were even some evidences about the ability of plasmids to enter nuclei through the NPC. How does a 5 kilobases large plasmid with a molecular weight of more than 3 megadaltons enter the NPC? Such a plasmid would have a diameter of at least 100nm in its compacted globular form[41]. Therefore, very likely the plasmid is struggled through the NPC in its unwinded decondensed form by a still unknown process. Since the plasmid nuclear entry is restricted to a few somatic cell types, it could be that in these cells particular shuttling factors (import machinery components?, transcription factors?) stick to DNA facilitating its nuclear transport. It could also be, that under certain physiological conditions these cells have more permessive nuclear pores, that enable the entry of DNA into the nuclei.

---

[37] For a recent review see: Nigg, E.A., Nucleocytoplasmic transport: signals, mechanisms and regulation, *Nature* **386**, 779-787 (1997)

[38] Wilke *et al.*, Efficacy of a peptide-based gene delivery systems depends on mitotic activity, *Gene Therapy* **3**, 1133-1142 (1996)

[39] Capecchi, M.R., High efficiency transformation by direct microinjection of DNA into cultured mammalian cells, *Cell* **22,** 479-488 (1980)

[40] Dowty *et al.*, Plasmid DNA entry into postmitotic nuclei of primary rat myotubes, *PNAS* **92**, 4572-4576 (1995)

[41] For a recent review see: Lasic Dan D.., *Structure and structure-activity relationships of lipid-based gene delivery systems*, book chapter in *Nonviral vectors for gene therapy,* Academic Press (1999)

Also the necessary steps that enable transfected DNA to reach the nucleus are not yet understood. In particular, is still unknow where and when DNA is released from its carrier (e.g., cationic lipids, polylysine, PEI). In the cytoplasm of cultured cells, lipid-DNA complexes have dimensions of 400-800 nm with 5 to 20 plasmids complexed in a single lipid-DNA particle. Obviously, for entering nuclear pores, plasmids have to be released from those huge complexes. However, a recent report described the detection of 500nm large PEI-DNA complexes in the nuclei of non-dividing cells, indicating that in some cases complexed plasmids can enter the nucleus[42]. Understanding the DNA nuclear import mechanism in detail is of primary importance and would probably allow the design of more efficient nonviral vectors.

2.2.3.3. NLS-mediated gene transfer

As previously discussed (see 2.2.2.2.), the nuclear envelope has been identified to be the major barrier for the gene transfer of foreign DNA. One of the most promising approaches that has been recently proposed to overcome this barrier, is the covalent linkage of NLS peptides (see 2.2.3.1.) to plasmid DNA. A recent report described indeed a nuclear accumulation of NLS-conjugated DNA fragments in digitonized cells (see 2.4.4.)[43]. A significative enhancement of expression of NLS-coupled transgenes has been also recently reported[44]. Each report described a different strategy for linking NLS to DNA. These strategies will be extensively described and discussed in Paper 1 (see 3.1.) and in chapters 2.4.3 and 2.4.4.

2.2.3.4. Viral strategies for DNA nuclear transport

Viruses employ a variety of strategies to enter the nuclear pore[45]. Deciphering these strategies and understanding the differences between naked and viral DNA transport could give new clues to manipulate the cytoplasmic and nuclear transport steps in many nonviral gene delivery protocols. In the current chapter I will briefly discuss some general features of viral nuclear import. I will also mention few selected nuclear transport strategies adopted by some virus families and by some plant pathogens.

Both Adenoviruses and Herpes Simplex Viruses are able to efficiently infect non-dividing cells[46]. To this purpose, they have evolved some common strategies to transport their large genomes (36 and 150 kb, respectively) into the nucleus of host cells: (1) use microtubule-associated motor mechanisms to move towards the nucleus, (2) associate to NLS-carrying proteins for nuclear targeting, (3) target the NPC in a protected, wrapped form, (4) transport the genome in a linear form for efficient transport trough the NPC. Some of these strategies should be adopted in the future for the development of better nonviral vectors. As a matter of fact, a recent report described a 1000-fold enhancement of transfection obtained with linearized NLS-coupled transgenes (see 2.4.3.).

---

[42] Labat-Moleur *et al.*, An electron microscopy study into the mechanism of gene transfer with lipopolyamines, *Gene Therapy* **3**, 1010-1017 (1996)

[43] Sebestyén *et al.*, DNA vector chemistry: the covalent attachment of signal peptides to plasmid DNA, *Nature Biotechnology* **16**, 80-85 (1998)

[44] (1) Zanta *et al.,* Gene delivery: a single nuclear localition signal peptide is sufficient to carry DNA to the cell nucleus, *PNAS* **96**, 91-96 (1999); (2) Branden *et al.,* A peptide nucleic acid-nuclear localization signal fusion that mediates nuclear transport of DNA, *Nature Biotechnology* **17**, 784-787 (1999)

[45] For a recent review see: Whittaker G.R. and Helenius A., Nuclear import and export of viruses and virus genomes, *Virology* **246**, 1-23 (1998)

[46] For a recent review see: Sebestyén, M.G. and Wolff, J.A., *Nuclear transport of exogenous DNA*, book chapter in *Nonviral vectors for gene therapy,* Academic Press (1999)

Another intriguing nuclear import strategy present in nature is the one adopted by the plant pathogen *Agrobacterium tumefaciens*. The *Agrobacterium* single-stranded DNA intermediate T-strand is composed of a complex (12.6 nm x 3.6 μm) with a single VirD2 peptide at its 5'-end and multiple VirE2 peptides along its length. Both VirD2 and VirE2 contain NLSs, and it is thought that the DNA docks to the NPC via the VirD2 peptide, at which point transport is initiated. The multiple VirE2 peptides then mediate the translocation of the 20 kb large DNA fragment through the NPC. One day components of this extremely efficient plant pathogen may be mixed with elements of viral gene delivery systems to create an optimal virus-like gene transfer vehicle (see 2.2.1.7.). As a matter of fact, a DNA fragment complexed to VirD2 and VirE2 has been already successfully imported into the nucleus of mammalian cells[47].

---

[47] Ziemienowicz *et al.*, Import of DNA into mammalian nuclei by proteins originating from a plant pathogenic bacterium, *PNAS* **96**, 3729-33 (1999)

2.3. STEROID MEDIATED GENE DELIVERY (SMGD)

2.3.1. Steroid Hormone Receptors

Nuclear Receptors[48], such as the receptors for steroids and thyroid hormones, retinoids and vitamin D3, are ligand-inducible transcription factors present in vertebrates, arthropods and nematodes. They regulate complex physiological events that trigger key steps during development, control maintenance and homeostasis, and induce or inhibit cellular proliferation, differentiation or death. To date, more than 150 different proteins have been identified as members of the nuclear receptor superfamily, which is often further divided into steroid and nonsteroid (thyroid/retinoid/vitamin D) receptor families. Steroid hormone receptors (SHRs) include the Glucocorticoid Receptor, the Mineralocorticoid Receptors, the Progesterone Receptor, the Androgen Receptor and the Estrogen Receptor. SHRs exert their influence during embryonic development and in adult homeostasis as hormone-activated transcriptional regulators. Their modular structure, consisting of a DNA binding domain (DBD), nuclear localization signals, a ligand binding domain (LBD) and several transcriptional activation functions (AFs), is conserved with other members of the nuclear receptor superfamily. Unique to the SHRs is their ability upon activation to bind to palindromic DNA sequences, called hormone response elements (HREs), exclusively as homodimers.

In absence of hormones, SHRs are generally localized in the cytoplasm associated with a large multiprotein complex of chaperones which maintains the receptors in an inactive but ligand-accessible conformation. Upon ligand binding, the receptors become conformationally active. Activated SHRs dissociate from the chaperones, translocate into the nucleus, bind to specific HREs and interact with components of the basal transcriptional machinery and with sequence-specific transcription factors. Is not so clear whether dissociated unligated SHRs are then exported from the nucleus in the cytoplasm. The shuttling of the receptors between the cytoplasm and the nucleus comprises an equilibrium which in presence of steroids is shifted towards the nucleus.

2.3.2. The principle of SMGD: Nuclear Receptors as vehicles for DNA

One drawback of nonviral vectors is that they do not allow DNA to be transported into the nucleus efficiently (see 2.1.3. and 2.2.3.2.). As discussed above (see 2.3.1.), SHRs are continously shuttling between the cytoplasm and the nucleus. Therefore, SHRs can be considered as natural ferries for steroid molecules. A question which consequently arises is whether the shuttling propriety of the SHRs could be exploited to improve the nuclear uptake of DNA. In other words, if a DNA molecule could be attached to a steroid receptor, then the resulting DNA/SHR complex should be transported into the nucleus.

The initial idea soon gave rise to a potential new gene transfer technology which was called Steroid Mediated Gene Delivery (SMGD). The concept of the SMGD approach is illustrated in Figure 2.

Another factor limiting the efficacy of gene transfer vectors is the inability to target genes specifically to a desired cell type. Most targeting techniques developed to date enable

---

[48] For a comprehensive review see: Mangelsdorf *et al.,* The Nuclear Receptor Superfamily: The Second Decade. *Cell* **83**, 835-877 (1995).

DNA to be internalized into the cytoplasm of the desired cell via specific surface ligand docking (see 2.2.1.6.). A strategy which has been neglected is the possibility of specifically transferring therapeutic genes into the nucleus of particular cell types, regardless of how the DNA is delivered to the cells. In this context, the SMGD approach could significantly improve the specificity of nonviral vectors. In fact, the final general aim of SMGD is to offer a selective advantage to the therapeutic treatment of cells that express a specific steroid receptor. Examples of possible targets are discussed in chapter 2.4.5.

2.3.3. The initial purposes of SMGD

The first nuclear receptor mentioned in the context of SMGD was the Mineralocorticoid Receptor (MR). The original application of SMGD in humans was in the context of a very rare illness called Apparent Mineralocorticoid Access (AME). Patients affected by AME suffer from hypertension, hypokalemia and sodium retention. This illness is a monogenic disease and is characterized by a defect in the gene encoding 11β-hydroxysteroid dehydrogenase[49] (11β-OHSD). The steroid hormones Cortisol and Aldosterone have an equal affinity for MR. 11β-OHSD inactivates Cortisol by conversion to Cortisone conferring to MR specificity for Aldosterone. This biochemical reaction is only relevant in mineralocorticoid target tissues, especially in distal kidney tubular cells. Thus, targeting 11β-OHSD to those cells has the potential to cure patients affected by AME. For that purpose, those cells would be the suitable target for the selective gene delivery of 11β-OHSD. In this context, the SMGD strategy could be applied to achieve a selective transfer of 11β-OHSD only in MR-positive distal kidney tubular cells and not in the MR-negative surronding cells. The success of the applied gene therapy treatment could be easily monitored by following urinary potassium excretion and sodium retention with increasing blood pressure and the suppression of renin and aldosterone.

Because of SMGD's initial purposes, the first months of my PhD studies were devoted to the identification of genomic clones containing the 11β-OHSD gene. For this purpose, I designed the necessary detection probes which were used to screen mouse and human P1 genomic libraries[50]. P1 clones are derived from P1 bacteriophages and can have DNA inserts of 100-110 kb. The final aim was to identify clones which spanned the whole locus surrounding the 6.2 kb 11β-OHSD gene. The use of large genomic segments was thought to be instrumental in view of the final clinical approach, that was the transfer of the whole locus in the target cells. The presence of the 11β-OHSD's regulatory sequences would have increased the chance of a correct long-term expression of the therapeutic protein. Luckily, the library screening resulted in the detection of three mouse and two human positive clones.

As discussed later (see 2.4.5.), the initial purposes of SMGD changed during the time. In fact, althought AME would have been a valid model to test the feasibility of the SMGD approach in humans, it had the big drawback of being an illness readily curable with conventional therapies. Furthermore the incidence of AME is extremely low with less than one hundred affected individuals worldwide. This brought into question the economic feasibility and clinical relevance of developing a novel therapeutic strategy for this particular illness.

---

[49] The research team of our collaborator Prof. Felix Frey has for many years studied the kinetics and the dynamics of 11β-OH-glucocorticoids.

[50] The P1 library screenings were performed by the company Genomic Systems Ltd.

2.3.4. Modelling the SMGD with the Glucocorticoid Receptor (GR)

Before starting to think in terms of a clinical application, the initial step was to demonstrate the overall feasability of the SMGD approach. Although other nuclear receptors could have been chosen, the SMGD was modelled with the well characterized Glucocorticoid Receptor (GR) system, because the necessary expression vectors, receptor mutants, commercial ligands for derivatization and the necessary know-how[51] were available. Of particular importance was the availability of chimeric GR-GFP proteins. GFP (green fluorescent protein) is a polypeptide that has intrinsic fluorescence when activated by UV-light and can be easily followed intracellularly by fluorescence microscopy . The cellular localization of the GR-GFP chimera in presence or absence of ligands has been previously studied in Ruscolab, confirming the previously discussed shuttling propriety of the Glucocorticoid Receptor[52]. The simplicity of the developed assay (no requirements of specific staining or sequential antibody incubation as in the case of epitope-tagged proteins) was extremely useful in testing the nuclear translocation potential of the synthesized steroid derivatives (see also 2.3.5., 2.3.7. and 3.1.).

Because of SMGD's initial application using the Mineralocorticoid Receptor (see 2.3.3.) and because of conflicting reports regarding its shuttling behaviour, I cloned GFP C-terminally of MR and I studied the cellular localization of the resulting MR-GFP chimera. Surprisingly, I was unable to observe any shuttling of the chimeric protein. In absence and in presence of Aldosterone, MR-GFP was localized in both the cytoplasm and the nucleus. Only two years later a report appeared[53] in which the shuttling of a GFP-MR chimeric protein (GFP cloned N-terminally of MR) was clearly described. Unfortunately, in that report the construction of a MR-GFP chimera (GFP cloned C-terminally) has not been neither described nor discussed. However, from my results it is possible to speculate that the C-terminally positioned GFP prevents the MR from interacting correctly with the ligand or with the nuclear import and/or export machinery. This faulty interaction would disable the MR-GFP chimera from shuttling correctly. Interestingly, the shuttling behaviour of GR can be observed with GFP positioned either C- or N-terminally.

2.3.5. The "first generation" of steroid derivatives

To model the SMGD, we first had to design and synthesize the necessary molecules for the steroid-decoration of plasmid DNA (see 2.3.2. for SMGD's principle). In particular, we had to develop special bifunctional steroid derivatives composed of a steroid molecule linked via a chemical spacer to a DNA-interacting compound. For each of these components, we had to take into account a multitude of variables for the design of the final steroid derivatives. Some of these variables are listed in Figure 3.

---

[51] The GR has been widely studied by Prof. Sandro Rusconi who became involved in the cloning and functional characterization of that protein during his postdoctoral studies. He continued this work when he returned to Zürich and is pursuing these studies in his current position in Fribourg.

[52] Andrea Nawrocky, "Construction of fluorescent GR chimeras to study receptor localization in living cells", Diplomawork (1996) by Prof. Rusconi, Institute of Biochemistry, University of Fribourg

[53] Fejes-Tóth *et al.*, Subcellular localization of mineralocorticoid receptors in living cells: effects of receptor agonists and antagonists, *PNAS*, **95**, 2973-2978 (1998)

In this chapter, I will only mention the variables that are relevant for the understaning of my work. Other variables will be discussed by some of my collaborators in future publications[54].

The big challenge in derivatizing a ligand is to synthesize a molecule which still retains a significant affinity for its cognate receptor. In this context, the ideal situation for designing a steroid derivative would entail knowing the three-dimensional structure of the Ligand Binding Domain (LBD) of the corresponding Steroid Hormone Receptor (SHR). As discussed previously (see 2.3.4.), we aimed to model the SMGD with the Glucocorticoid Receptor (GR). Unfortunately, at that time no SHR LBD crystal structure was available. However, the LBD's crystal structure of both an unliganded (RXRα) and a liganded Nuclear Receptor (RARγ) had been recently solved. Furthermore, just before the beginning of the SMGD project, a report appeared in which a sequence alignment of all Nuclear Receptor LBD's had been performed[55]. Based on the available crystal structures and the results of the alignment, the authors were able to predict a common structure for the LBD of all nuclear receptors. The authors also proposed a model for the ligand-binding interaction of GR with Dexamethasone, a well know GR-agonist. According to that model, the hormone is totally wrapped around by polypeptide residues of the LBD, with the exception of a little "hole" through which the ligand can be approached from outside. We had the chance to get directly from the authors an electronic version of that model. Since we were convinced about its validity, we started to design our first steroid derivatives based on the modelled interaction. The most favourable positions for steroid derivatization seemed to be 4 and 6 (see Figure 4), since a chemical group attached to one of those positions would have sticked out of the LBD's "hole". With the help of experienced chemists[56], we started to investigate for possible chemical reactions for the linkage of a chemical spacer at those positions. According to the chemists opinion, all the possible reactions were particularly complicated requiring sophisticated reagents and apparatuses, difficult to find even in well- equipped chemistry laboratories. At that point, we seriously started to doubt the feasibilty of any derivatization at those chemical positions.

The steroid derivatization of other positions seemed to be less complicated. In particular, postion 21 (see Figure 4) seemed to be a good candidate, since several GR-ligands for derivatization at that particular position were available. In this context, a former collaborator[57] had the idea to chemically link the DNA-intercalating agent Ethidium Bromide at position 21 of the steroid molecule Cortisol-21-hemisuccinate. The resulting compound was named Cortisol-21-Ethidium (Cl-21-Et) and constitues the prototype of our "very first" generation of steroid derivatives[58]. As discussed later (see 2.3.6.), thanks to Cl-21-Et we got the first encouraging results which helped everybody to believe further in the SMGD approach.

---

[54] Bernasconi A. *et al.*, in preparation; Rebuffat *et al.*, in preparation.

[55] Wurtz J.-M. *et al.*, A canonical structure for the ligand-binding domain of nuclear receptors, *Nature Structural Biology* **3**, 87-94 (1998).

[56] Prof.Gossauer and Dr.Engel, Chemistry Department, University of Fribourg

[57] Dr. Ivo Galli, European Patent Office, The Netherlands

[58] The bifunctional molecules described and tested in the first paper (see 3.1.) were arbitrarily declared as "first generation" steroid derivatives. In reality, these molecules are members of a

Thanks to the previously described assay (see 2.3.4.), I observed that Cl-21-Et was able to induce nuclear translocation of GR. This observation was an indirect proof about the ability of GR to bind to Cl-21-Et. Although good news, this result was in contrast to the GRs ligand-binding interaction model. According to that model, a substituent attached to position 21 would point to the inner part of the LBD. In fact, a steroid derivatization at position 21 would almost have an opposite orientation with respect to subsituents linked the most favourable positions 4 and 6 (see Figure 4). The answer to these conflicting results appeared in a report which described the crystal structure of Estrogen Receptor's LBD, the first ever solved for a SHR[59]. In this structure, the positioning of the ligand was turned by 180° with respect to the model previously proposed. Several months later, the crystal structure of Progesterone Receptor's LBD confirmed the orientation of the hormone in the ligand-pocket[60], inducing us to definetely give up the idea to derivatize positions 4 and 6. Therefore, we continued to derivatized the steroids at position 21.

2.3.6. The first encouraging results

Cl-21-Et (see 2.3.5.) was the first steroid derivative that was tested in combination with other nonviral vectors. In a very crude experiment, Cl-21-Et was simply mixed with plasmid DNA carrying a reporter gene. The resulting steroid-DNA complex was then used for transfection experiments, using different gene transfer procedures. To measure a possible "enhancement of transfection", the expression levels of the steroid-complexed and uncomplexed DNA were compared in cycling cells which naturally expressed high levels of GR. We were very happy to observe that the steroid-DNA complex mediated three to four fold higher expression levels than uncomplexed DNA, using Calcium Phosphate as a transfection agent (see Figure 5). Similar enhancements of transfection were found by transfecting the cells with Polyethylenimine (see 2.2.1.4.), indicating that the observed effect was independent from the adopted gene transfer procedure.

At that time we were very excited about the obtained results. However, we had no evidence that the enhancement of transfection was GR-mediated, rather than simply caused by the presence of the many lipophilic steroid molecules in the transfection cocktails. As a matter of fact, several reports appeared in which enhanced expression levels were obtained, simply by adding steroids such as Estradiol or Dexamethasone in the transfection mixtures[61]. To exclude this possibility, the expression levels of the steroid-complexed and uncomplexed DNA were compared in cells that do not express functional GR. Therein, we did not observe any enhancement of expression. This result induced us to think that the previously observed enhancement of transfection was GR-mediated. As discussed later (see 2.4.2.),

---

forth or even a fifth generation of steroid derivatives !!!

[59] Brzozowski *et al.*, Molecular basis of agonism and antagonism in the oestrogen receptor, *Nature*, **389**, 753-758 (1997)

[60] Williams *et al.*, Atomic structure of progesterone complexed with its receptor, *Nature*, **393**, 392-396, (1998)

[61] Jain *et al*, Estradiol enhances gene delivery to human breast tumor cells, *J Mol Med*, **76**, 709-714 (1998); Braun *et al*, In vitro and in vivo effects of glucocorticoids on gene transfer to skeletal muscle, *FEBS*, **454**, 277-282 (1999); Bernasconi *et al*, Cortisol increases transfection efficiency of cells, *FEBS*, **419**, 103-106 (1997)

the true breakthrough came when adenoviral vectors encoding GR were available. These enabled us to develop an experimental system in which cells were externally supplemented with GR.

2.3.7. SMGD's "winning horse": Dr.Wehrli!

For the very "first generation" of steroid derivatives (see 2.3.6.) we attached the hormone Cortisol to the DNA-intercalating agent Ethidium. These molecules, even if representing a first heroic attempt to demonstrate the feasibility of the SMGD approach, have two big drawbacks. The first one, is the very low affinity for the GR, that is a hundred fold loss of affinity compared to the natural ligand Cortisol. The second one, is mediated by the nature of the DNA-interaction. The fact the Ethidium can not be covalently linked to DNA, does not permit the determination of the number of steroid molecules attached to the steroid-decorated plasmid. We solved the first problem by choosing a ligand with a higher affinity for GR, namely Dexamethasone. The second problem was solved by changing the nature of the DNA-interaction, that is by linking the steroid derivative to DNA in a covalent manner. For this purpose, we chose Psoralen, a photoreactive molecule that can be crosslinked to DNA by UV-light irradiation.

The ideal steroid derivative needed to be stable under typical cell culture and physiological conditions. Therefore the choice of the bonds which linked its different components was particularly critical. In this context, we had the big chance to get the support of an experienced chemist, Dr. Hans Wehrli. Dr. Wehrli is a sixty years old chemist, which worked for more than twenty years for Ciba-Geigy. Because of the reorganization within that company due to its fusion with Sandoz[62], Dr. Wehrli was one of the victims of the "early retirement program". Since he worked for quite a while in the "steroid chemistry department", we could not expect a better collaborator. What a luck for us and for him! The most important innovation introduced by Dr. Wehrli, was the introduction of two Urethane bonds (see Figure 1B of Paper 1, in chapter 3.1.) to link the chemical spacer to the other two components of the steroid derivatives, that is the hormone molecule and the DNA-interacting compound. By testing the Urethane bond under different conditions, we were amazed about its stability and its resistance. These features were absent in the "first generation" of steroid derivatives (see 2.3.5.).

---

[62] In 1996, Ciba-Geigy fused with Sandoz giving rise to the "giant" Novartis.

2.4. DESCRIPTION AND SIGNIFICANCE OF THE PERSONAL CONTRIBUTIONS

2.4.1. The covalent attachment of steroids to DNA

As previously discussed (see 2.3.7.), we abandoned the strategy of producing steroid-decorated plasmids via a DNA-intercalating agent. Instead of Ethidium, the "new generation" of steroid derivatives had the crosslinking reagent Psoralen as a DNA-interacting compound. We synthesized a large number of similar bifunctional steroid derivatives. These molecules had either Cortisol or Dexamethasone as a steroid hormone molecule and differed in the composition and in the lenght of their chemical spacers. Out of this initial pool of steroid derivatives, we selected a compound composed of Dexamethasone conjugated to Psoralen via a 8 atoms long spacer, shortly denominated DR9NP. The chemical structure of DR9NP is showed in Figure 1B in chapter 3.1.. Compared to the other synthesized steroid derivatives, DR9NP showed a better solubility in various solvents and had a superior affinity for the GR. In vitro binding studies performed by my collaborators, revealed indeed very similar dissociating constants for DR9NP and the strong binding hormone Dexamethasone. Thanks to the previously described assay (see 2.3.4.), I was able to observe that DR9NP and Dexamethasone induced a similar strong nuclear translocation of GR. This observation is described in in Figure 1C of Paper 1 (see 3.1.).

An important question still to be answered was whether the steroid derivatives having Psoralen as a DNA-interacting compound could have been attached to plasmid DNA. In this context, the first molecule to be tested was the steroid derivative "Cortisol-Psoralen", shortly named CR5NP. To analyze its crosslinking potential I first UV-irradiated a plasmid DNA in presence of CR5NP. The excess of unreacted CR5NP molecules was removed thanks to several organic extractions and precipitation steps. The irradiation and the purification procedures were performed using the same conditions described in the literature for Psoralen. The treated plasmid was linearized and analyzed by alkaline gel electrophoresis. Under those conditions, crosslinked DNA fragments remain double stranded and are visible as slow migrating bands, whereas uncrosslinked DNA fragments become single stranded and are visible as fast migrating bands. I was very glad to observe that the CR5NP-treated plasmid migrated slower than the untreated plasmid, indicating that CR5NP could have been crosslinked to DNA. Similar results were also found for other steroid derivatives, demonstrating the potential of steroid-conjugated Psoralen for the covalent linkage of steroids to DNA.

Similar analysis have been subsequently performed for the steroid derivative DR9NP and are described in Figure 3 of Paper 1 (see 3.1.). In particular, a plasmid carrying a reporter gene was UV-irradiated at different concentrations of DR9NP. Therefore, I obtained different steroid-decorated plasmid preparations. The fact that DR9NP was covalently linked to DNA enabled me to estimate for each preparation the average number of steroid molecules attached to the DR9NP-crosslinked plasmids. In a parallel experiment, I also determined for each preparation the residual reporter gene activity. Since we were crosslinking the plasmid randomly, including the biologically active regions, we were expecting an activity loss for a fraction of the plasmids. By irradiating the plasmids at low DR9NP concentrations only 10-20% of the plasmids had at least one crosslinked molecule. This low crosslinking efficiency correlated with a slight loss of gene activity. At higher DR9NP concentrations, the plasmids carried one to five steroid molecules per plasmid. These still low crosslinking efficiencies correlated with residual reporter activities of 90% and 36%, respectively.

As discussed later (see 2.4.3.), DR9NP enabled us to obtain the proof-of-principle for the SMGD approach. However, the above described results highlighted two important drawbacks of DR9NP. The first is its low crosslinking efficiency, 100-fold lower than Psoralen. The second one is that it can be crosslinked only randomly on a plasmid, interfering with the transcriptional activity of the encoded reporter gene. Higher and better positioned steroid decorations should be obtained with alternative steroid derivatives and strategies. Some of these are described in Figure 6. Among these, particular attractive is the idea to use Peptide Nucleic Acid (PNA) molecules as carriers for steroids (see strategy e) in Figure 6). As a matter of fact, PNA has been already used to decorate plasmids with NLS peptides (see 2.2.3.).

2.4.2. Three important experimental "revolutions"

As previously discussed (see 2.3.6.), the first steroid-DNA complexes were obtained by mixing a few micrograms of plasmid DNA with *picomoles* of Cl-21-Et. In contrast, the crosslinking reaction of plasmid DNA with DR9NP (see 2.4.1.) generally required *nanomoles* of steroid derivative, that is 1000-fold more hormone starting material! Since the DR9NP synthesis was time-consuming and its yield was limited to a few milligrams, we had to dramatically reduce the investment of DR9NP-crosslinked DNA. For this purpose we switched to a more convenient and efficient transfection procedure, that is lipofection in smaller wells. Thanks to this first "revolution", we were able to transfect genetic material in the nanograms-range.

To test whether GR would be the real cause of any "enhancement of transfection" mediated by DR9NP-crosslinked reporters, we had to develop an experimental system in which cells could be externally supplemented with GR. For parallel purposes one of the team collegues in Fribourg[63], designed special adenoviral vectors encoding GR (see Figure 2 B of Paper 1 in 3.1.). These adenoviruses allowed us to overexpress GR in receptor-negative cells. Thanks to this second "revolution", we significantly increased the probability for steroid-decorated plasmids to interact with nuclear receptors.

Thanks to the DNA-crosslinking propriety of DR9NP, we were able to explore more deeply the observed "enhancements of transfection". In particular, an internal control was added in each experiment which aimed to measure the gene activity of steroid-decorated reporters. This internal control consisted in the addition of a naked plasmid encoding a second reporter gene, in the transfection cocktails (see 3.1.). Thanks to this third "revolution", we were always able to normalize the readout of the gene activity values.

2.4.3. The proof-of-principle

My major contribution is, with no doubt, to have obtained the necessary results to demonstrate the feasability of the SMGD approach. In other words, I demonstrated for the first time, that nuclear receptors can be exploited as gene transfer vehicles. This proof-of-principle is extensively described in Paper 1 (see 3.1.). Within this work, I explored the possibility of covalently attaching steroids to DNA. In particular, I tested the SMGD strategy with the bifunctional steroid derivative DR9NP. My major achievement was to demonstrate that the expression and the nuclear accumulation of of steroid-decorated reporter plasmids are comparably enhanced in presence of GR. In this context, if we compare my results with the achievements obtained with the NLS-coupled transgenes (see 2.2.3.3.), we will notice that my work is the first that shows a clear correlation between increased gene expression an facilitated gene delivery (see Table 2). In fact, the 3-fold higher levels of nuclear accumulation are consistent with the 2- to 3-fold enhancement of expression. This correlation could be interpreted as a facilitated nuclear transport of steroid-decorated plasmids which results in an equivalent enhancement of expression.

---

[63] Dr. Stefano Brenz Verca, Group Prof. Rusconi

An extensive comparison between the SMGD and the NLS-mediated gene transfer will be extensively discussed in Paper 1 (see 3.1.). However, further important aspects will be mentioned below.

It isn't so clear whether the number of targeting signals per decorated transgene is generally relevant for its facilitated nuclear delivery. Branden and co-workers observed an enhancement of expression in presence of 11 NLS per plasmid. However, in presence of 5 NLS per transgene, no effect has been observed. In contrast, Zanta and co-workers observed a great enhancement of expression in presence of only one NLS, placed at the very end of the reporter gene. Zanta's decorated transgene had a size of 3.5 kb and was linear. It is likely that the size and the topology of the decorated transgene may play an important role in determing its nuclear import efficiency. In addition, the position of the targeting signals on the transgene could directly influence both its gene activity and its nuclear transport. The DNA molecule I used had a size of 10kb and was circular. According to the above hypothesis, shorter and linear steroid-decorated DNA fragments could be transfered with greater efficiency in nuclear-receptor positive cells. In this context, ideal candidates would be synthetic oligonucleotide such as antisense mRNAs and chimeraplasts. To date, an important drawback of these molecules is indeed their impossibility to be targeted to the nucleus of a particular cell type. To solve this problem, special steroid derivatives could be conjugated to oligonucleotides. In conclusion, the SMGD approach has the great potential to improve efficiency and specificity of both large and small therapeutic nucleic acids.

2.4.4. Detection of plasmid DNA in the nucleus of intact cells

Only a few research teams explored the nuclear accumulation potential of NLS-coupled DNA (see Table 2 in 2.4.3.). This type of study has been mostly performed using an *in vitro* nuclear transport system involving digitonin-permeabilzed cells. In this system, cytoplasms are emptied by permeabilization of cells membranes with digitonin (cells are "digitonized"). The cells are subsequently supplemented with cellular extracts containing factors of the nuclear import machinery. Using this system, Sebastyen and co-workers were able to observe nuclear accumulation of NLS-coupled DNA fragments. However, no nuclear accumulation has been observed by introducing the same NLS-decorated DNA by cytoplasmic microinjection or transfection of intact cells. An important difference between the *in vitro* model and an intact cell is the presence of the unaltered, full cytoplasmic content of the latter. It is possible that digitonin disrupts the cytoskeleton or other cellular structures which are responsible for the cytosolic sequestration of NLS-decorated DNA in intact cells.

We could not use digitonized cells to test the nuclear accumulation potential of the steroid-decorated DNA. To use this system, we had to supplement digitonized cells with a high number of functional GR, which is almost impossible to maintain *in vitro*. Therefore, the most straightforward experiment has been performed, that is compare and quantify the cellular localization of naked and steroid-decorated plasmids under the conditions used for the expression studies (see Table 1 and Figure 5 of Paper 1 in chapter 3.1.). To this purpose, plasmids were Rhodamine-labeled, lipofected and analyzed by confocal microscopy. Rhodamine-labeled vectors appeared like punctuate red signals. As expected, the majority of the signals localized in the cytoplasm. However, already in the negative controls (absence of GR and/or absence of steroid decorations) 3 to 5 per cent of the cells had nuclear localized signals. I was very happy to notice that in presence of GR, steroid-decorated DNA mediated 3-fold higher levels of nuclear signals than naked DNA. As already discussed (see 2.4.3.), this result allowed us to obtain the SMGD's proof-of-principle, since the increase of nuclear accumulation correlated with a comparable enhancement of expression.

Conflicting reports appeared about the possibility to detect nuclear localized plasmids in living cells. The fact that some of my confocal images were projections of forty different optical sections, allowed me to analyse the observed nuclear signals by three-dimensional viewing (see Figure 6 of Paper in chapter 3.1.). By analysing three different projections of apparent nuclear signals, I was able to distinguish signals that really localized in the nucleus (signals within the nucleus in all three projections) from signals that overlapped with, but actually were not in the nucleus (signals out of the nucleus in at least one projection). This observation showed that the strict two-dimensional analysis (one projection only) of nuclear signals can give rise to ambigous cellular localizations. On the other hand, the same observation confirmed that transfected plasmid DNA can be localized in the nucleus of living cells.

2.4.5. How could SMGD influence human gene therapy?

The SMGD approach has been designed to improve both the efficiency and the specificity of nonviral vectors. We demonstrated that the transfection efficiency of steroid-decorated transgenes is increased by a factor three in nuclear receptor-positive cells (see 2.4.3.). This improvement was sufficient to demonstrate the feasability of the SMGD strategy (proof-of-principle), but would probably be too low to offer a selective advantage for the therapeutical treatment of cells that express a specific steroid receptor. On the other hand, we have to consider that the selectively enhanced gene transfer has been obtained with poorly decorated-transgenes (only 1 to 5 steroid molecules for the decoration of a 10 kb plasmid with a molecular weight of 7 megadaltons!). Furthermore, the adopted decoration-strategy partially disabled the transcriptional activity of the encoded reporter gene (see 2.4.1.). Moreover, it is likely that the steroid derivatives had a lower binding affinity for the GR, once crosslinked to DNA. The synthesis of steroid derivatives with longer chemical spacers could partially solve this problem. From this point of view, our achievements have to be considered not only satisfactory, but even heroical!

The nuclear transfer of DNA is particularly limited in somatic cells, mostly because these cells are non-proliferating. As previously discussed (see 2.2.3.) an increased nuclear targeting could dramatically improve the gene transfer efficiency in this type of cells. This hypothesis has been already confirmed using NLS-coupled transgenes. In fact, by coupling a reporter plasmid to a NLS-containing peptide dimer (M9-ScT), Subramanian and co-workers obtained a 63-fold enhancement of expression in confluent endothelial cells[64]. Similar experiments perfomed in cycling cells mediated then times smaller values (see Table 2 in 2.4.3.). Analogously, we aim to test the SMGD in mitotically arrested cells, expecting a greater effect.

We clearly showed the specificity of the SMGD, since the enhancements of expression were observed only in nuclear receptor-positive cells. In this context, the most thinkable targets could be the steroid receptors preferentially expressed in some type of tumours. A concrete example is represented by the Androgen Receptors which are expressed at increased levels in advanced hormone independent prostate cancer. Prostate cancer is one of the most diagnosed cancer forms in males. No effective therapy exists for advanced or metastatic prostate cancer. In fact, chemotherapy is ineffective and androgen ablation is pallative and non-curative. Analogously, the Estrogen Receptor and the Progesterone Receptor could be targeted in breast cancer cells. Several potential therapeutical strategies are thinkable, including selective transfer of tumor suppressor genes or metastatis suppressor genes, shutdown of oncogenes and genetic induction of apoptosis[65]. Similarly, it may be also possible to target therapeutic oligonucleotides, such as antisense mRNAs, triple helix and chimeraplasts to a particular cell population. As a matter of fact, a recent report described the successful targeting of androgen receptor-positive prostatic carcinoma cells via special designed PNA-dihydrotestosterone conjugates[66]. By designing the PNA complementary to the c-myc oncogene, the authors were able to target the c-myc mRNAs and therefore decrease the cellular levels of the MYC protein.

In conclusion, the most concrete application of the SMGD strategy in humans will very likely be the selective treatment of tumours that express a particular steroid receptors. The potential is enormous, since candidates are the prostate cancer and the breast cancer, two of the world wide most diagnosed tumoural forms. To this purpose, the SMGD principle has to be first extended to other nuclear receptors and tested *in vivo*. The molecules that will first find a clinical application will probably be steroid-conjugated therapeutic oligonucleotides. An application of larger therapeutic nucleic acids is only thinkable when improved steroid-decorated transgenes will be designed in order to achieve significatively higher levels of preferential expression. In this case, some steroid derivatives could also find a niche as transfection reagents for hardly transfectable cell lines (e.g., primary cells) that express a particular steroid receptor. The SMGD could also be combined with other nuclear targeting strategies, such as the NLS-mediated gene transfer (see 2.2.3.3.). Which efficacy and specificity would a transgene have, if decorated with both steroids and NLS peptides? We could also imagine combined decorations of steroids with cellular targeting ligands, such as the asialoglycoproteins (ASGP; see 2.2.1.6.). How efficient would such a decorated-transgene be expressed in cells that express high levels of both the ASGP receptor and GR, such as hepatocytes? A thinkable *in vivo* application, could be the combination of SMGD with nanospheres (see 2.2.1.8.). Would the steroid-decoration of encapsulated transgenes improve the transfection efficiency of nanospheres in certain cells? Would it be possible to externally decorate nanospheres with steroids? Would it be feasable to specifically target particular tissue types with such steroid-decorated nanospheres?

---

[64] Subramanian *et al.*, Nuclear targeting peptide scaffolds for lipofection of nondividing cells, *Nature Biotechnology*, **17**, 873-877 (1999)

[65] For a recent review see: J.C. Reed, *Apoptosis as a goal of cancer gene therapy,* book chapter in *The development of human gene therapy,* Cold Spring Harbor Laboratory Press (1999)

[66] Boffa *et al.*, Dihydrotestosterone as a selective cellular/nuclear localization vector for anti-gene peptide nucleic acid in prostatic carcinoma cells., *Cancer Research,* **60**, 2258-62 (2000)

With some immagination, several combinations of SMGD with other gene transfer technologies are thinkable. Some of these will probably exist one day, enabling nonviral vectors to achieve efficacies and specificities that can be currently obtained with viral vectors only.

DNA size and form	**Targeting signals per reporter**	**Enhancement of expression**	**Increased nuclear accumulation**	**Reference (year)**
10 kb, linear	200 NLS	NO	only in digitonized cells	Sebastyen (1998)
3.5 kb, linear	1 NLS	10- to 1000-fold	N.D.	Zanta (1999)
10 kb, circular	11 NLS	3- to 8-fold	N.D.	Branden (1999)
10 kb, circular	1 to 5 steroids	2- to 3-fold	3-fold in living cells	Ceppi (2001)

Table 2. Summary and comparison of the results obtained with steroid-decorated and NLS-coupled transgenes.

(See 2.2.3.3. for the reported literature references)

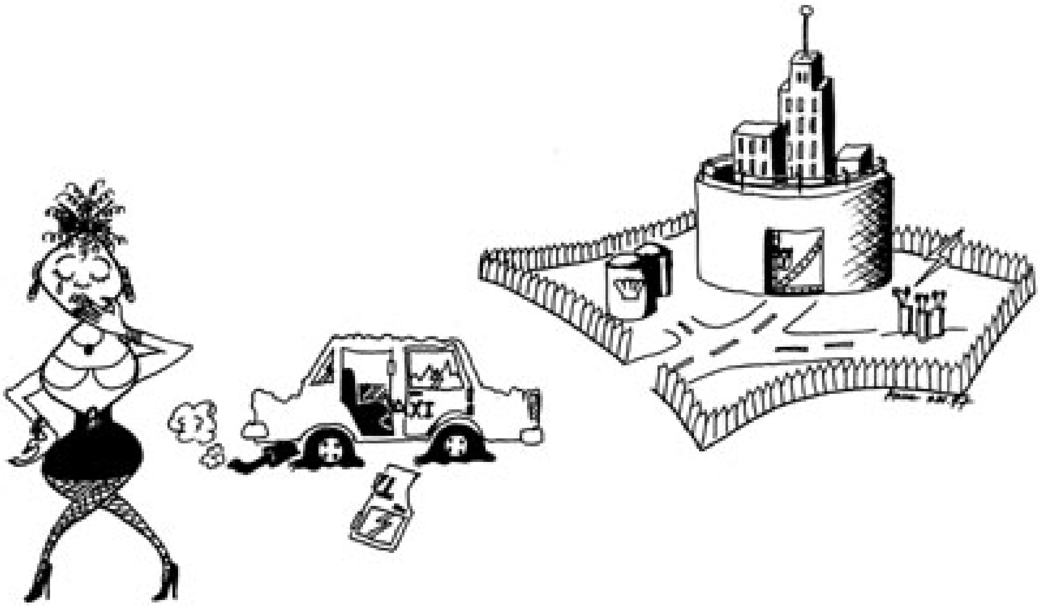


FIGURE 1. The "perfect" gene transfer vector doesn't exist yet … (author of the drawing: Anne Genilloud, member of the "Ruscolab", 1997)

Conceptually, gene therapy treatments seem to be achievable quite easily. In practice, considerable obstacles have emerged, mostly caused by the inefficacy of current gene transfer systems (vectors). The drawing shows a crying curvilinear lady (left). Her unhappiness is due to the fact that the taxicab (center) that has to bring her downtown (right) does not really look safe. The taxi is even broken. With some phantasy we could imagine that the curvilinear lady represents DNA, the broken cab represents one of the current imperfect vector and the city represents a cell nucleus. The bottomline of this drawing is that the nucleus is generally a problematic destination to reach for DNA, especially if in combination with nonviral vectors.
Symbols: crying sexy lady, DNA; broken cab, vector; fence; cell membrane; city surrounded by a thick wall; nucleus with nuclear envelope. Various cell organelles are also schematized inside the fence.

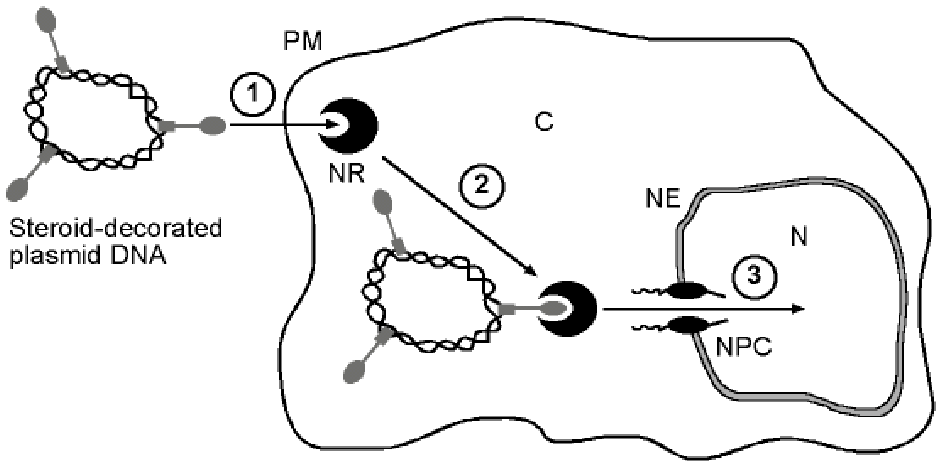
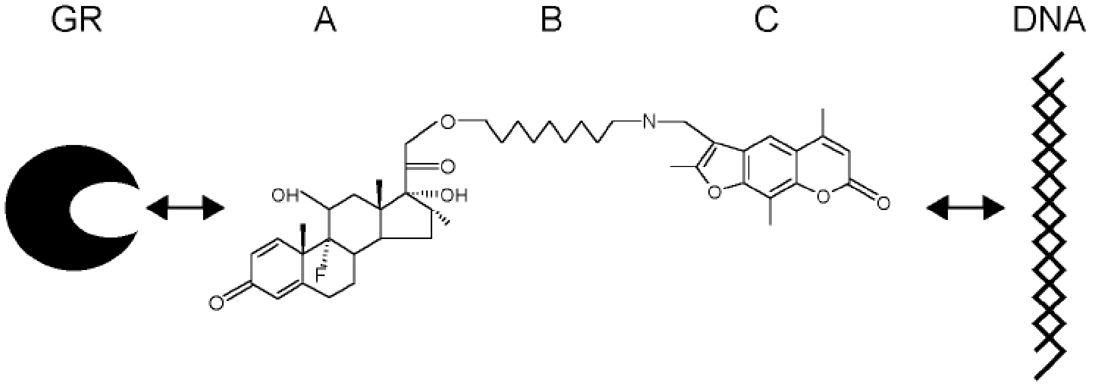


FIGURE 2. The principle of Steroid Mediated Gene Delivery (SMGD)

Steroid Nuclear Receptors (NRs) are cellular proteins that are continously shuttling between the cytoplasm and the nucleus. In absence of any ligands (steroids) the NRs are mainly localized in the cytoplasm, whereas in presence of ligands the receptors translocate into the nucleus. A question that arises is whether the shuttling property of NRs could be exploited to enhance the cytoplasmic-nuclear transport of DNA, very inefficient in current nonviral vectors. The idea of the SMGD gene transfer technology is indeed to steroid-decorate reporter or therapeutic DNA thanks to special developed bifunctional steroid derivatives (see Figure 3). The steroid derivatives bind to plasmid DNA, either by ionic interaction or via an intercalating or a crosslinking reagent. The resulting steroid-decorated plasmids are transported into the cytoplasm by conventional transfection procedures (1). Cytoplasmically located nuclear receptors bind the steroid moiety of steroid-decorated plasmids (2). The steroid recognition results in nuclear translocation of the whole complex (3). Abbreviations: PM, Plasma Membrane; NR, Nuclear Receptor; C, Cytoplasm; NE, Nuclear Envelope; NPC, Nuclear Pore Complex; N, Nucleus.



A: Steroid molecule
- chemical position for derivatization
- steroid hormone type
- maintanance of affinity for receptor
- nuclear translocation inducer

B: Chemical spacer
- length
- stability
- solubility
- chemical reactivity

C: DNA-interacting compound
- ionic interaction molecule
- intercalating agent
- crosslinking reagent
- incorporating molecule

FIGURE 3. A typical bifunctional steroid derivative and variables for the steroid-decoration of plasmid DNA

All the developed bifunctional steroid derivatives are composed of a steroid molecule (A) linked via a chemical spacer (B) to a DNA-interacting compound (C). For each of these components there were many variables, that we explored in designing different steroid derivatives. A list containing the names and the properties of the synthesized and characterized compounds is reported in the *Appendix*. Because of its superior affinity to the Glucocorticoid Receptor (GR) if compared to other steroids like Cortisol, almost all compounds contained the synthetic hormone Dexamethasone. The tested chemical spacers had a length that varied from 6 to 23 residues and had different compositions and solubility properties.Three different DNA binders were tested: a) the intercalating agent EtBr, b) the ionic interacting compound Spermine and c) the crosslinking reagent Psoralen. The compounds had quite different binding affinities for GR. Out of the initial pool of steroid derivatives, we selected the Dexamethasone-Psoralen conjugate DR9NP (see Figure 1B in chapter 3.1), because of its superior affinity for GR and because of the possibility to covalently link the molecule to DNA.

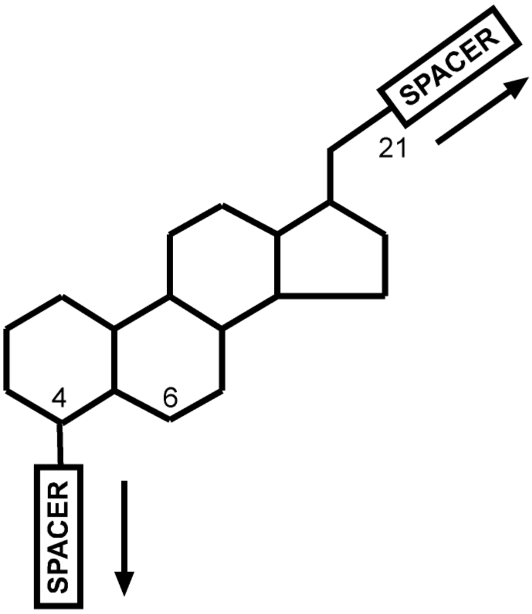


FIGURE 4. Possible chemical positions for steroid derivatization

Two positions were taken into account for linking the chemical spacer to the steroid backbone (see Figure 3); position 4 and position 21. The former was compatible with the early proposed model for the ligand-binding interaction of the Glucocorticoid Receptor with its strong binding ligand Dexamethasone (see text for details and literature references) . Only in a later phase of the SMGD project, this model resulted to be wrong inducing us to give up the idea of derivatizing position 4. In contrast, the intention of derivatizing position 21 was consistent with the later resolved Ligand Binding Domain's crystal structures of the Estrogen Receptor and the Progesteron Receptor. In these structures, the positioning of the ligand was turned by 180° with respect to the model previously proposed. Consequently, all the steroids were chemically derivatized at position 21.

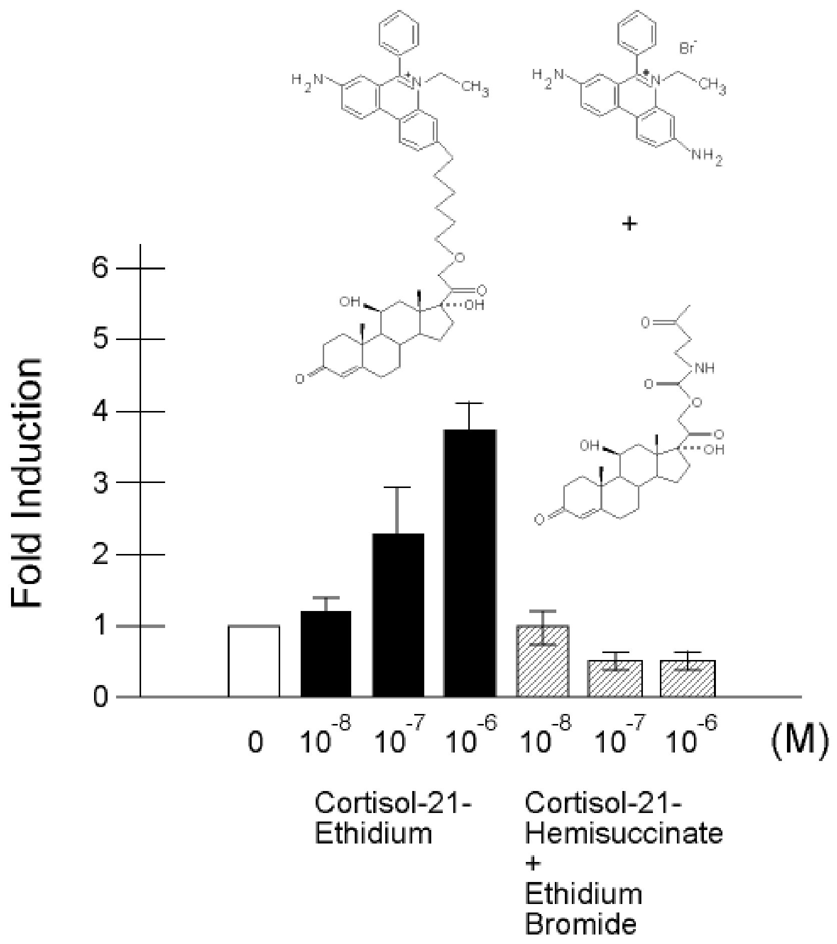


FIGURE 5. Expression levels of transfected reporter plasmid in GR-positive cells under various conditions

5 µg of the CMVLacZ reporter plasmid were pre-incubated for 10 minutes at RT with 0.1 nmoles (final concentration: 10^{-8}M), 1 nmoles (10^{-7}M) or 10 nmoles (10^{-6}M) of the steroid derivative Cortisol-21-Ethidium (Cl-21-Et) or to its chemical precursors Cortisol-21-Hemisuccinate and Ethidium Bromide (negative control). With these mixtures proliferating GR-positive rat 3Y1 cells were transfected using the Calcium Phosphate gene transfer procedure. The transfection efficiencies of complexed and uncomplexed reporter DNA were compared by analysing their LacZ activities 48 hours after transfection. Cl-21-Et-complexed reporter mediated until 4-fold higher expression levels if compared to uncomplexed DNA or the negative controls. Similar enhancements of transfection were observed also in another GR-encoding cell line (human A549-1) and were absent in GR-negative cells (monkey CV-1).
Symbols: empty bar, uncomplexed plasmid; filled bar, Cl-21-Et-complexed plasmid; hatched bar, plasmid mixed to the chemical precursors of Cl-21-Et.

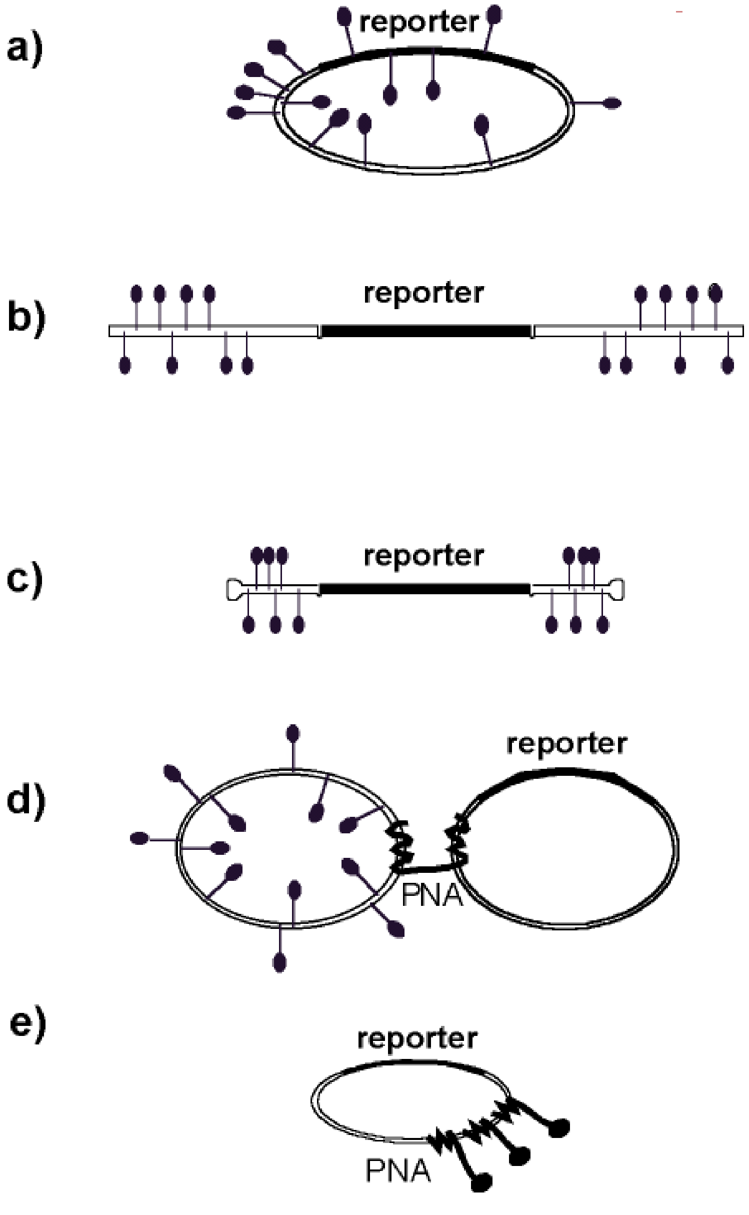


FIGURE 6. Strategies to steroid-decorate therapeutic or reporter DNA

a) In the simplest approach, the Steroid Derivative (SD) is directly crosslinked to the reporter gene. The disadvantage is that in a fraction of plasmids, the reporter function will be impaired. **b)** To preserve the reporter integrity, the SD is separately crosslinked to a ligatable DNA fragment. **c)** Instead of a restriction fragment, a specially engineered oligonucleotide can be designed to be first loadable with SD and then ligated to the transgene. **d)** In this system, the SD is crosslinked to a separate DNA fragment or plasmid. The decorated fragment is tethered to the transgene via a bifunctional triple-helix forming oligonucleotide, which recognizes two distinct regions. In the ideal situation, we envisage the use of PNA instead of conventional nucleic acids to drive the linking reaction. **e)** Ultimately, the steroid could also be directly chemically linked to the PNA moiety.

3. PAPERS

3.1. PAPER 1: "SELECTIVELY ENHANCED TRANSFER OF STEROID-DECORATED TRANSGENES IN NUCLEAR RECEPTOR-POSITIVE CELLS"

(Submitted to *Nature Biotechnology*)

Selectively enhanced transfer of steroid-decorated transgenes in nuclear receptor-positive cells

Maurizio Ceppi (1), Stefano Brenz Verca (1), Ibrahim Merdol (2), Alessio Bernasconi (3), Alexandre Rebuffat (3), Hans Wehrli (3), Brigitte Frey (3), Felix J. Frey (3) and Sandro Rusconi (1*)

1 Biochemistry institute, UNIFR, Pérolles, CH-1700 Fribourg, Switzerland
2 Histology Institute, UNIFR, Pérolles, CH-1700 Fribourg, Switzerland
3 Nephrology Dept., Inselspital, CH-3010 Bern, Switzerland

* Corresponding Author
Tel +41 26 300 8656
Fax +41 26 300 9735
e-mail: sandro.rusconi@unifr.ch

Keywords: chemical crosslinking; glucocorticoid receptor; nonviral gene transfer; nuclear import; psoralen; steroid-mediated gene delivery.

Abstract

Aiming at improving both the efficiency and the specificity of nonviral gene transfer, we designed the Steroid Mediated Gene Delivery (SMGD) approach. The idea behind the SMGD strategy is to facilitate the nuclear uptake of transfected DNA with the help of nuclear receptors, which are natural shuttles for steroid molecules. We modelled the SMGD with the Glucocorticoid Receptor (GR) system. The essential step was to chemically derivatize steroids to allow their covalent linkage to plasmid DNA while maintaining their availability for the GR. To this purpose, we synthesized and tested a large number of bifunctional steroid derivatives and selected the compound that we shortly denominate DR9NP for the experiments presented in this work. We show that GR efficiently translocates into the nucleus upon exposure to few nanomolar amounts of DR9NP. Furthermore, the expression of DR9NP-decorated reporter plasmids was reproducibly enhanced 2- to 3-fold in presence of GR. The effect was occurring only in cells expressing the GR, was independent from the transactivation potential of GR and correlated with an equivalent plasmid nuclear accumulation. The final general aim of the SMGD approach is to offer an additional selective advantage to the gene transfer-based treatment of somatic tissues that express a specific steroid receptor.

Introduction

Nonviral gene therapy vectors are generally designed to allow the genetic material to cross the cell membrane of mammalian cells with high efficiency. However, only a small portion of the delivered nucleic acids undergoes nuclear translocation. The nuclear envelope has been identified to be a major barrier for the gene transfer of foreign DNA (1-3), specially in non-dividing cells (4). Among the most promising strategies that have been proposed to overcome this barrier we shall mention the linkage of the SV40 nuclear localization signal (NLS) to reporter DNA (5-7). Other obstacles to nuclear accumulation of plasmid DNA include the entrapment of DNA in the endolysosomal compartment (8) and the degradation of DNA by cytosolic nucleases (9). Viruses have evolved sophisticated molecular mechanisms for the nuclear delivery of their genes (reviewed by Whittaker et al. (10)), a feature that explains in part the higher transduction efficiency of viral vectors. Another factor limiting the efficacy of nonviral vectors is the inability to specifically target genes to a desired cell type. Most targeting techniques developed to date enable DNA to be internalised into the cytoplasm of the desired cells via specific surface ligand docking (11). A step which has been rather neglected until recently is the possibility to preferentially favour the transfer therapeutic genes into the nucleus of particular cell types, independently of the DNA delivery method.

In order to improve both the efficiency and the specificity of nonviral gene transfer, we designed the Steroid Mediated Gene Delivery (SMGD) approach that is illustrated in Fig. 1A. The rationale behind SMGD is to improve the nuclear uptake of transfected DNA with the help of nuclear receptors, which are natural shuttles for steroid molecules. Nuclear receptors such as the glucocorticoid receptor translocate to the nucleus once activated by incoming steroids (12). The basic strategy is to chemically derivatize steroids and to link them covalently to plasmid DNA. The resulting steroid-decorated plasmids are first introduced in the cell by conventional transfection procedures (Fig. 1A, point 1). Cytoplasmically located nuclear receptors are then expected to bind the steroid moiety of steroid-decorated plasmids (Fig. 1A, point 2), resulting ultimately in nuclear translocation of the complex (Fig. 1A, point 3). Our approach is analogous to the recently reported nuclear import strategy of the HIV-1 genome (13). In that report, the authors suggested that the pre-integration complex central DNA flap interacts with cellular or viral proteins that shuttle between the cytoplasm and the nucleus of the host cell. According to this model, this interaction would enable the linear HIV-1 genome to be crawled through the nuclear pore.

In the present study, we modelled the SMGD approach with the well characterized Glucocorticoid Receptor (GR) system (14, 15). To this purpose we synthesized and tested several bifunctional steroid derivatives, all composed by a steroid molecule covalently linked to a DNA interacting compound (Bernasconi et al., in preparation). The aim of this work was to test the feasibility of the SMGD using the steroid derivative DR9NP (Fig. 1B), which comprises a dexamethasone moiety and a psoralen function and has been selected from our initial collection of synthesized hormone derivatives (Rebuffat et al., in preparation) . Our work unequivocally demonstrates that the expression and the nuclear uptake of DR9NP-decorated reporter plasmids are selectively enhanced in presence of GR. This study is the first that shows that nuclear receptors can be exploited as gene transfer vehicles.

Results

Synthesis of the steroid derivative DR9NP. The steroid hormone Dexamethasone (Dex) was conjugated to the DNA-crosslinking molecule Psoralen (Aminotrioxsalen) via a 9 atoms long spacer. The resulting steroid derivative was named for simplicity DR9NP to outline its 9 atoms-long spacer, the dexamethasone and the psoralen moieties and the urethane bonds (Fig. 1B). DR9NP (patent pending) has the characteristic to contain two urethane bonds (OCONH) which link the spacer to the two polycyclic structures. The stability of the urethane bonds has been verified under acidic (pH 5) and alkaline (pH 9) conditions, by proteinase K (pH 7.8, 50 µg/ml) and dispase (2.4 U/ml) digestion and after incubation with cellular extracts and with DMEM / 10% FCS (Bernasconi et al., in preparation). All the tests indicated that DR9NP is stable under typical cell culture conditions.

Similar bifunctional steroid derivatives were synthesized, differing from DR9NP in the spacer length and composition, but DR9NP has been selected from this initial collection of hormone, because of its excellent solubility in various solvents and its superior affinity for GR (Rebuffat et al., in preparation).

DR9NP induces nuclear translocation of GR. GR(Ala) is a previously characterized dominant negative mutant of the glucocorticoid Receptor (GR) (16). GR(Ala) maintains competence for hormone binding, nuclear translocation and DNA binding, but is incapable for transactivation (16). We previously observed a more pronounced cytoplasmic localization of GR(Ala) than GR in absence of steroid hormones (16), making GR(Ala) an ideal candidate to test the nuclear translocation potential of steroids. For this purpose we generated the chimeric protein GR(Ala)GFP (Fig. 2A, construct 1). The cellular localization of GR(Ala)GFP was analyzed in presence or in absence of DR9NP. HeLa cells were transiently transfected with GR(Ala)GFP, fixed with formaldehyde/glutaraldehyde and inspected by fluorescence microscopy in the presence of UV-light. In absence of hormones GR(Ala)GFP was mainly localised in the cytoplasm (Fig. 1C), whereas in the presence of Dex (Fig. 1D) or DR9NP (Fig. 1E) the chimeric protein was localised in the nucleus indicating that the two steroids have a similar binding affinity for the Glucocorticoid Receptor. In vitro binding studies revealed indeed dissociating constants (K_D) of $5x10^{-8}$ M for DR9NP and 10^{-9} M for Dex (Rebuffat et al., in preparation).

DR9NP can be crosslinked to plasmid DNA. Psoralen is a photo reactive molecule which can be crosslinked to DNA upon long-wave (365 nm) UV irradiation (reviewed by Hearst et al.(17)). Under those conditions, DNA is typically reacted with 1 Psoralen molecule per 100 base pairs (17). We first tested by NMR analysis whether DR9NP is still intact after UV-irradiation and we found no difference between the NMR profiles before and after UV-irradiation (Bernasconi et al., in preparation). To test whether DR9NP can be crosslinked to DNA, the plasmid CMVLacZ (Fig. 2A, construct 2) was UV-irradiated in absence and at four different DR9NP concentrations (0 M, 10^{-8} M, 10^{-7} M, 10^{-6} M and 10^{-5} M). After irradiation, unreacted DR9NP molecules were removed by Chloroform/ Isoamyl alcohol extraction. The resulting DR9NP-crosslinked CMVlacZ preparations (D8, D7, D6 and D5) and the negative control D0 were linearized by restriction enzyme digestion, alkali-denatured and analyzed by gel electrophoresis (Fig. 3A). Under these conditions, crosslinked DNA fragments remain double stranded and are visible as slow-migrating bands (dsDNA), whereas uncrosslinked DNA fragments become single stranded and are visible as fast-migrating bands (ssDNA). As expected D0 yields exclusively ssDNA (lane 1), while D8, D7 and D6 produce increasing amounts of dsDNA (lanes 2,3 and 4). The preparation D5 remains essentially double stranded (lane 5). The percentage of crosslinked DNA has been calculated for each vector preparation (empty circles in Fig. 3B and Experimental Procedures). Only 10% of D8 vectors and 20% D7 vectors have at least one crosslink. In contrast 90% of D6 vectors have at least one crosslink and D5 vectors have an average of five crosslinked DR9NP molecules. A 100-fold higher crosslinking efficiency was obtained with native Psoralen (data not shown).

The reporter gene activity of each preparation has been determined by measuring the amount of beta-Galactosidase (ß-Gal) in CV-1 cells transiently transfected with equal amounts of the corresponding DNA (filled circles in Fig. 3B). When 6% (D8), 13% (D7), 58% (D6) of the plasmids are crosslinked, there is only a moderate loss of reporter gene activity. In contrast when 99% (D5) of the plasmids are crosslinked, the gene activity is reduced to 36%. The loss of gene activity is likely due to the fact that a portion of the reacting DR9NP molecules crosslinks the reporter within the biological active regions (Fig. 3C).

The beta-Galactosidase expression of DR9NP-crosslinked CMVlacZ is enhanced in presence of GR. To test whether GR would affect the transfection efficiency of DR9NP-crosslinked CMVLacZ we first had to develop an experimental system in which cells could be externally supplemented with GR. For this purpose we generated two adenoviral transfer vectors, one encoding for wild type GR (AdGR, construct 5 in Fig. 2B) and one encoding for the mutant GR(Ala) (AdGR(Ala), construct 6 in Fig. 2B). CV-1 cells were chosen as targets for infection , as this cell line does not express GR endogenously (18, 19). By infecting CV-1 cells with an adenovirus encoding GFP (AdGFP, construct 7 in Fig. 2B) at a multiple of infectivity (MOI) of 3, 100% of the cells expressed the reporter gene, indicating that CV-1 cells can be infected efficiently by adenoviruses (our unpublished data).

In a first experiment, CV-1 cells were first infected with AdGFP (Mock) or AdGR and subsequently lipofected either with D0 or D6. To enable full expression of the adenovirus encoded GR, cells were transfected 19 hours after the infection (see Fig. 4A for time course). Uncrosslinked CMVLuc was added to each transfection cocktail for readout normalisation. Cells were harvested and the activities of ß-Gal and Luc were determined simultaneously. The normalized expression levels are shown in Fig. 4B. In absence of GR there is no difference in the expression levels from the D0 and D6 preparations (compare bars 1 and 2), whereas in presence of GR, D6 mediates 2-to 3-fold higher ß-Gal expression than D0 (compare bars 3 and 4). An analogous experiment has been performed by comparing the expression levels of D0 and D5 (Fig. 4D). In absence of GR, the activity of D5 is reduced to 40% compared to D0 (compare bars 7 and 8) confirming the loss of gene activity observed previously (Fig. 3B). In presence of GR, D0 and D5 have similar expression levels (compare bars 9 and 10), meaning that also D5 is subject to a 2-fold to 3-fold enhancement of expression (compare bars 8 and 10). No significant enhancements of expression were observed with the vector preparations D8 and D7 where only a minority of the molecules bears a crosslinked steroid (data not shown). Comparable enhancements of expression were reproducibly obtained in a cell line which express GR endogenously (human lung carcinoma cells A549-1, data not shown), indicating that a DR9NP-mediated increased transfection efficiency can be obtained also at physiological concentrations of GR.

To explore whether the transactivation potential of GR rather than its nuclear translocation is responsible for the observed enhancement of expression, the reporter gene activity of the different vector preparations was analyzed in presence of GR(Ala) (Fig. 4D). As discussed previously, this mutant GR is incapable of sustaining transactivation. We found that, similar to GR, GR(Ala) enables D6 to express higher ß-Gal levels than D0 (compare bars 5 and 6) and rescues D5 to display expression levels comparable to D0 (compare bars 11 and 12). This observation renders unlikely the possibility that the observed enhancement of expression is caused by an increased transcriptional activity of the DR9NP-crosslinked vectors and reinforces the interpretation that the effect is caused by a facilitated nuclear delivery.

Enhanced expression of DR9NP-crosslinked CMVlacZ correlates with increased plasmid nuclear accumulation. To follow the cellular localization of the vectors, CV-1 cells previously infected with AdLacZ (Mock), AdGR or AdGR(Ala), were lipofected with Rhodamine-labeled D0 or D5 and imaged with a confocal laser scanning microscope. A representative picture of a central optical section of transfected cells is shown in Fig. 5A where Rhodamine-labeled aggregates appear like punctuate white signals. The majority of the signals is localised in the cytoplasm or in the perinuclear region of the cells and only few signals are localised in the nuclei (arrows in Fig. 5A). The cellular localization of the signals was quantified for each condition by averaging the scored localization of signals in many cells for each experimental condition (see legend to Fig. 5 and Experimental Procedures). The details of all the counted signals and the percentages of cytoplasmic, perinuclear and nuclear signals are reported in Table 1. In the mock-infected cells there is no significant difference in the cellular localization of D0 and D5 (compare columns B and D). In presence of GR, the D5 preparation produces a 3-fold higher percentage of nuclear signals than D0 (13% in column H vs. 4% in column F). Likewise, GR(Ala) mediates 3-fold higher levels of nuclear signals for D5 if compared to D0 preparation (10% in column N vs. 3% in column L). In presence of GR or GR(Ala), no significant difference is detectable in the percentage of perinuclear or cytoplasmic signals (compare column H with column F and column N with column L). The percentages of nuclear localized signals are graphically represented in Fig. 5B. The 3-fold higher levels of nuclear accumulation observed for DR9NP-crosslinked CMVlacZ in presence of GR, although modest, are consistent with the 2-fold to 3-fold enhancements of expression observed previously (Fig. 4). This correlation is best interpreted as resulting from a facilitated nuclear transport of DR9NP-decorated plasmids which results in an equivalent enhancement of expression.

Proof of nuclear localization of transfected plasmid DNA. Conflicting reports appeared recently regarding the ability to visualise nuclear localized plasmid DNA after transfection of cultured cells. A report described the impossibility to detect any nuclear plasmid using different microscopic techniques, that is light microscopic evaluation of fluorescently labeled DNA and electron microscopic evaluation of gold-labeled DNA (1). Another report described the detection of nuclear localized fluorescent plasmids in 20% - 30% of the transfected cells (20). These contrasting results induced us to explore more deeply our observed putative nuclear localized signals. For this experiment, CV-1 cells, previously infected with AdGR, were lipofected with Rhodamine-labeled D5. To better visualise cellular structures, the cells were stained with acridine orange and then imaged with a confocal laser scanning microscope as for the previous experiments. Images were composite of 35 optical sections obtained at 200 nm intervals. A typical wide field image is shown in Figure 6A where Rhodamine-labeled vectors appear as before like punctuate red signals. From this perspective, signals are visible in the cytoplasm (open arrows), in the perinuclear region (closed arrows) or within the nucleus (rectangle) of the cells. We further verified the localization of the nuclear signals by tri-dimensional viewing. Fig. 6B represents an enlarged view of the cell highlighted in Fig. 6A. A vertical portion of the cell which includes three apparent nuclear localized signals (dashed vertical lines) has been rotated clockwise by 90 degrees and is projected in Fig. 6C. From this perspective, two signals are in the nucleus and one signal is in the perinuclear region. Analogously, a horizontal portion of the cell which includes the nuclear signals (dashed horizontal lines) has been rotated clockwise by 90 degrees and is projected in Fig. 6D. Also from this perspective the same two signals are in the nucleus and the same single signal appears to coincide with the perinuclear region. We collected similar observations for uncrosslinked plasmids and also in absence of GR (data not shown) and confirmed the data presented in Table 1 also under those more stringent conditions. These observations demonstrate that the majority of the punctuated signals seen as overlapping with the nucleus area after transfection of Rhodamine-labeled plasmids can be unequivocally located within the nucleus.

Table 1. Cellular localization of naked and DR9NP-decorated plasmid DNA in presence or absence of GR.

Adenovirus[1]	Mock		Mock		AdGR		AdGR		AdGR(Ala)		AdGR(Ala)	
Preparation	D0		D5		D0		D5		D0		D5	
Signals[2]	Σ	% ±SD	Σ	% ±SD	Σ	% ±SD	Σ	% ±SD	Σ	% ±SD	Σ	% ±SD
	A	B	C	D	E	F	G	H	I	L	M	N
Cytoplasmic[3]	654	55 ± 5	494	55 ± 9	618	53 ± 4	343	39 ± 13	545	56 ± 7	384	48 ± 6
Perinuclear	470	40 ± 6	366	41 ± 8	484	43 ± 4	356	48 ± 14	402	42 ± 6	323	42 ± 6
Nuclear[4]	50	4 ± 1	41	5 ± 1	49	4 ± 1	110	13 ± 4	29	3 ± 1	79	10 ± 1
TOTAL	1174		901		1151		809		976		786	

[1] The experimental outline and the different conditions are described in Fig. 5.

[2] "Signals" are the punctuate white signals visible in the black and white pictures described in Fig. 5 and correspond to the Rhodamine-labeled vectors. Σ is the sum of the counted signals within 150 cells evaluated for each condition. % is the average of the percentages of cytoplasmic, perinuclear or nuclear localized signals and SD is the standard deviation of these percentages.

[3] Examples of cytoplasmic, perinuclear and nuclear localized signals are showed in Fig. 6A. The subdivision into these broad classes is arbitrarily defined by the authors.

[4] The percentages of nuclear localized signals are graphically represented in Fig. 5B.

Discussion

In this work we described the Steroid Mediated Gene Delivery (SMGD) approach, a novel technique to improve both the efficiency and the specificity of nonviral gene transfer. In particular, we explored the possibility to exploit nuclear receptors as ferries for plasmid DNA. Although other nuclear receptors could have been chosen, we modelled the SMGD with the Glucocorticoid Receptor (GR) system, because the necessary expression vectors, receptor mutants and commercial ligands for derivatisation were available. For the experiments described here, we used the bifunctional steroid derivative DR9NP (Fig. 1B). We first demonstrated that GR translocates into the nucleus upon exposure to DR9NP (Fig. 1C). Secondly, we showed that DR9NP can be crosslinked to plasmid DNA (Fig. 3A). Finally, our major achievement was to demonstrate that the expression and the nuclear accumulation of DR9NP-decorated reporter plasmids are comparably enhanced in presence of GR and that this is independent of its transactivation potential (Figs. 4, 5, and 6).

Several recent reports proposed similar approaches to improve the nuclear import of plasmid DNA. In particular, different strategies have been described to covalently link the SV40 T-antigen nuclear localization signal (NLS) to reporter DNA. The attachment of an NLS-bearing peptide to plasmid DNA increased nuclear accumulation of the conjugated DNA in digitonin-permeabilised cells (5). Another report described the preparation of an NLS-coupled linearized reporter construct (6). In that work, the authors state a 10-fold to 1'000-fold enhancement of transfection but did not verify the effect on actual DNA transport into the nucleus by physical methods. Recently, a correlation between the effect of NLS on both DNAs nuclear import and expression has been reported, reinforcing the feasibility of this approach (21). A further report described a 3- to 8-fold enhancement of transfection obtained by conjugating plasmid DNA with a PNA-NLS fusion (7). This result is comparable to the 2- to 3-fold enhancement of expression observed for the pilot SMGD approach reported here (Fig. 4B). Taken collectively, these results suggest that linearized NLS-coupled reporters provide higher transfection efficiency than circular NLS-coupled plasmids. It is thus possible that after passing a certain size-threshold, linearized DNA is probably better imported into the nucleus than circular DNA. This hypothesis is supported by some nuclear import strategies present in nature. For instance, the genome of large DNA viruses, such as Herpes Simplex virus and Adenovirus is transported into the nucleus of host cells as a linear fragment coupled to different NLS-containing proteins (10). Analogously, the *Agrobacterium tumefaciens* T-DNA is imported in the nucleus of plant cells as a linear single-stranded DNA coupled to the NLS-containing proteins VirD2 and VirE2 (22). To our knowledge, so far there has been no report describing a direct comparison between the transfection efficiencies and nuclear import efficiency of linear versus circular NLS-coupled reporters. This type of study is currently in progress for our steroid-decorated reporters.

Although a promising strategy, the delivery of NLS-coupled transgenes does not allow to target DNA specifically to a desired cell type, since the factors comprising the import machinery interacting with NLS are apparently expressed ubiquitously (23). The final general aim of the SMGD is indeed to offer a selective advantage to the therapeutical gene transfer in target cells that express a specific steroid receptor. Potential targets could be nuclear receptors present in some type of tumours or degenerating tissues (24, 25). For example Androgen receptors could be targeted in prostate cancer (26, 27) and Progesterone or Estrogen receptors could be targeted in breast cancer cells (28, 29). As a therapeutical strategy, it may be possible in this way to transfer a tumour suppressor gene or any antitumoral factor selectively to these cancer cells. Analogously, it may be also possible to favour the accumulation of synthetic oligonucleotides such as antisense, ribozymes, and chimeraplasts to selected cell layers and tissues. Different steroid derivatives which could be conjugated to these molecules are under development. Other nuclear receptors expressed in somatic cells, such as those for retinoic acids, vitamin D, thyroid hormones, peroxysome proliferators etc., may also be targeted by an extended

SMGD procedure. Since the nonviral gene transfer in non-dividing cells is even less efficient (4), a greater effect of nuclear targeting is expected in this type of cell (30). We are currently testing this hypothesis in artificially mitotically arrested cells. Obviously, also direct DNA binding by shuttling transcription factors instead of complicated crosslinking with ligands, can in principle be exploited to favour nuclear accumulation. We have momentarily renounced to this type of experiments since it is arduous to prove that the enhanced expression is indeed due to transport rather than to direct transcriptional effects and it is also difficult to avoid unspecific binding to cryptic sites within the constructs. For instance, a recent report described the cell-specific import of plasmid DNA (31). In that work, a DNA element containing binding sites for the transcription factor SRF was inserted in a reporter plasmid. The plasmid was allegedly preferentially localized in the nucleus of SRF expressing cells. The authors also obtained a 5-fold to 10-fold enhancement of expression, but did not explore whether the effect was caused by the transactivation potential of SRF rather than its nuclear translocation. In addition, the inserted DNA element contained the smooth muscle gamma actin (SMGA) promoter which has an intrinsic transcriptional enhancer activity (32). A further report described that the presence of the Epstein-Barr Virus *oriP* sequence on a plasmid increased 100-fold the expression of the encoded reporter gene in cells expressing EBNA1 (33). The authors concluded that the increase of gene expression resulted from the additive effects of plasmid nuclear import and *oriP*-EBNA1 transcriptional enhancement but could not cleanly dissociate these effects. In our work we observed higher expression levels of steroid-decorated reporters even in presence of a mutant GR which is totally unable to transactivate (Fig. 4D). Furthermore, the 2- to 3-fold enhancement of expression correlated with an equivalent plasmid nuclear accumulation (Fig. 5B). This correlation suggests that the effect is caused indeed by a facilitated nuclear delivery and renders alternative explanations rather unlikely.

In this work, we were also able to demonstrate by 3D-reconstruction of images that transfected plasmid DNA can be localized into the nucleus of cultured cells. Formally, we were expecting diffused staining inside the nucleus. However, punctuated signals similar to ours have been reported for nuclear imported plasmids in digitonin-permeabilised cells (34) and for HIV-1 genomes in the nuclei of infected cultured cells (13). More diffuse patterns have been found for DNaseI treated DNA, for oligonucleotides and for NLS-bearing proteins (34). Taken together, these results suggest that the diffused nuclear staining observed in several reports (8) may be caused by the nuclear import of degraded nucleic acids rather than intact plasmid DNA. However, we cannot exclude that the punctuate staining simply depends on the adopted transfection reagent (cationic lipids) and/or the little amount (30-70 ng) transfected nucleic acids. To clarify this, we are currently following the fate of cytoplasmically microinjected labelled DNA under the various conditions (decorated versus native, in presence versus absence of GR etc.).

The observed punctuated signals have apparent diameters that vary from 200 to 800 nm (Fig. 6B). It has been proposed that 5 to 20 plasmids are complexed in a single DNA/lipid particle (35). However, from our experiments, it is not possible to determine the number of plasmids that may generate each single signal. Since it is still not clear whether DNA uncoating takes place preferentially in the cytoplasm or in the nucleus (36), we cannot exclude that a fraction of the nuclear localized plasmids is still bound to some residual cationic lipids or that it re-aggregates after internalisation.

In conclusion, this study demonstrates for the first time that a nuclear receptor can be used as a gene delivery helper vehicle. Our work is still at the level of a proof-of-principle and several strategies are envisageable to further improve the efficiency of the SMGD approach. Besides trying to synthesize bifunctional derivatives that better maintain affinity for the cognate receptor, we are aiming at a more localised decoration of the transgene. in fact, the major drawback of our "first generation" steroid derivatives is that they can be crosslinked only randomly on a plasmid, interfering with the transcriptional activity of the encoded reporter gene. We are currently developing a "second generation" of steroid derivatives which could be conjugated to biologically inactive regions of reporter plasmids. In particular, we are exploring the possibility to use triple-strand forming PNA ([20] and references therein) as carrier for steroid molecules. With this strategy we should obtain plasmids with higher and better positioned steroid-decorations compared to DR9NP. A higher number of clustered conjugated steroids per plasmid should increase the probability for nuclear receptors to interact with the complex. This should in turn enhance the nuclear transport of steroid-decorated DNA. Future studies will also elucidate whether other nuclear receptors could be exploited as ferries for DNA. To this purpose, the "second generation" of steroids will also include ligands for the estrogen, androgen, progesterone and mineralocorticoid receptor. These molecules may constitute powerful tools for clinical applications aiming at the selective macromolecular treatment of target tissues that express a specific steroid receptor.

Experimental protocols

Construction of reporter plasmids and recombinant adenoviruses.

Reporter plasmids: The plasmid CMVGR(Ala)GFP (Fig. 2A, construct 1) was constructed by fusing a GFP(S65T) mutant (37) C-terminally to a rat glucocorticoid receptor (GR) bearing a frame shift mutation in its N-terminus transforming a natural poly-glutamine stretch into a poly-alanine stretch (16). The resulting GR(Ala)GFP fusion protein ORF is constitutively expressed under the control of the human CMV promoter and is followed by the rabbit ß-globin splice signal and 3' polyadenylation signal and a SV40 origin of replication. Plasmids CMVLacZ (Fig. 2A, construct 2) and MMTVLacZ (Fig. 2A, construct 3) carry an expression cassette for ß-Galactosidase (ß-Gal) driven by the human CMV promoter and MMTV promoter, respectively (S. Wieland and S. Rusconi, unpublished). In both plasmids the ß-Gal ORF is followed by the rabbit ß-globin splice signal and 3' poly-adenylation signal. Plasmid CMVLuc (Fig. 2A, construct 4; kind gift of M. Imhof) expresses firefly luciferase (Luc) under the control of the human CMV promoter and has an SV40 polyadenylation signal. All the plasmids were purified by CsCl. Plasmid sequences and construction details are available on request.

Recombinant adenoviruses: All recombinant adenoviruses were generated as cloned full length recombinant genomes (referred to as "virusmids") in E. coli (38) from a PacI flanked, E1E3 deleted adenovirus type 5 backbone (vmRL-CMV1; Brenz Verca, et al., in preparation). AdGR (Fig. 2B, construct 5) and AdGR(Ala) (Fig. 2B, construct 6) were generated by recombining vmRL-CMV1 with the corresponding expression vectors for wild type and Ala-mutant Glucocorticoid Receptor, respectively. To avoid toxicity problems by expressed transcription factors, we engineered the insert into a repressible cassette that allows silent propagation in a Tet-Repressor expressing packaging cell line (S. Brenz Verca, in preparation). The construction of AdGFP (Fig. 2B, construct 7) from virusmid vmRL-CMV1 is described in detail elsewhere (39). AdGFP expresses Clontech's EGFP mutant under control of the human CMV promoter. AdLacZ (Fig. 2B, construct 8) was constructed on an analogous way from virusmid vmRL-CMV1. Viruses were propagated and purified by standard methods (40). The lysates were titrated as described elsewhere (S. Brenz Verca, in preparation) by an end-point cytopathic assay (41, 42), aliquoted and stored at –70 °C.

Preparation of DR9NP-crosslinked DNA.

The steroid derivative DR9NP (6-{[(dexamethasone)carbonyl]amino}hexyl(2,5,9-trimethylpsoralen)methylcarbamate; patent pending) was synthesized as described (43). DR9NP was resuspended in Ethanol to a concentration of 10^{-3}M. Five different irradiation cocktails were made by mixing 100 µg CMVLacZ with 0, 0.04, 0.4, 4 or 40 nmoles DR9NP in 4.2 ml H_2O. The final concentrations in the cocktails were 25 ng/ml for DNA and 0M, 10^{-8}M, 10^{-7}M, 10^{-6}M or 10^{-5}M for DR9NP, respectively. Each cocktail was transferred into a well of a 6-well tissue culture plate (TPP). The plate was then covered by its lid and put on top of an ice-bed in a plastic box inside a crosslinker apparatus (BIO-LINK, BLX) equipped with four UV lamps (type T-8.L, l=365 nm). The cooled plate was then irradiated for 1 hour with an approximate UV-intensity of 12 J/cm^2. The crosslinked products were extracted twice with Chloroform/Isoamyl alcohol (24:1), precipitated with ethanol and resuspended in TE (10 mM Tris-HCl, 1 mM EDTA). For characterization, the resulting DR9NP-crosslinked CMVLacZ preparations were linearized by restriction enzyme digestion, alkali-denatured and analyzed by gel electrophoresis.

Cell culture, adenoviral infections, transfections and reporter assays.

Cell culture: HeLa cells and CV-1 cells were grown in DMEM (GIBCO) supplemented with 2.5% Fetal Calf Serum 2.5% New Born Calf Serum, 1% Penicillin/Streptomycin and 1% L-Glutamine. Cells were maintained at 37 °C in a 5% CO_2 100% humidified air atmosphere.

Adenoviral infections: 100'000 CV-1 cells per well were seeded in 24-well tissue culture plates to reach 60-80% confluence during transfection. At time point 0h (see Fig. 4A for experimental outline), cells were incubated with DMEM (3% FCS) supplemented with adenoviral particles to reach a Multiple Of Infectivity (MOI) of 14. At time point 15h, cells were rinsed twice with Tris Buffered Saline (TBS).

Transfections: At time point 19h, cells were lipofected with the transfection reagent TRANSFAST (Promega) following the manufacturer's instructions, with a DNA to cationic lipid charge ratio of 1:2. Briefly, 0.1 µg naked or DR9NP-crosslinked CMVlacZ, 1 µg CMVLuc and 0.9 µg sonicated calf thymus DNA as carrier were covered with a mixture containing 12 µl TRANSFAST and 2 ml serum-free medium. 200 µl of this transfection cocktail, containing 10 ng CMVLacZ and 100 ng CMVLuc, were added to each well. In the samples that included more than 10 ng LacZ reporter plasmid, the amount of carrier DNA was proportionally diminished. After 1 hour, the cells were rinsed twice with TBS and supplemented with prewarmed complete medium.

Reporter assays: At time point 48h, cells were harvested and the activities of ß-Galactosidase (ß-Gal) and Luciferase (Luc) were determined simultaneously with the chemiluminescent gene reporter assay Dual Light (Tropix, Perkin Elmer) following the manufacturer's instructions. Typical measured values were between 10^6 and 10^7 RLU / mg protein for Luc and 10^5-10^6 RLU / mg protein for ß-Gal. For normalization, the values obtained for ß-Gal were divided by the values obtained for Luc.

Nuclear translocation assay.

HeLa cells were trypsinized and seeded at around 20-30% confluence into small (diameter of 5 cm) tissue culture dishes. Cells were allowed to adhere to the dish for 4-6 hours. Cells were then transfected by calcium phosphate with 5 µg CMVGR(Ala)GFP, 1 µg of an expression vector encoding for SV40 T-antigen and 4 µg of sonicated calf thymus DNA as carrier. The precipitate was allowed to deposit on the cells for 15 hours (37 °C, 5% CO_2). The cells were then rinsed twice with TBS, supplemented with fresh medium and further incubated under standard conditions. 48 hours after transfections, cells were fixed with 2% Formaldehyde / 0.2% Glutaraldehyde and inspected in the presence of UV-light, using a Zeiss Axiovert fluorescence microscope. Where mentioned, Dexamethasone or DR9NP have been added 1 hour before fixation, to get a final steroid concentration of $5x10^{-7}M$ in the cell medium.

DNA-labeling, confocal microscopy and image analysis.

Naked and DR9NP-crosslinked CMVLacZ plasmids were labeled with Rhodamine using the Label-It reagent (Panvera) following the manufacturer's instructions. Briefly, the plasmid (5 µg) was diluted to a final concentration of 0.1 mg/ml, and 50 µl of the labeling reagent were added. The labeling reaction proceeded for 1 hour at 37 °C, followed by Microspin Column purification (Sephadex G50) to remove incorporated fluorescence. According to the manufacturer, by using the labeling reagent to DNA ratio of 1:1 (v:w), a labeling efficiency of one Rhodamine molecule per 20-60 base pairs should be achieved. The overall efficiency of Rhodamine labeling was qualitatively compared by agarose electrophoresis in absence of EtBr. CV-1 cells cultured on slides (placed in the wells of a 24-well tissue culture plate) were lipofected with the Rhodamine-labeled vectors as described above (see *Cell culture, adenoviral infections, transfections and reporter assays*). Considering the losses during the extraction procedure, the amount of transfected Rhodamine-labeled DNA was estimated to be between 30 and 40 ng per well. 24 hours after transfection, the cells were fixed with 4% Formaldehyde and examined using a BIO-RAD 1024, argon / krypton confocal laser scanning fluorescence microscope (LSCM, BIO-RAD Microsciences Division Ltd), with K1 and K2 dual filter blocks. To quantitatively evaluate the cellular localization of the Rhodamine signals (see Fig. 5 and Table 1), low magnification (x 20 objective), central optical sections of 500 nm were taken. The original coloured pictures were converted electronically to black and white pictures, so that Rhodamine-labeled vectors appeared like punctuate white signals (see Fig. 5A). For the precise cellular localization of Rhodamine signals (see Fig. 6), higher magnification (x 60 or 100 oil objective), 30 to 50 Z-series consecutive optical sections of 200 nm thickness were taken. To visualize nuclei and general cellular structures, cells were previously stained with acridine orange. The LSCM was set to All-Lines of Excitation of the argon / krypton laser, with acridine orange stained cells detected at 605 nm and Rhodamine signals detected at 680 nm. Each optical section was cleaned using a 3x Kalman averaging filter to improve the signal / noise ratio. The possibility of fluorescence bleed from one fluorochrome to the other was controlled by initially visualising individual fluorochromes in single channel mode using either a 568 nm or a 647 nm excitation filter for acridine orange or Rhodamine, respectively. Collected z-series optical sections were superimposed using the Laser-Sharp-Processing software (BIO-RAD) to enable the reconstructed three-dimensional relationships to be visualised as two-dimensional photomicrographs. Confocal optical sections were also imported into the image analysis software Imaris (Bitplane AG, CH), where cut planes of the confocal images were viewed in both the x and y axis to determine the precise localization of the signals. No filters or image correction were applied to the images.

Literature References

1. Zabner, J., Fasbender, A.J., Moninger, T., Poellinger, K.A. & Welsh, M.J. Cellular and molecular barriers to gene transfer by a cationic lipid.*J Biol Chem* **270**, 18997-9007 (1995).
2. Capecchi, M.R. High efficiency transformation by direct microinjection of DNA into cultured mammalian cells.*Cell* **22**, 479-88 (1980).
3. Felgner, P.L., et al. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure.*Proc Natl Acad Sci U S A* **84**, 7413-7 (1987).
4. Wilke, M., Fortunati, E., van den Broek, M., Hoogeveen, A.T. & Scholte, B.J. Efficacy of a peptide-based gene delivery system depends on mitotic activity.*Gene Ther* **3**, 1133-42 (1996).
5. Sebestyen, M.G., et al. DNA vector chemistry: the covalent attachment of signal peptides to plasmid DNA.*Nat Biotechnol* **16**, 80-5 (1998).
6. Zanta, M.A., Belguise-Valladier, P. & Behr, J.P. Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus.*Proc Natl Acad Sci U S A* **96**, 91-6 (1999).
7. Branden, L.J., Mohamed, A.J. & Smith, C.I. A peptide nucleic acid-nuclear localization signal fusion that mediates nuclear transport of DNA.*Nat Biotechnol* **17**, 784-7 (1999).
8. Coonrod, A., Li, F.Q. & Horwitz, M. On the mechanism of DNA transfection: efficient gene transfer without viruses.*Gene Ther* **4**, 1313-21 (1997).
9. Lechardeur, D., et al. Metabolic instability of plasmid DNA in the cytosol: a potential barrier to gene transfer.*Gene Ther* **6**, 482-97 (1999).
10. Whittaker, G.R. & Helenius, A. Nuclear import and export of viruses and virus genomes.*Virology* **246**, 1-23 (1998).

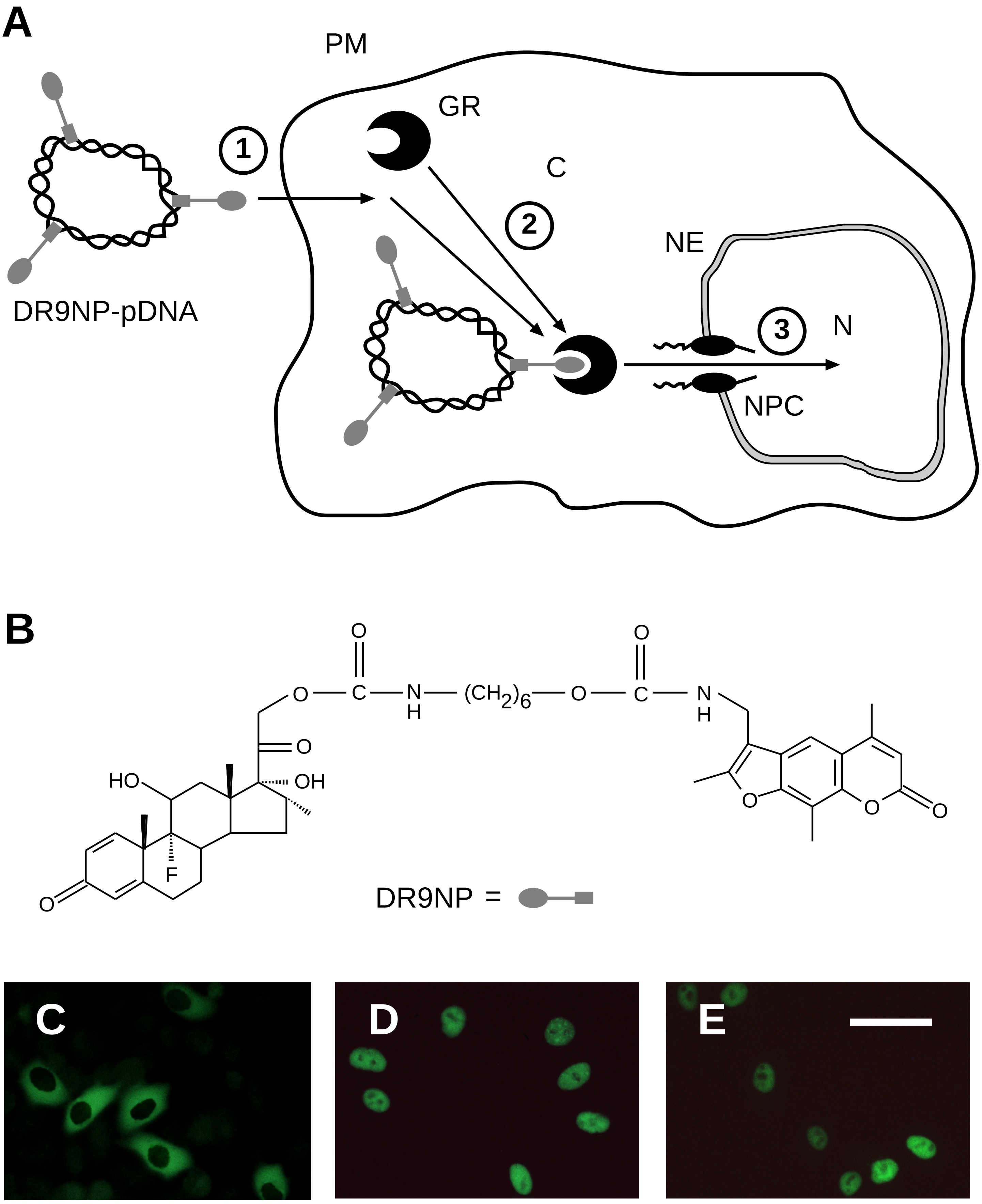
11. Wagner, E. Ligand-polycation conjugates for receptor-targeted gene transfer. in *Nonviral vectors for gene therapy* (ed. Huang/Hung/Wagner) 208-227 (Academic press, 1999).
12. Mangelsdorf, D.J., et al. The nuclear receptor superfamily: the second decade.*Cell* **83**, 835-9 (1995).
13. Zennou, V., et al. HIV-1 genome nuclear import is mediated by a central DNA flap.*Cell* **101**, 173-85 (2000).
14. Wright, A.P., et al. Structure and function of the glucocorticoid receptor.*J Steroid Biochem Mol Biol* **47**, 11-9 (1993).
15. Sackey, F.N., Hache, R.J., Reich, T., Kwast-Welfeld, J. & Lefebvre, Y.A. Determinants of subcellular distribution of the glucocorticoid receptor.*Mol Endocrinol* **10**, 1191-205 (1996).
16. Lanz, R.B., Wieland, S., Hug, M. & Rusconi, S. A transcriptional repressor obtained by alternative translation of a trinucleotide repeat.*Nucleic Acids Res* **23**, 138-45 (1995).
17. Hearst, J.E. Psoralen photochemistry.*Annu Rev Biophys Bioeng* **10**, 69-86 (1981).
18. Giguere, V., Hollenberg, S.M., Rosenfeld, M.G. & Evans, R.M. Functional domains of the human glucocorticoid receptor.*Cell* **46**, 645-52 (1986).
19. Miesfeld, R., et al. Genetic complementation of a glucocorticoid receptor deficiency by expression of cloned receptor cDNA.*Cell* **46**, 389-99 (1986).
20. Zelphati, O., Liang, X., Hobart, P. & Felgner, P.L. Gene chemistry: functionally and conformationally intact fluorescent plasmid DNA.*Hum Gene Ther* **10**, 15-24 (1999).
21. Ludtke, J.J., Zhang, G., Sebestyen, M.G. & Wolff, J.A. A nuclear localization signal can enhance both the nuclear transport and expression of 1 kb DNA.*J Cell Sci* **112**, 2033-41 (1999).
22. Ziemienowicz, A., Gorlich, D., Lanka, E., Hohn, B. & Rossi, L. Import of DNA into mammalian nuclei by proteins originating from a plant pathogenic bacterium.*Proc Natl Acad Sci U S A* **96**, 3729-33 (1999).

23. Kohler, M., et al. Cloning of two novel human importin-alpha subunits and analysis of the expression pattern of the importin-alpha protein family.*FEBS Lett* **417**, 104-8 (1997).
24. Wu, G.D. A nuclear receptor to prevent colon cancer.*N Engl J Med* **342**, 651-3 (2000).
25. Henderson, B.E. & Feigelson, H.S. Hormonal carcinogenesis. *Carcinogenesis* **21**, 427-33 (2000).
26. Brinkmann, A.O. & Trapman, J. Prostate cancer schemes for androgen escape [news; comment].*Nat Med* **6**, 628-9 (2000).
27. Reid, P., Kantoff, P. & Oh, W. Antiandrogens in prostate cancer.*Invest New Drugs* **17**, 271-84 (1999).
28. Kurebayashi, J., et al. Expression levels of estrogen receptor-alpha, estrogen receptor-beta, coactivators, and corepressors in breast cancer.*Clin Cancer Res* **6**, 512-8 (2000).
29. Speirs, V., Malone, C., Walton, D.S., Kerin, M.J. & Atkin, S.L. Increased expression of estrogen receptor beta mRNA in tamoxifen- resistant breast cancer patients.*Cancer Res* **59**, 5421-4 (1999).
30. Subramanian, A., Ranganathan, P. & Diamond, S.L. Nuclear targeting peptide scaffolds for lipofection of nondividing mammalian cells.*Nat Biotechnol* **17**, 873-7 (1999).
31. Vacik, J., Dean, B.S., Zimmer, W.E. & Dean, D.A. Cell-specific nuclear import of plasmid DNA.*Gene Ther* **6**, 1006-14 (1999).
32. Kovacs, A.M. & Zimmer, W.E. Cell-specific transcription of the smooth muscle gamma-actin gene requires both positive- and negative-acting cis elements.*Gene Expr* **7**, 115-29 (1998).
33. Langle-Rouault, F., et al. Up to 100-fold increase of apparent gene expression in the presence of Epstein-Barr virus oriP sequences and EBNA1: implications of the nuclear import of plasmids.*J Virol* **72**, 6181-5 (1998).

34. Hagstrom, J.E., et al. Nuclear import of DNA in digitonin-permeabilized cells.*J Cell Sci* **110**, 2323-31 (1997).
35. Lasic, D.D. Structure and structure-activity relationships of lipid-based gene delivery systems. in *Nonviral vectors for gene therapy* (ed. Huang/Hung/Wagner) 69-89 (Academic press, 1999).
36. Labat-Moleur, F., et al. An electron microscopy study into the mechanism of gene transfer with lipopolyamines.*Gene Ther* **3**, 1010-7 (1996).
37. Heim, R., Cubitt, A.B. & Tsien, R.Y. Improved green fluorescence.*Nature* **373**, 664 (1995).
38. Chartier, C., et al. Efficient generation of recombinant adenovirus vectors by homologous recombination in Escherichia coli.*J Virol* **70**, 4805-10 (1996).
39. Heider, H., Brenz Verca, S., Rusconi, S. & Asmis, R. Comparison of lipid-mediated and adenoviral gene transfer in human monocyte-derived macrophages and COS-7 cells.*Biotechniques* **28**, 260-5, 268-70 (2000).
40. Graham, F. & Prevec, L. Manipulation of adenovirus vectors. in *Methods in molecular biology* (ed. Murray, E.J.) 109-128 (Humana Press, Clifton, NJ, 1991).
41. Burleson, F., Chambers, T. & Wiedbrauk, D. Virology: A laboratory manual.*Virology: A laboratory manual* (Academic Press, London, NY, San Diego, 1992).
42. Precious, B. & Russell, W.C. Growth, Purification ant titration of adenoviruses. in *Virology: a practical approach* (ed. Mahy, B.W.J.) (IRL press, Oxford, 1991).
43. Frey, F., Frey, B., Wehrli, H.-U. & Rusconi, S. *Conjugates of DNA interacting groups with steroid hormones for use as nucleic acid transfection agents*. PCT/CH WO 00/11018 (2000)

Acknowledgments

We are indebted to R. Heim (UCSD) and M. Imhof (EPFL Lausanne), respectively for the gift of the GFP cDNA and expression vector for luciferase. Our thanks go also to M. Bueno and B. Huse (UNIFR, Biochemistry) for the construction of S65T mutant of GFP and assembly of recombinant adenoviral vectors for GR, respectively. The contributions of P. Matthey (UNIFR, Biochemistry, for excellent technical assistance), P. De Los Rios (UNIFR, Physics, for useful mathematical coaching) and M. Woodle (GTI-Novartis, for crucial conceptual and technical discussions) are also gratefully acknowledged. This work has been supported by the Canton of Fribourg, the Canton of Bern, the Inselspital, the private funds of the 'Ruscolab', and in large part by the Swiss National Research Foundation, grants Nr: 4037-49003 and 4037-044802, both belonging to the National Research Program NFP37 'somatic gene therapy' www.unifr.ch/nfp37.


A
PM
GR
C
①
②
③
NE
N
NPC
DR9NP-pDNA
B
O
C
N
H
(CH2)6
O
HO
OH
F
DR9NP =
C
D
E

No hormone

+ Dexamethasone

+ DR9NP

Figure 1. The principle of SMGD and structure and properties of the bifunctional steroid derivative DR9NP. (A) Schematic representation of the cellular events involved in SMGD. Several DR9NP molecules are crosslinked to plasmid DNA (pDNA). The resulting complex DR9NP-pDNA is transported into the cytoplasm by conventional transfection (1). Cytoplasmically located GR binds to the steroid moiety of DR9NP-pDNA (2). Steroid recognition results in nuclear translocation of GR and associated DR9NP-pDNA (3). Abbreviations: PM, Plasma Membrane; GR, Glucocorticoid Receptor; C, Cytoplasm; NE, Nuclear Envelope; NPC, Nuclear Pore Complex; N, Nucleus. (B) DR9NP (see Experimental Procedures for extended nomenclature) is composed by the steroid hormone Dexamethasone (fluorinated polycyclic structure at the left) linked via urethane linkage to a 9 atoms long spacer to the DNA-crosslinking molecule Psoralen (polycyclic structure at the right). (C,D,E) Cellular localization of transiently transfected GR(Ala)GFP in absence of hormone (C), in presence of either $5x10^{-7}$ M Dexamethasone (D) or $5x10^{-7}$ M DR9NP (E). Scale bar: 50 µm.

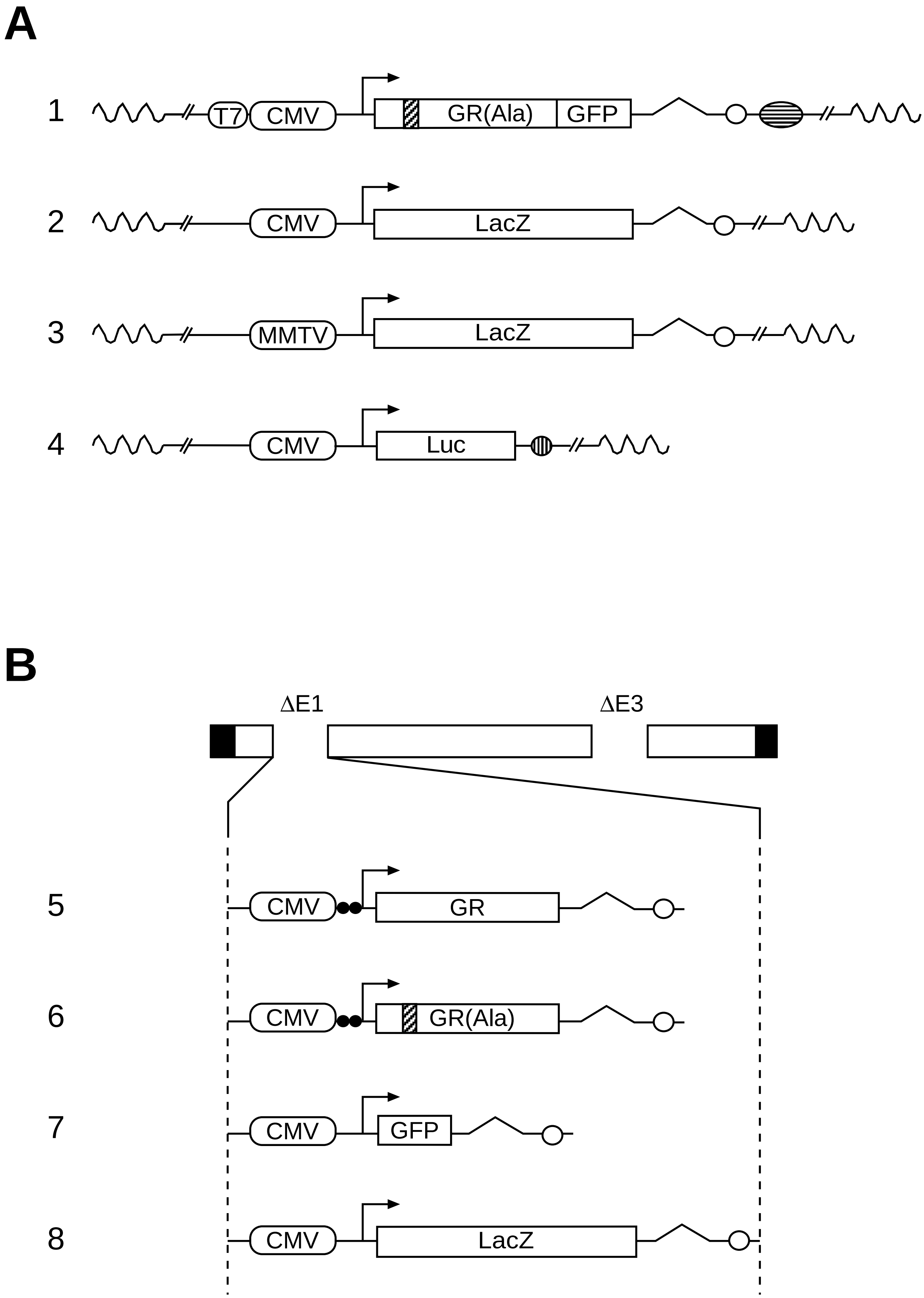

A
1
T7
CMV
GR(Ala)
GFP
2
CMV
LacZ
3
MMTV
LacZ
4
CMV
Luc
B
ΔE1
ΔE3
5
CMV
GR
6
CMV
GR(Ala)
7
CMV
GFP
8
CMV
LacZ

Figure 2. Utilised plasmids and recombinant adenoviruses. (A) Reporter plasmids. The linear maps outline the essential biological elements but are drawn out of scale. Vector names: 1, CMVGR(Ala)GFP; 2, CMVLacZ; 3, MMTVLacZ; 4, CMVLuc. (B) Recombinant adenoviruses. The genome of the recombinant Ad5 is schematised out of scale. Vector names: 5, AdGR; 6, AdGR(Ala); 7, AdGFP; 8, AdLacZ. Symbols and abbreviations: wavy line, plasmid sequence; T7, T7 bacteriophage RNA polymerase promoter; CMV, Cytomegalovirus promoter/enhancer; bent arrow, transcription start; hatched box, poly-alanine stretch; GR(Ala)GFP, fusion of GR(Ala) and GFP; broken line, rabbit beta-globin splice signal; empty circle, rabbit beta-globin 3' polyadenylation signal; hatched oval, SV40 origin of replication; LacZ, Beta-Galactosidase gene; MMTV, Mouse Mammary Tumour Virus inducible promoter; Luc, Luciferase gene; hatched circle, SV40 polyadenylation signal; filled box, adenoviral inverted terminal repeat (ITR); ΔE1, deletion of adenoviral E1 region; ΔE3, deletion of adenoviral E3 region; filled circles, 2x tet-operators; GR, Glucocorticoid Receptor gene; GR(Ala), GR dominant negative mutant (16); GFP, Green Fluorescent Protein gene.

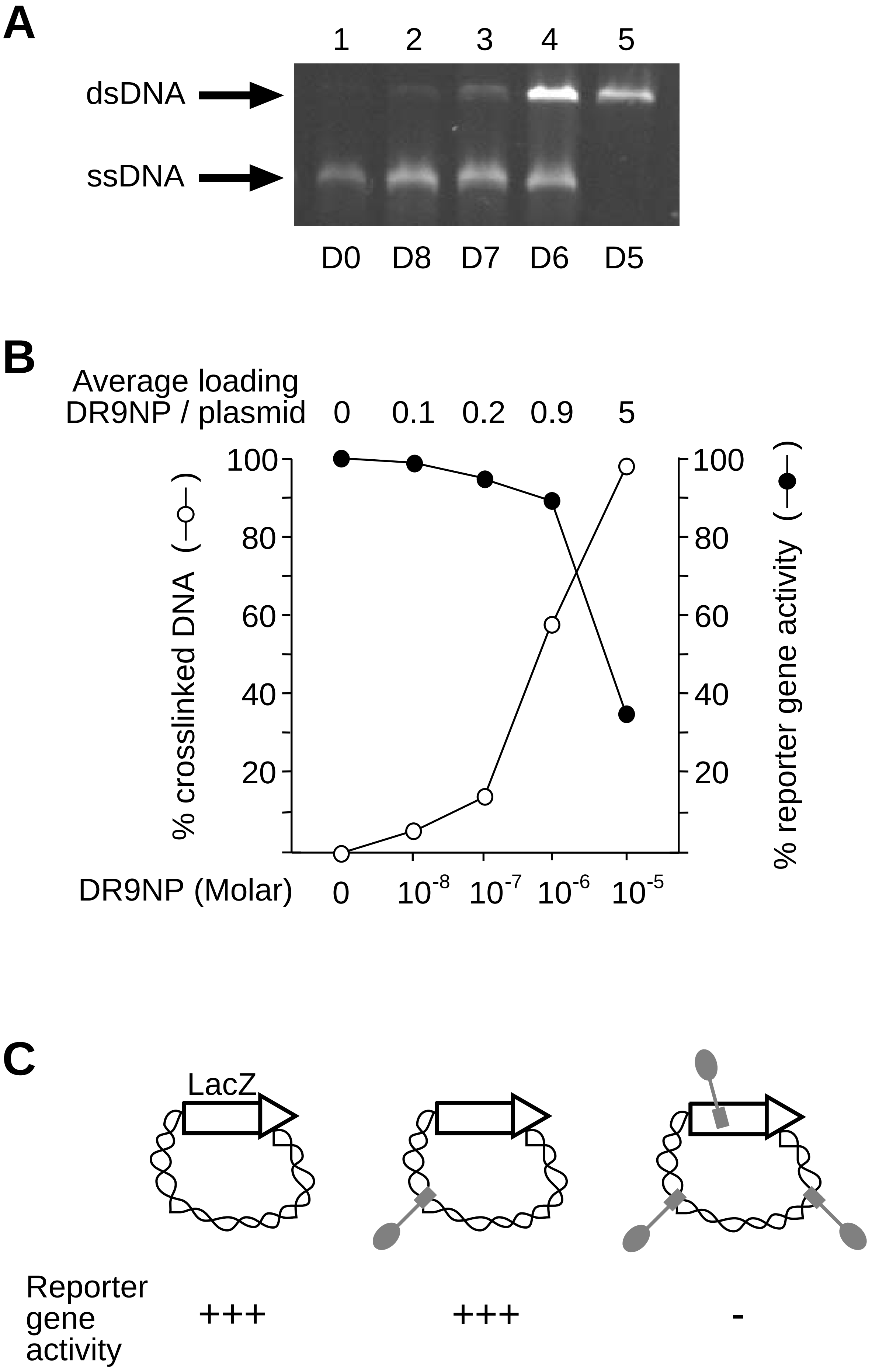

A
1 2 3 4 5
dsDNA
ssDNA
D0 D8 D7 D6 D5
B
Average loading
DR9NP / plasmid 0 0.1 0.2 0.9 5
% crosslinked DNA
% reporter gene activity
100
80
60
40
20
DR9NP (Molar) 0 10^{-8} 10^{-7} 10^{-6} 10^{-5}
C
LacZ
Reporter gene activity
+++
+++
-

Figure 3. Structural and functional characterization of DR9NP-crosslinked CMVLacZ. (A) 1% Agarose gel electrophoresis of linearized alkali-denatured CMVLacZ (10 kb), UV-crosslinked in presence of various concentrations of DR9NP. The resulting DR9NP-crosslinked CMVlacZ preparations were named D0 (UV-irradiation in absence of DR9NP, lane 1), D8 (in presence of 10^{-8} M DR9NP, lane 2), D7 (10^{-7} M DR9NP, lane 3), D6 (10^{-6} M DR9NP, lane 4) and D5 (10^{-5} M DR9NP, lane 5). (B) Fraction of DR9NP-crosslinked CMVLacZ (empty circles) and percentage of residual ß-Galactosidase (ß-Gal) reporter activity (black circles). The intensity of all bands shown in (A) has been quantified by densitometry. The percentage of crosslinked DNA has been calculated for each vector by dividing the intensity of the dsDNA by the sum of the intensities of the dsDNA and ssDNA signal. The reporter gene activity was determined by transiently transfecting CV-1 cells with equal amounts of the different preparations and by measuring the expressed ß-Gal with a chemiluminescent reporter gene assay system. The percentages of reporter gene activity have been normalized by dividing each activity by the one measured for D0, which is defined as 100%. The average number of crosslinked DR9NP molecules per plasmid has been calculated mathematically. If we assume that the DR9NP molecules are crosslinked on pDNA following the rules of Poisson's distribution, then the probability p to have n crosslinks is defined as $p(n,L) = (L^n / n!) * e^{-L}$, where L = average number of crosslinks. For p=0, $L= -\ln\{p(0)\}$, where $p(0)=1 - (\% \text{ crosslinked DNA} / 100)$, which corresponds to the probability to have no crosslinks. (C) Illustration of the three classes of possible molecules in crosslinked preparations. The reporter gene activity of CMVLacZ without any crosslink (left), with DR9NP crosslinked outside the reporter gene (center) and with at least one crosslink in the LacZ gene (right). Symbols: +++, active; -, inactive.

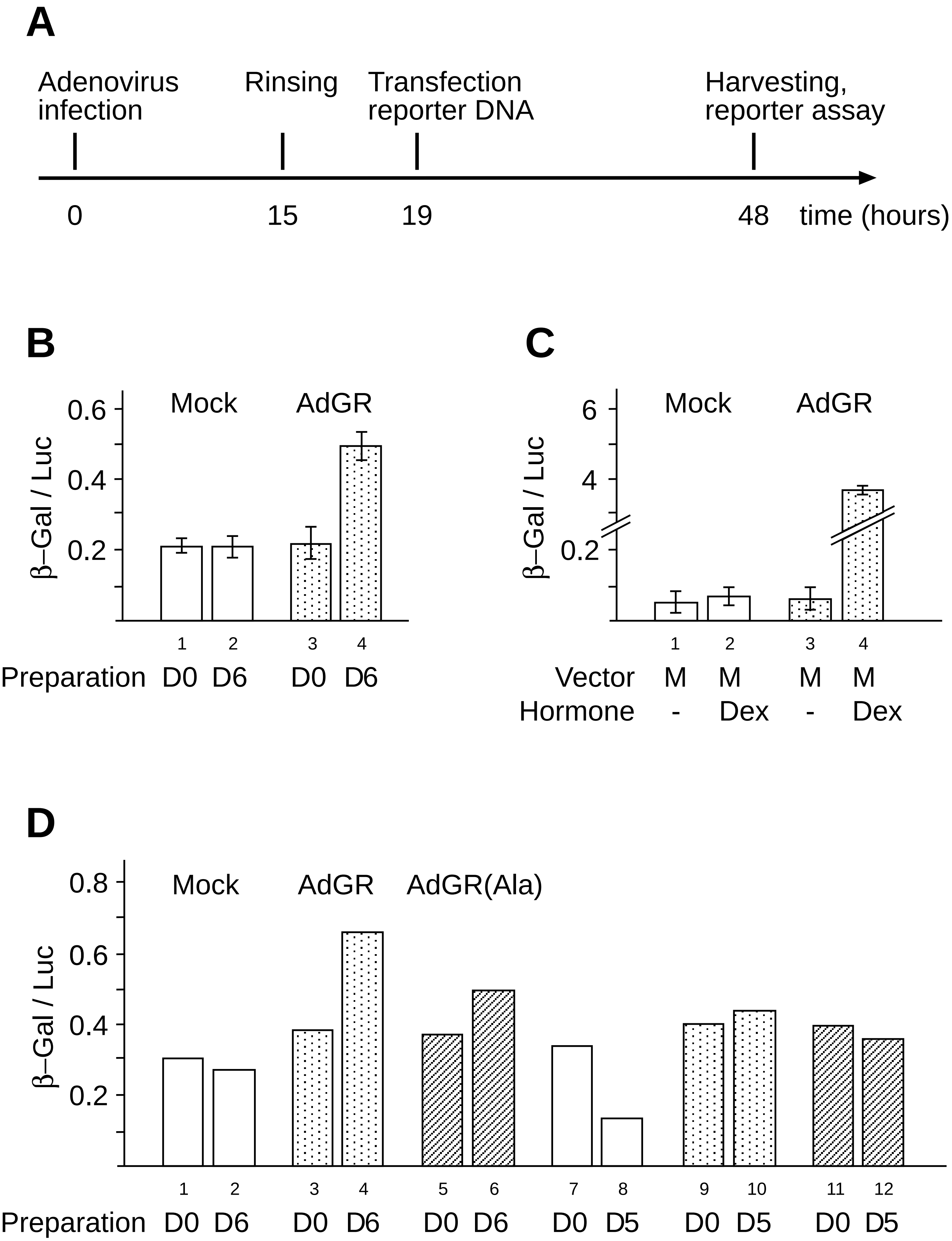

A
Adenovirus infection
Rinsing
Transfection reporter DNA
Harvesting, reporter assay
0
15
19
48
time (hours)
B
β–Gal / Luc
0.6
0.4
0.2
Mock
AdGR
1
2
3
4
Preparation
D0
D6
D0
D6
C
β–Gal / Luc
6
4
0.2
Mock
AdGR
1
2
3
4
Vector
M
M
M
M
Hormone
-
Dex
-
Dex
D
β–Gal / Luc
0.8
0.6
0.4
0.2
Mock
AdGR
AdGR(Ala)
1
2
3
4
5
6
7
8
9
10
11
12
Preparation
D0
D6
D0
D6
D0
D6
D0
D5
D0
D5
D0
D5

Figure 4. The activity of DR9NP-decorated reporter plasmid is enhanced in presence of GR. (A) Experimental outline. At time point 0h, CV-1 cells cultured in 24-wells plates were infected either with AdGFP (Mock) or AdGR both at MOI = 14. Cells were rinsed (15h) and subsequently lipofected (19h) with either 10 ng uncrosslinked CMVlacZ (D0) or 10 ng DR9NP-crosslinked CMVlacZ (D6). 100 ng uncrosslinked CMVLuc were added to each transfection cocktail as an internal reference. Cells were harvested (48h) and the activities of ß-Gal and Luc were determined simultaneously with a chemiluminescent reporter gene assay system. Typical measured values were 10^6 - 10^7 RLU / mg protein for LUC and 10^5 – 10^6 RLU / mg protein for ß-Gal. (B,C,D) Histograms representing normalized ß-Gal / Luc values. (B) Enhancement of reporter activity for the DR9NP-decorated CMVlacZ in presence of GR. (C) Monitoring the activity of the adeno-transduced GR. After infection either with AdGFP (Mock) or AdGR at MOI = 14, CV-1 cells were lipofected with 300 ng MMTVLacZ, where the reporter gene LacZ is under the control of the GR inducible MMTV promoter (construct 3 in Fig. 2A). After incubation in presence or absence of Dex, the cells were harvested and the reporter gene activities were quantified. In absence of GR, Dex has no influence on the reporter gene activity and only basal levels of ß-Gal are detectable (bars 1 and 2). In contrast, in presence of GR, Dex mediates a 80-fold enhancement of reporter expression (compare bars 3 and 4), indicating that GR is expressed and is functional. The presence of GR in the infected cells has been also confirmed by immunodetection studies (our unpublished data). (D) Comparison of D0, D5 and D6 reporter activities in absence of GR or in presence of GR and GR(Ala). Results in (B) and (C) are the averages (± SD) of 3 independent experiments. Symbols and abbreviations: h, hour; MOI, Multiple Of Infectivity; ß-Gal, ß-Galactosidase; Luc, Luciferase; M, MMTVLacZ; Dex, Dexamethasone; empty bar, Mock infected; shaded bar, AdGR infected; hatched bar, AdGR(Ala) infected.

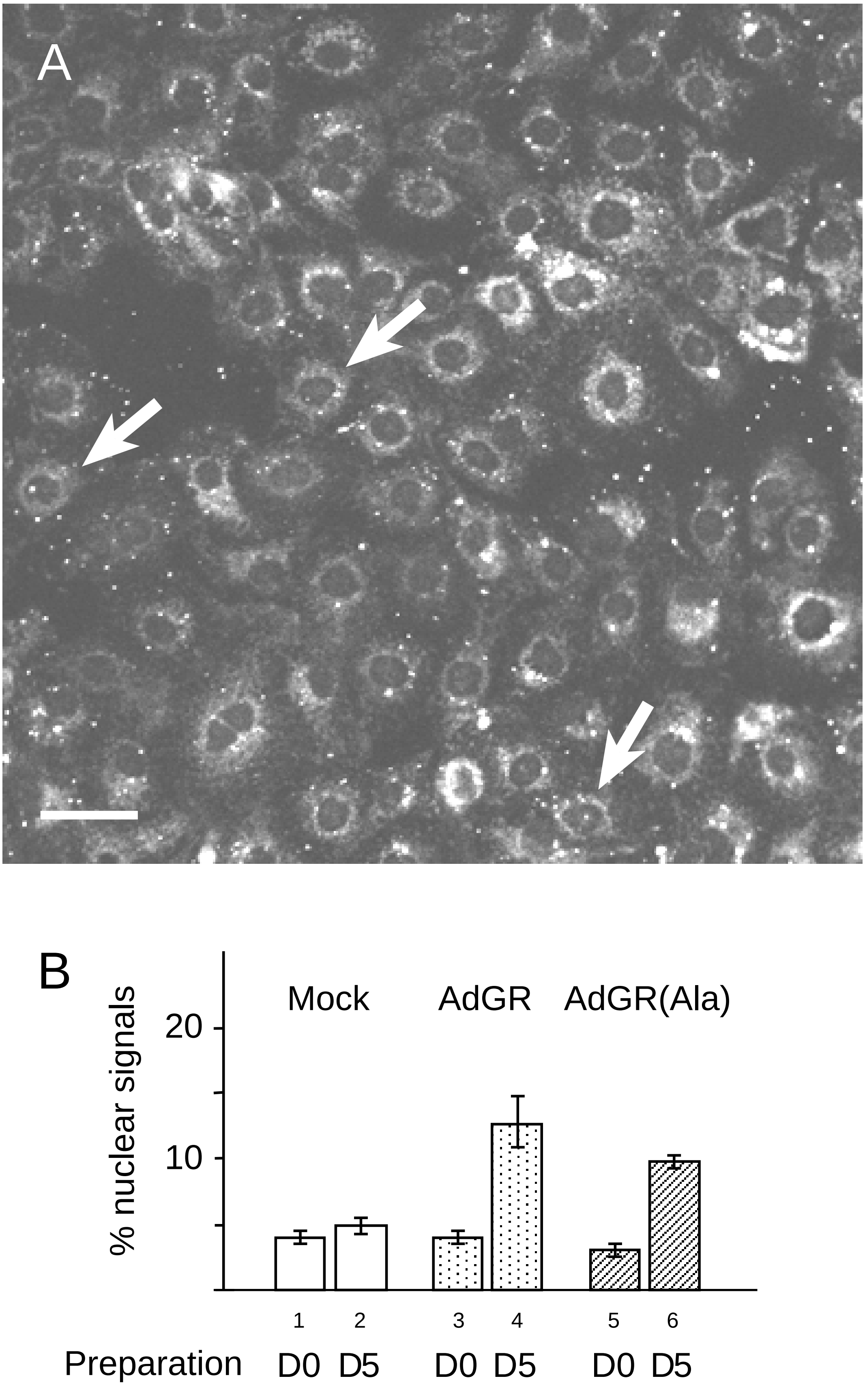

A
B
% nuclear signals
20
10
Mock
AdGR
AdGR(Ala)
1
2
3
4
5
6
Preparation
D0
D5
D0
D5
D0
D5

Figure 5. The nuclear accumulation of DR9NP-decorated plasmid DNA is enhanced in presence of GR. CV-1 cells cultured on slides were infected with AdLacZ (Mock), AdGR or AdGR(Ala) at MOI 14 and subsequently lipofected with Rhodamine-labeled D0 or D5. The experimental time schedule was the same as described in Figure 4. Cells were fixed with 4% Formaldehyde and imaged with a confocal laser scanning microscope. The resulting images were wide field pictures of central optical sections. For the quantitative evaluation, the original coloured pictures were converted to black and white pictures, so that Rhodamine-labeled vectors appear like punctuate white signals. (A) Confocal microscope image of CV-1 cells lipofected with Rhodamine-labeled D5 without any infection. Arrows indicate examples of cells with clear nuclear signals. Scale bar: 50 µm. (B) Histogram of evaluated pictures as in (A). Percentage of CV-1 cells with nuclearly localized D0 or D5 under different conditions. For each condition 5 pictures were taken randomly. For the quantitative evaluation, the cellular localization of signals within 150 cells (30 cells for each picture) has been determined. The cellular localization of the signals has been subdivided into three classes: cytoplasmic, perinuclear or nuclear. Representative examples of each class are shown in Fig. 6A. The reported percentage is the average (±SD) of percentages of nuclear signals. The details of all counted signals are reported in Table 1.

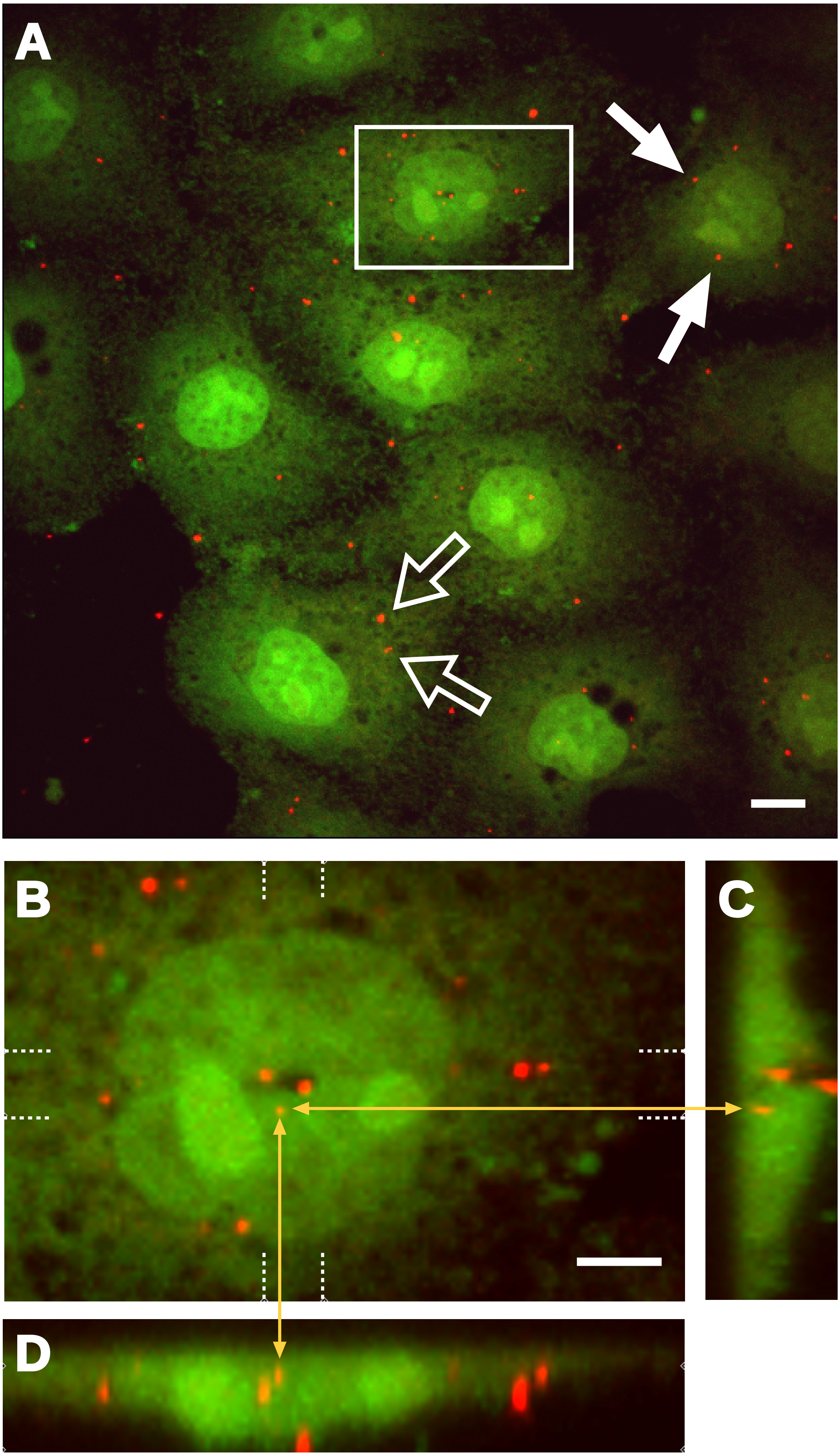

A
B
C
D

Figure 6. Bi- and tri-dimensional viewing of nuclear localized plasmid DNA after transfection. CV-1 cells cultured on slides were infected with AdGR and subsequently lipofected with Rhodamine-labeled D5. Cells were fixed with 4% Formaldehyde and stained with acridine orange to visualize nuclei and general cellular structures. Cells were imaged with a confocal laser scanning microscope. The images are composites of 35 optical sections obtained at 200 nm intervals through the Z-axis of the cells. Rhodamine-labeled vectors appear like punctuate red signals. (A) Wide field confocal microscope image. The rectangle highlights a cell with 3 apparent nuclear localized signals. Closed arrows indicate examples of perinuclear localized signals. Open arrows indicate examples of cytoplasmic localized signals. (B) Enlargement of the cell highlighted in (A). Dashed vertical lines define a 3.5 µm broad portion which has been rotated clockwise by 90 degrees through the Y-axis of the cell and is projected in (C). Dashed vertical lines define a 4 µm broad portion which has been rotated clockwise by 90 degrees through the X-axis of the cells and is projected in (D). The yellow arrows indicate the different projections of a nuclear localized signal. Scale bars are 10 µm (A) or 5 µm (B, C, D).

3.2. PAPER 2: "VECTORS FOR GENE DELIVERY"

Book chapter in *Gene Therapy for Rheumatoid Arthritis*, Ed. S. Gay , 2000, Humana Press

VECTORS FOR GENE DELIVERY

Sandro Rusconi & Maurizio Ceppi
Inst for Biochemistry
UNIFR Pérolles
1700 Fribourg, Switzerland
sandro.rusconi@unifr.ch

In: *Gene Therapy for Rheumatoid Arthritis*, Ed. S. Gay , 2000, Humana Press

Foreword

Gene therapy bases its rationale on the transfer of genetic components (genes or fragments thereof) into somatic cells, with the aim of preventing, correcting, or healing various types of disorders. After this introductory sentence you expect us to start telling you some of the marvellous achievements in setting up the tools that allow this transfer. However, before entering into the intricate details of vectorology please allow us the following clear-cut statement: “as of today there is no perfect or general vector for gene therapy and there won’t be probably any in the foreseeable future”. We hope that with this statement in mind, it will be easier for the readers to understand why there is still such a multitude of seemingly disparate efforts in establishing appropriate vehicles for the gene transfer.

Therefore, the efficacy of gene therapy largely depends on the properties of the chosen ‘vector’ for gene transfer and expression. The reader should be also reminded that there is still some ambiguity in the denomination ‘vector’ because this concept can be understood either as the mere cis-elements that compose the transferred sequence (that is the nucleic acid sequence arrangement) or as the vehicle/method which is utilised for the transfer of the required gene.

1. PROBLEMS INFLUENCING VECTOR'S CHOICE

1.1 Nucleic acids as medicine: megadaltons instead of kilodaltons

In gene therapy the drug is a segment of either DNA or RNA and this imposes major constraints in the delivery. In conventional pharmacology the drugs are molecules of limited size (hundreds of Daltons) that either freely enter into cells due to their lipophilic character or are hydrophilic and destined to either act in the extracellular space or to be imported through specific biological channels. The classical pharmacological drugs are designed to act over a relatively short time and their therapeutic concentration is usually controlled by re-administration. A termination of the administration results in a dilution and termination of the pharmacological effects. Nucleic acids do not share many of the above properties: they have a large molecular size (1 megadalton for a segment of 1500 base pairs), are destined to work in the cell nucleus but are neither lipophilic, nor can count on a physiological import system. Once delivered into the nucleus they either integrate and persist for the rest of the cell's life or are maintained episomally for variable amounts of time. Therefore, the usual pharmacological strategies only marginally apply to the delivery of these monstrous molecules. For instance, in order to render them permeable to the cell membrane, one has to either compact them into lipid-containing particles or into viral envelopes or capsids. This means that the units of delivery are no longer single, soluble molecules but relatively large (100-500 nm) and only partially soluble aggregates. This latter aspect makes the work with nucleic acids as medicines very arduous and still poorly reproducible in the complexity of a living organism. To conclude these considerations we will mention that in the jargon of the gene therapists, the transferred gene is also usually referred to as the 'transgene'. The use of this term will hopefully simplify the reading of the further paragraphs.

1.2. Correcting disorders derived from loss-of-function or gain-of-function

Genetic and acquired disorders result from an imbalance of metabolic functions, which are ultimately controlled by the genetic layout of the affected cells. The nature of the delivery vector and the properties of the transgene will largely depend on whether the therapy is aimed at inhibiting or supplementing (or enhancing) metabolic functions.

In most monogenic disorders, the phenotype is caused by a single loss-of-function that depending on the hyerarchical position of the affected gene can cause a simple or a very pleiotropic defect. For instance in cystic fibrosis or in muscle dystrophy, the lack of function of the corresponding genes results in rather circumscribed phenotypes. In this case the therapy shall be aimed at the organs that cause the most debilitating symptoms. Thus, it may be necessary to count on a rather well targeted delivery of the therapeutic supplemental gene. When trying to correct another monogenic disorder such as lack of factor VIII, the vector does not necessarily need to be targeted to the original tissue (the liver), since the corrective factor will be secreted virtually from any targeted tissue.

When trying in general to compensate loss-of-function disorders, the level of gene transfer will also not necessarily need to be 100% since in most cases a small percentage of cured cells will exert a corrective function (see also under 1.5). Therefore, there will be lower requirements in terms of efficiency of gene transfer on the chosen vector. For these reasons, monogenic disorders, though relatively rare and unattractive from the marketing point of view, have received a fair amount of attention by academically- or industrially-based gene therapists. On the other hand, when trying to control disorders derived from gain-of-function such as most hyper-proliferatory diseases (cancers, auto immune disorders etc) it will be more important to reach transfer levels close to 100% or to ensure at least that the transfected cells initiate a feed-back control on the still untransfected partners and produce a 'bystander effect' (1,2). Thus, the type of vector and the construction of the transgene have to be adapted to this task.

1.3. The choice of cis-elements: constitutive or regulated expression

The first level at which the properties of a vector are defined is the assembly of the regulatory and coding elements. In most of proof-of-principle experiments in which the transgene was a reporter gene, the promoter of choice was taken from the panel of conventionally strong constitutive champions (CMV, RSV or SV40). These promoters could be also considered for clinically valid therapeutic vectors, although their strength is strongly cell-specific. Therefore, in 'second generation' experiments we have witnessed the use of tissue-specific promoters, although with erratic results (3-6). In fact, the use of a genuinely tissue-specific control would circumvent the need of precisely targeting the delivery, since the regulation would be brought at the transcriptional level. However, our understanding of tissue specificity of transcription is restricted to relatively short cis-acting elements, whereas in chromosomal genes locus regulation occupies probably relatively extensive sequences (7,8). This is very relevant, since the currently available strategies that ensure long term expression are based on random integration in the host genome. This random integration is different in each individual transformed cell and leads to unpredictable position effects that influence the expression of the transgene (4,9-11).

As learned from conventional transgenic animal models obtained by pronuclear microinjection (in which the transferred DNA also randomly integrates), in the majority of the cases the transgenes are silenced, and only a fully equipped locus is 'protected' from these erratic influence of the flanking regions.

Such large DNA segments are not compatible with the packaging capacity of most current vectors, therefore we are momentarily 'condemned' to use surrogate mini-regulatory elements (12,13).

For some disorders, another important goal is the search of bio-sensing cis-regulatory elements that can respond to metabolic status such as hypoxia (14,15), glucose levels (16), etc. These elements will be indispensable in the assembly of artificial glands that are designed to respond to natural balances of metabolites, thereby producing factors such as insulin or Epo in physiologically relevant and homeostatically controlled amounts.

Finally, an interesting collection of externally controllable cis-elements can be found in the literature, such as promoters that can be regulated by insect hormones (17), steroid antagonists (18-20), rapamycin or tetracycline derivatives (21,22). The advantage of these systems is that the action of the transgene can be pharmacologically regulated. These have been used with variable success in animal models, where the administration of the external drugs was shown to exert the anticipated effects on the gene expression (18). Four parameters are important in these vectors: a) the magnitude of control, b) the potential for immunogenicity of the regulatory factor, c) the cross-reaction of the controlling drug with resident metabolism, d) the half-life of the drug, the regulatory gene product and the target gene product which altogether determine the rapidity of the onset and fading of the response. The systems that use vertebrate regulators such as receptor mutants that respond to steroid antagonists have some advantage in their low immunogenicity, but have some disadvantage in potential cross-reaction with resident receptors. On the other hand, the immunogenicity of the popular tet-regulatory system, which has a high magnitude of regulation but utilises a procaryotic regulatory factor, has not yet been fully assessed. This means that so far there is still a lot to optimise in this field and that no perfect system is yet available.

1.4. The three fundamental questions in gene delivery: efficiency, specificity and persistence

1 gram of tissue contains an average of 1 billion cells and the interstitial passages are rarely larger than 150-200 nm. These numbers should suffice to illustrate the first big problem in gene delivery: efficiency of transfer. To this we should add the non-specific binding of particles by the extra-cellular matrix and the consequent dilution of active principle. Finally, we should remember that there is no specific import for nucleic acids though the cell membrane and through the nuclear envelope (see 1.1 and 1.8). Therefore, it becomes evident that the best current vehicles are bio-particles that have naturally evolved the capacity to solve many of these problems: the viruses (see 1.8; 2.2 and 2.3).

Since it is not always possible to guarantee absolute specificity of gene expression (see above 1.3), we have sometimes to delegate the specificity to the delivery particle. To this aim several strategies have been designed (see 2.3.2 and 2.4.2 below).

Finally we should consider that when correcting chronic or progressively degenerative disorders, the transgene must persist and be active over a very long time, preferably for indefinite time. This is one of the most difficult tasks. Even if we manage to concoct the best regulatory regions that will prevent gene silencing through random integration in the genome (see 1.7), we cannot prevent the transformed cells to be lost by natural shedding such as in rapidly growing epithelia. This forces us to choose among two alternatives. a) target master stem cells that will be maintained throughout the renewal of the target tissue; b) accept the discomfort of periodical re-administration of the transgene. With partial exception of the bone marrow, and in spite of the spectacular recent advances in stem cell research, we are not yet able to guarantee the efficient transformation of pluripotent precursors and so far we are forced to consider re-administration as inevitable for the long term correction of most chronic disorders. Re-administration brings with itself all the unpredictabilities of the immune reactions, specially but not exclusively when working with viral vectors (see below). Thus, we can affirm that at the state of the art, there is still no single or clear solution to the long term treatment of chronic conditions.

1.5. Efficiency of transfer and persistence of expression: not always 100% required

The former paragraph could lead a pessimist to the conclusion that chronic conditions will never be treatable by gene therapy. The good news come from the fact that for some conditions such as haemophilia (23) or cystic fibrosis and many others, a fraction of the natural levels of expression is sufficient to achieve therapeutic effects. When attempting to correct those conditions, it is sufficient to guarantee between 5 and 10% of transformation of the target tissue. Therefore, the corresponding vehicles do not need to sustain a 100% transfer, although the problem of persistence of the transformed cells is still relevant.

On the other hand, there are treatments that require neither high efficiency nor specificity nor persistence of the transfer. One example is DNA-based vaccination (24,25), where a permanent effect is achieved upon transient expression of a transgene. Another spectacular example is the corrections of critical limb ischemia (26) where the ectopic and transient expression of naked DNA injected intramuscularly brings about sufficient VEGF signal to rescue ischemic tissue. Analogous protocols are currently considered for treatment of other cardio-vascular conditions where a short term treatment can produce long lasting beneficial effects. These examples should suffice to illustrate the concept that efficiency, specificity and persistence are not a major requirement in all cases of gene-assisted therapy, and this is of encouragement for all those who believe in this type of interventions.

1.6 Specificity: strategies, satisfactions and frustrations

The choice of the physical strategy for delivery determines the requirements to the vehicle. Gene transfer can be achieved *ex vivo (*for example in bone marrow explants) and in this case, the specificity and immediate immunogenicity (see below) of the vector are less relevant. In other protocols, specificity can also be achieved *in vivo* by local application (inhalation, double balloon catheter, intramuscular, intratumoral, brain stereotactic injection etc.). Also in this case the properties of the gene carrier are focussing on efficiency rather than on specificity, since this latter is defined by the administration protocol. Only in systemic delivery (intravenous injection) the problem of targeting becomes relevant. In the simplest cases, one can exploit the natural tendency of some organs such as liver and kidney to accumulate particulated drugs (27-30). However, these organs are not necessarily the targets in all disorders and this poses some serious problems of readdressing the accumulation of the transgene-bearing vehicle (see targeting under 2.3.2 and 2.4.2). The problem is double, since, not only one has to devise specific docking elements on the carrier particles, but one has also to circumvent non-specific accumulation in the above mentioned organs (see one example in (31)).

1.7. Persistence and integration: to be or not to be? In either case you'll pay a fee

As commented above a reproducible and efficient method for inserting the transgene into a defined chromosomal location is still lacking. This situation causes two side-problems: gene silencing from position effects (see also 1.3) and random insertional mutagenesis (that will be further commented under 3.2). Therefore there is not yet a satisfactory protocol that ensures indefinite persistence of the transgene without causing the two above-mentioned effects. This problem will be solved only when either locus-specific integration can be achieved (as originally hoped with the AAV vectors (32,33)) or when self-replicating and segregating artificial chromosomes (34) will be available. Until then, when we choose an integrating vector for our therapeutic protocol, we must be aware that we shall benefit of its potential to make the gene to persist but at the same time to randomly disturb resident functions and to be subject to uncontrollable position effects.

Of course we can choose to utilise a non-integrating vector such as an RNA virus, an Adenovirus (35) or an Herpes virus (36). In these cases, the transgene persists for a while but is not co-replicated when the host cell proliferates and is destined to be lost. Therefore the dilemma is in the choice of accepting the benefits of chromosomal integration and pay the fee of random silencing and insertional mutagenesis, or avoid these latter but paying the fee of non-permanent transformation.

1.8. Viral versus non-viral, who wins?

The non-viral modes of gene transfer include physical, chemical and biochemical protocols. Among the physical methods direct injection of naked DNA (37), pressure-mediated transfer (38,39), electrically enhanced transfer (40,41) and biolistic bombardment (42,43) have showed various degrees of efficiency. The chemicals/biochemical protocols include the use of cationic lipids and different compaction methods and each company or research lab claims to have the best results, although it is rare to see direct and extensive cross-comparisons in the published papers. Recently, the biochemical methods in which viral proteins are included to spike liposomes (33,44-46) have received increased attention, since they seem to promise enhanced gene transfer coupled to increased targeting. Although we can observe that in cell cultures DNA can be delivered to more than 99% of the cells, only a minor portion (3-10%) will ultimately transiently express the transgene (our unpublished results). This discrepancy is due to the second barrier in gene transfer: the nuclear envelope (37,47). It is hypothesised that the majority of the transfected DNA is degraded in the cytoplasm and is not reaching the nucleus. Among the strategies that have been recently proposed to reinforce this second transfer we shall mention the attempts to link to the DNA oligopeptides containing nuclear localisation sequences (48,49, and references therein). In our laboratory we are exploring the possibility of using resident nuclear shuttles to favour the import of the transgenes into the target cells. The strategy has been named SMGD (Steroid Mediated Gene Delivery, Figure 1) and aims at using intracellular nuclear receptors as ferrying vectors for the transfected DNA. Nuclear receptors such as the steroid receptors have nanomolar affinities for specific ligands and are nucleophilic, therefore appear to be excellent candidates for efficient and specific shuttles for macromolecules that display et their surface the cognate ligands. To achieve this, we had to devise strategies to chemically 'decorate' the transgene with ligands. So far, we have obtained encouraging results with model compounds interacting with the glucocorticoid receptor (Ceppi et al. in preparation).

2. SMALL PARADE OF CURRENTLY POPULAR VECTORS

2.1 The simplest way: delivering naked or 'biochemically dressed' DNA

2.1.1 Structures and methods

Attempts to deliver naked DNA by direct intramuscular injection have been pioneered by J Wolff (47,50, and references therein). The initial encouraging results have prompted a series of emulatory protocols aimed at exploiting this simple delivery system for gene-based vaccination. The mechanism of DNA uptake by muscles is only tentatively explained (50) has so far precluded the rational design of improvements of the efficiency. In spite of its simplicity, this method has been the first ever bringing clinically relevant results in the treatment of critical limb ischemia with ectopic expression of VEGF (26).

Besides these straightforward but highly empirical approaches, a number of ways to enhance the uptake of DNA has been adapted from the long standing experience with cell-cultures. This has led to the reformulation of various combinations of liposomes, lipoplexes and polylipoplexes (51) and to other sophisticated receptor-ligand mediated internalisation systems (52). These efforts have built an important platform of technologies for general and specific gene transfer, although the efficiency of these transfer methods is still several logs inferior to the virally-based modes.
In general, to achieve anything between 0.1 and few percent of transfected cells, one has to employ a 10exp[4] to 10exp[5] molecules per cell, whereas with viral transfer 1 to 10 particles per cell can give up to 100% transfer. This poses also the problem of the kinetics of delivery. When added in one shot, the excess molecules are either lost, degraded or can generate non-specific immune reactions. Therefore, non viral transfer usually implies the continuous delivery over a period of time. Recent advances in the design of biodegradable microspheres or encapsulating biolpolymers that progressively liberate trapped DNA has shed new perspectives on this strategy (28,53-56). Still, when working with non-viral delivery, one has to deal with the meager efficiency of nuclear transfer of the transfected DNA (see above). This problem subsists independently to the delivery method and is one of the reasons of the large ratio (active molecules per cell) that is required to achieve reasonable transformation rates. Finally, there is no specific mechanism for integration into the host genome, and this relegates non-viral transfer to the realm of transient treatments, unless some strategy to improve integration or persistence of the transgene can be established. For these reasons, transfer of naked DNA is currently only indicated for DNA-based vaccinations, which nevertheless held a phenomenal potential in the prevention of infectious diseases and cancer (25).

2.1.2 Targeting

The use of microsphere-aided delivery (see above) can help in specific augmentation of local concentration of the active molecules, thus providing a sort of topical specificity. Other ways of simple targeting are offered by the body anatomy which permits molecular treatment of mucosae, epithelia etc. When injected systemically, each liposome formulation displays minor differences in the preferences of organ accumulation (28,30). However, the common tendency is that formulations accumulate in liver and kidney and are not able to pass blood-brain barrier (36,57). This natural condition will be an important hurdle in designing specifically targeted formulations. Drastic protocols such as liver by-pass (31) are good for a proof-of-principle of how to short-circuit this problem but are too laborious and invasive to be clinically implementable in a generalised manner.
A major effort has also been devoted to the identification of molecular components of the endothelial zip-code system (58,59). Once understood, this tissue-specific marking of the vascular system could provide an elegant system of local accumulation of active particles.
At the cellular level, the best candidates protocols in particle targeting are the ones that exploit affinity of ligands for surface receptors (52). Some important improvements have been achieved by preparing 'virosomes' (33,44) in which viral proteins are decorating the surface of liposomes. If proven to be reproducibly infectious and compatible with large scale preparation, these hybrid particles can pave the way to the generation of

"artificial viruses" that may enjoy the advantages of in vitro assembly and avoid some of the disadvantages of biologically assembled viruses (commented under 2.5.3).

2.1.3 Advantages and disadvantages

The production of in vitro assembled particles can be better controlled and guaranteed be devoid of adventitious infectious pathogens. Therefore these formulations are pharmacologically safer than biologically assembled infectious particles. Secondly, there will be less constraints on the size of the transgene, which is a major problem in biologically assembled viral particles (see 2.3). Furthermore, the composition of synthetic particles can be designed to be devoid of immunogenic elements. Therefore, in vitro formulations containing nucleic acids can be considered suitable for multiple re-administrations, a property that is not yet guaranteed with the most popular viral vectors. The lack of immunogenicity should however not be overemphasised, because DNA that has been conventionally amplified in bacterial systems (the most convenient basis of molecular large scale preparation) acquires some intrinsic immunogenicity because of the loss of methylation of CpG -rich sequences (60-63) and perhaps also because of some other newly acquired bacterial methylation patterns (62, our unpublished observations). Those problems can be theoretically solved, although there is no clear picture of the mid- and long-term reaction of an immune-competent organism subjected to repeated DNA delivery that does not go through the digestive tract.

The major disadvantage of non viral transfer methods remains obviously the intrinsic low efficiency of transfer. The improvements of the last five-ten years have increased the transfer rate perhaps by a factor of ten, but there are several logs to catch up with the viral transfer (see 2.1.1). If no breakthrough comes into the field, one has to assume that non-viral gene transfer will be restricted to the treatment of conditions where efficiency of the transfer is not a crucial parameter.

2.1.4 Suitability and examples

Paradoxically to its intrinsic inefficiency, non-ßviral gene delivery has been the first treatment to demonstrate unequivocal therapeutic value. This occurred in the seminal experiments by the research team of Jeff Isner (26), in which the expression of a pro-vascularising factor (VEGF) through simple intramuscular injection of a suitable recombinant plasmid has rescued necrotising limbs already in phase I trials. We are looking forward to confirmations of these encouraging data in phase II and phase II trials, as well as their extension of other treatments in the cardiovascular field. The trials of Victor Dzau and colleagues (39) are another success story in non viral gene-assisted treatment. Artery bypass based on vein grafts currently fails in a high proportion due to the aberrant growth of the intima, induced by the higher blood pressure in the transplanted vein. The team of Dzau has pre-treated the graft with a simple pressure-mediated gene transfer and shown that the transferred genes were able to inhibit the hyper-proliferation of the smooth muscle cells, thus reducing significantly the occlusion of the graft. The transgenes chosen were either growth-inhibitory genes or decoy-oligonucleotides that transiently titrate transcriptional factors that are essential for the expression of pro-proliferatory genes.

We conclude this paragraph by mentioning the discovery of the interesting properties of the herpes viral protein VP22 (64). This protein is capable of cell-to-cell transfer and can translocate into cells even when added to the extracellular medium. This property extends to some VP22 chimeric proteins (65, and references therein). This may be just the tip of the iceberg of several new protein-mediated macro-molecular transfer systems that may solve some of the current problems of non-viral transfer, provided they can be designed to be invisible to the immune system.

2.2. Replication-defective and replication-competent viruses

2.2.1 General features of replication-defective recombinant viruses.

Viruses have evolved since millions of years to become professional gene-porters. They can exploit the most sophisticated molecular mechanisms to escape immune surveillance, to specifically dock to target tissues, to enter through cell membranes, to resist intracellular degradative enzymes, to deliver nucleic acids to the nucleus or to organise specialised compartments for genome replication and expression, to integrate into the host genome or to remain latent for several years in host organisms and to be even passaged through the germ line. Therefore, there is no better vehicle that can be envisioned by gene therapists to efficiently transfer the preferred therapeutic gene. Viruses have however a very nasty property: they are pathogenic because their capsid proteins are mostly immunogenic or toxic and their genetic reprogramming strongly disturbs the cell metabolism and cause diseases of various severity. A better knowledge of the viral genomes has permitted the distinction of segments necessary for the packaging into the capsid from those encoding replicative functions and capsid or envelope components. This has permitted to construct viruses that retain only fragments of their genome and are debilitated in some pathogenic functions. These defective viruses need to be amplified either in specialised packaging cells or in presence of helper viruses that provide *in trans* the missing functions. The situation is different for each virus and it would be too intricate to comment all the sequence geographies, thus I will take the example of the adenovirus to illustrate the steps undertaken to optimise transfer vectors for gene therapy. The adenoviral genome consists of 36 kb of linear DNA with inverted terminal repeats that are indispensable for replication and packaging into the capsid (35,66). The 'left' portion (E1/E2 in Figure 2A) contains the early genes whose expression is indispensable to prepare the conditions for genome replication (66). Other early functions are scattered in other regions and are dispensable for replication. The remaining 80% of the genome is occupied by the late genes, mostly expressed through the major late promoter (MLP, Figure 2A) and giving rise to variegated proteins through differential splicing. In packaging cells, the 'early' portion of the genome could be anchored into the chromosomes and shown to be functioning in trans. This permits the growth and assembly of viral genomes whose early segment is either missing or substituted by a transgene of interest (figure 2B). This scheme has been maintained in all the viruses of so called first and second generation. Those recombinant viruses have proven invaluable to demonstrate efficient gene transfer in animal models and also in patients (33,66-68). However, the remaining leaky segment encoding late genes confers a significant immunogenic potential to those generation I vectors. Therefore, the expression in immune competent animals is restricted to few weeks. Several strategies have been proposed to reduce this immunogenicity (66). The best solution so far has been offered by the so-called 'gutless' (also called 'helper dependent' or 'high-capacity' or 'third generation') adeno-vectors. In these constructs the entire late region is replaced by a ‘neutral’ DNA segment including the therapeutic gene(s) (Figure 2C) and the recombinant genome is grown and packaged in presence of a helper virus whose assembly is repressed by various strategies (66,67). After careful purification one can obtain significant titers of the recombinant vectors that are minimally contaminated (approximately 10exp[-4]) by the helper virus. Extremely encouraging results have been recently reported with these gutted Adenovectors that were shown to produce a permanent somatic gene alteration that can persist for several months (69,70) even in immunocompetent animals. We are confident that clinical trials involving gutted viruses will confirm the compatibility of these vectors with long term correction by gene transfer.

2.2.2 Principle of replication-competent viruses

Several viruses encode early proteins that interact with ubiquitous tumor suppressors such as P53 or retinoblastoma. By exploiting this situation, some research teams have developed recombinant viruses that maintain a conditional replicative potential whose fulfilment depends on the absence of tumor-suppressor functions. Selective or at least preferential replication has been reported for adenoviruses that retain the E1B gene (71-73), for HSV (74-77) or other RNA viruses (33). These viruses are capable of lithic growth in cells that are missing or underexpressing tumor suppressors and this property makes them attractive candidates for tumor treatments, leading to some interesting clinical protocols. Only the future will tell whether these expectations are well placed and whether these oncolytic viruses can be safely used either as stand-alone or as combination-treatment in tumor therapy.

2.3. bio-weapon 1: DNA viruses

2.3.1 Structures and methods

In the preceding paragraphs we have illustrated the principles behind recombinant Adenoviruses. Therefore we will not comment further on these developments. With analogous protocols two other important DNA viral carriers have been designed: the adeno-associated viruses (AAV) and the herpes viruses (HSV). These two are distinguished by diametrically opposite properties. The recombinant AAV particles have a very limited packaging capacity (3.5-4 kb) and integrate the recombinant DNA into the host genome (32,36,78). The intact AAV has the capacity of integrating specifically into a site on chromosome 19 but this property is missing in the emptied recombinant genomes, which integrate randomly. In spite of this random integration, the recombinant-AAV constructs seem to be refractory to gene silencing through position effects. this property is currently attributed to the AAV terminal repeats that seem to possess some kind of 'insulator' property that renders the intervening sequence rather independent of the integration context (79). The increasingly simple protocols for production of r-AAVs and the extremely low immunogenicity and toxicity of these particles has prompted a large number of investigations of therapeutic gene transfer into small and large animals (13,80-83). Therefore, these vectors promise to be a reasonable choice for the permanent transfer of small-sized constructs. The construction of HSV vectors is more laborious but offers several advantages. Particularly interesting is the possibility of generating recombinant genomes of very high capacity (up to 150 kb (33,36,84)). This opportunity could permit the transfer of large loci or of multiple regulatory cassettes for precise tuning of gene expression.

Recently, an interesting variation on the theme has emerged: the possibility of combining the advantages of two independent viruses. So for instance hybrid genomes that combine the great infectious capacity of Adenovectors and the possibility of integrating the transgene through a surrogate retroviral transposition have been proposed (32,33,73,78). Analogously, hybrid HSV-AAV vectors that combine the large capacity of packaging of HSV and the integration power of AAV have also been proposed (32,33,36,84). These hybrid viral vectors are probably still at their rudimentary stage, but represent exciting developments in a field that would otherwise have stagnated over the intrinsic limitations of each individual carrier.

2.3.2 Targeting /retargeting

Every virus has a natural tropism which is defined by the host molecular partners required for infection, replication and packaging. When working with recombinant viruses the primary factor is represented by the infection mechanism. This is dependent on the match between the cell surface receptors / co-receptors and the docking sites displayed at the surface of the infectious particle. There are essentially two ways of changing the infectivity tropism: one is to alter the docking proteins (for instance the adeno fibres (68,73,85,86)) by adding protein domains that represent ligands for alternative receptors; another consist in preparing Janus-type ligands that on one side interact with the original docking structure and on the other face they provide a new ligand (85). The latter strategy has the advantage of maintaining the same general structure of the vector and is probably preferable to the former strategy which can alter the properties of the capsid and requires specially engineered packaging cells for the amplification of the recombinant vector. Both strategies have been tried in cellular and animal models, but the success has been so far only moderate, since the new specificity imposed a high price in infectivity. These approaches will require substantial improvement before being suitable for serious clinical trials.

2.3.3. Advantages / disadvantages

The recombinant DNA viruses mentioned above share several advantages. They can be grown at very high titers (between 10exp[9] to 10exp[11] per ml), they have a very stable genome and they can efficiently infect both proliferating and non proliferating cells. This latter property is certainly the most attractive for somatic gene transfer, since many therapies would require the gene transfer in cells that do not proliferate such as neurons, endothelial cells etc. Adeno and HSV do not integrate their genome, and this can be considered as both advantageous and disadvantageous. The persistence as unintegrated genomes makes them immune to position effects (good news) but also implies the dilution of the recombinant genome upon proliferation of the infected cells (bad news). The recombinant AAVs combine the capacity of integrating in the host genome and a partial resistance towards position effects through their inverted terminal repeats (ITRs). However, a major disadvantage of AAV is the restricted packaging capacity (at most 4 kb of foreign DNA) while r-Adenos of the first generation can carry transgenes around 8 kb and 'gutted' adeno can accommodate more than 30 kb or HSV episomes can arrange up to 150 kb (36,84). The immunogenic potential of the DNA viruses is different fore each vector, with r-Adenos of the first generation being the most immunogenic and thus suitable only for unique treatments and for treatment of disorders where a certain immune reaction may even be advantageous such as cancer.

2.3.4. Suitability and examples

The literature on pre-clinical studies with DNA viral vectors is extremely wide and covers all possible disease models from cancer to infectious disorders. However, only very few reports bring convincing evidence of potential up-scalability to large animals. For several years, hyper-critical circles used to say that gene therapy has until now "only been good to cure mice". In spite of these sarcastic affirmations, progress in some areas has been rather spectacular. The natural tendency of adenoviruses to accumulate in the liver after systemic administration had prompted a series of interesting trials for the correction of metabolic disorders (33,66,73). Unfortunately, the tragic events linked with one of these trials (87-91) has slowed down the experimentation with further improved Adenovectors, including gutted adenos. On the other side, the transduction of recombinant AAV expressing blood clotting factors has been demonstrated to provide long term therapeutic benefits in both small and large animal models (33,80,82,83,92). Similarly, the use of HSV and gutted Adenoviruses in pre-clinical tests has shown convincing persistence of gene expression in many tissues (33,82). Thus, DNA-viruses remain powerful tools for the treatment of both acute and chronic conditions.

2.4. bio-weapon 2: RNA viruses

2.4.1. Structures and methods

RNA viruses tend to have a relatively small and less stable genome compared to DNA viruses. The unstability of RNA genomes is mainly due to the lack of proofreading in RNA replication and this results in error rates in the range of 1 mistake in 10'000 nucleotides. Most RNA viruses can only transiently persist within a cell, but the retroviruses can convert their RNA genome into a cDNA which is transposed into the host genome and thus can be virtually carried indefinitely within the host cell. This latter property has immediately evidenced retroviruses as preferred vectors for gene transfer. The inspiration of the ability of retroviruses to carry foreign genes certainly comes from the early discovery of murine and avian oncogenic retroviruses which can transduce cellular protooncogenes. The first attempts to construct engineered retroviruses was indeed based on murine retroviruses. The essential cis-elements that must be carried along with the engineered genome are the long terminal repeats (LTR), the packaging region and the primer annealing sequence (93). The genes for the necessary proteins (reverse transcriptase, capsid and envelope protein) can be transferred to host chromosomes in packaging cells. Once transfected with a plasmid encoding the engineered retroviral backbone, these packaging cells can produce infectious particles at titers around 10exp[6] 10exp[7] (93). The engineering of recombinant lentiviruses is more laborious since several regulatory proteins must be concertedly expressed to allow packaging (33,36,94). Currently the best system seems to be via co-transfection of trans-complementing plasmids (95). The complexity of the procedure is such that it cannot fully exclude the arising of recombinant genomes that are capable of autonomous replication (RCP, replication competent particles). The latent chance of the emergence of these RCPs has certainly strongly hampered the clinical implementation of protocols with lentiviral backbones, and this in spite of the obvious advantage of lentivirus over other retroviruses: their capacity of infecting quiescent cells (94).

2.4.2. Targeting /retargeting

The viral envelope (consisting of host cell membrane spiked by env proteins) can be virtually substituted or engineered at will. The currently used winning horse is the VSV env protein (93). Several attempts to engineer the env proteins to change their docking specificities have been reported. These attempts have invariably resulted in lower titers since there is little way to rationally redesign docking surfaces. Given the improvements in recombinant virus handling we can anticipate that new specificities will be obtained through genetic selection from combinatorial libraries rather than through rational design. Alternatively, changes of tropism can be obtained as for the Adenovirus, by coating a general env-protein with bifunctional ligands that on one hand mask the natural specificity and on the other hand contact alternative docking sites (see also 2.3.2). Unfortunately, it is not guaranteed that all docking sites will be suitable for viral internalisation, which is a rather complicated process.

2.4.3. Advantages / disadvantages

The major advantage of retroviral backbones is that they can be assembled free of viral-protein coding genes. This means that after integration a properly engineered provirus will not encode for immunogenic viral proteins, thus guaranteeing a long term survival of the transduced cell. The major advantage of lentiviruses is the capacity of infecting non-proliferating cells (see also 2.4.1), a situation which is advantageous for the treatment of differentiated tissues or slowly turning-over tissues such as the CNS, endothelium, or bones.

A major disadvantage of retroviruses is the low titer (two to four logs lower than the one of DNA viruses. A standard in vivo therapy may require between 10exp[12] an 10exp[13] infectious particles. For DNA vectors this means processing volumes of less than 1 litre, whereas tens to hundreds of litres may be necessary to obtain the same amount of retroviruses. This situation may become a strong drawback for the industrial preparation of clinical materials. The more subtle disadvantage of retroviruses is the intrinsic low-fidelity of replication. Assuming 1 mutation for every 10 kb, every third copy of a 3 kb transgene sequence will contain at least one mismatch. This can result to the loss of the biological activity in a large fraction of the transduced cells. Finally, as with AAV systems, the random integration into the host genome and the rather limited (about 9 kb) packaging capacity pose problems of long-term maintenance of gene expression (see above 1.7) and of insertional mutagenesis.

2.4.4. Suitability and examples

The very first bona fide gene therapy clinical trial (treatment of ADA deficiency (96)) was indeed performed with recombinant murine retroviruses. Since then retroviruses have been used to carry compensatory genes or toxic genes in many clinical protocols. However none of these attempts could demonstrate significant therapeutic effects, specially because the experiments were confined to phase I status, where the major focus is the measurement of side-effects. From this point of view the retroviruses have at least confirmed their relatively low toxicity. The major draw-back of recombinant murine retrovirus is their inability to infect quiescent cells, but this property is rescued in the lentiviral systems (see 2.4.1), which have shown to be equally versatile and yet capable of infecting post-mitotic cells (94). The improvements in assembly will soon permit to produce recombinant lentiviruses guaranteed free of replication-competent particles, and this will open their option for clinical trials.

Finally, the simplicity of the original murine retroviral system already used in the very first (and essentially failed) gene therapy attempts by Anderson or Bordignon (96,97), has not discouraged the team of Alain Fischer who ultimately has brought the very first historical example of permanent radical cure of a genetic disorder by the ex-vivo transduction of bone marrow transplants with vectors supplementing the IL2-receptor gene which is defective in a rare form of SCID (82,98). The two young patients of Dr. Fischer are the best results that gene therapy has scored within its short adventure, and for the public opinion are more worth than thousands of pre-clinical 'mouse therapies' or reporter assays. Resting on these encouraging indications, gene therapy based on simple viral vectorology may indeed pave the way to several efficacious intermediate clinical applications. Whether or not this strategy will persist in the long term, it depends only on further improvements, but we can say that, if wisely used, the today's vectors already have a significant potential for the treatment of chronic disorders.

2.5. Macromolecular weapons: a short click at the *www.fantasticoligo.com* site

2.5.1. Structures and methods

Gene therapy does not require the transfer of full genetic complements to become effective. Small gene fragments, in form of synthetic single stranded or double stranded oligonucleotides can exert a powerful control on gene expression. The most popular form of oligonucleotide-assisted therapy is done with antisense sequences that are destined to block either the maturation or the translation of specific mRNAs. Protocols aimed at tumor control through oligonucleotides that down regulate the production of protoonogenic proteins or anti-apoptotic factors have achieved the phase III (99). A further sophistication is the use of oligonucleotides that have a ribozyme function (100) and are able to specifically hydrolyse target mRNAs. Also double stranded 'decoy' oligonucleotides that compete with genomic sequences by binding transcription factors have been successfully used in the clinic (39).

A further level of sophistication is represented by oligonucleotides that are capable of forming triple-helix structures with specific target sequences. For triplex formation, the only a-priori requirement for the target sequence is that purines and pyrimidines should be segregated on the two strands (101, and references therein). Oligonucleotides capable of repressing or stimulating gene expression have been designed in this manner (102,103). Recently, triplex forming oligos capable of guiding molecules that induce specific repair of target sequences have been proposed (104). The major disadvantage of oligonucleotides is their short survival within a cell. Therefore, these protocols imply either continuous supply of the therapeutic oligo or single treatment of acute conditions. Several modifications of the desoxy-ribonucleotide backbone have been proposed to augment the resistance of oligos toward degradative nucleases. The most spectacular modification is probably the so called PNA (protein-nucleic acid) backbone, in which the carrier polymer no longer a phosphate-ribose chain (105,106, and references therein). A great advantage of PNA is certainly the lower negative charge of the polymer chain, which enormously stabilises either the double or the triple stranded structures that it undergoes with target sequences. PNA has been shown to be easy to handle and to permit also accumulation into cellular subcompartments such as mitochondria (107), a condition which is not met by other nucleic acid transfer methods. We are certain that PNA will become the polymer of choice for the locus-specific accumulation of active principles.

Finally, a bizarre family of chimeric oligonucleotides called 'chimeroplasts' or 'chimeraplasts ' has been reported to be capable of inducing specific gene repair at remarkable frequency (104,108-111). Although the exact mechanism and the prerequisites imposed on the chimeric oligonucleotide structure remain poorly rationalised, the efficiency with which repair if single base pair mutations has reached up to 40% under some circumstances (109). There is still some controversy about the general applicability of the chimeraplasty, about its real efficiency and safety, and about the number of molecules that must accumulate into a cell to obtain satisfactory frequencies of repair. Also, for a certain time this technology could not be reproduced by research teams independent of the original discoverer. However, recent reports indicate that this technique may have finally worked in different laboratories (111). If indeed broadly applicable, chimeraplasty may become the method of choice for the treatment of many disorders caused by small genetic defects (single nucleotide mutations). The greatest advantage of this technique is that it promises to be exquisitely site-specific, thus to generate much less if at all undesired side-effects. if chimeraplasty can be ameliorated to achieve close to 100% repair, it may become a technique that reopens the option of germ line interventions, with all the bulky complement of ethical problems accompanying this dossier. In fact, taken at face value, chimeraplasty is the only available technology that fully deserves the denomination of 'gene therapy' in its strict sense.

2.5.2. Targeting

Owing to its principle, oligonucleotide-mediated therapy does not need to be strictly targeted at the level of delivery, since it is intrinsically aimed at precise interactions through base-pairing. However, given the very high costs of oligonucleotides, it will certainly be pharmacologically advantageous if the therapeutic oligos can be specifically delivered or accumulated to target tissues. We envisage that our SMGD protocol (see 1.8) can be also adapted to ameliorate the accumulation of oligonucleotides in a tissue-preferential manner. Local delivery is so far the most popular option, although decoration with specific ligands for internalising receptors has been very promising, at least with liver-directed therapy with chimeraplasts (112).

2.5.3. Advantages / disadvantages

Compared to intact genes, oligonucleotides have molecular sizes of several orders of magnitude smaller (few thousands Daltons). This smaller size renders them more similar to conventional drugs, although they do not easily permeate through cell membranes. A second advantage is that oligonucleotides do not require biosynthetic steps for their preparation, an thus can be more easily formulated under pathogen-free conditions.

The main disadvantage is that oligos tend to be degraded after a relatively short time, implying that the treatment of persisting diseases will require repeated administrations. The chimeraplasts are indeed able to produce a permanent effect but their current range of correction is limited to single point mutations.

2.5.4. Suitability and examples

In spite of the limitations mentioned above, oligonucleotides have been maintaining their therapeutic promises. After transfer of double stranded decoy oligonucleotides that titrate transcription factors controlling cell proliferation, V. Dzau and colleagues has been able to significantly reduce the incidence of intima-growth in vein transplants (39). In this case, the merit of the approach is that the transient treatment is sufficient to bring a permanent effect, since it sustains the non-degenerative adaptation of veins to the higher pressure. We have also already mentioned the spectacular correction rate obtained in rat models of a liver disorder (109) with chimeroplasty. Here also, a short treatment permits a long term therapeutic effect. Several antisense oligo approaches against protooncogenes are now in advanced clinical testing. However, we have to wait these tests to assess the validity of this approach. Personally, we are quite sceptical about these anti-cancer therapies, specially since they don't offer a priori any bystander effect (see 1.2) and therefore seem less suitable for tumour eradication.

3. FINAL HURDLES AND CONCLUSIONS

3.1. Immune response and re-administration

This book is focussing on the molecular treatment of a disorder with auto immune and inflammatory components. In this kind of treatment it is absolutely imperative that the procedure should not imply reagents or manipulations that could unnecessarily activate the cellular or humoral immune system be it specific or innate. Therefore, the work with viral vectors of any kind should be considered with substantial caution. However, also non viral gene transfer could pose several problems when using entire genes, due to the innate reaction against unmethylated CpG-rich motifs. In general, re-administration is almost unavoidable with the current technology and this does not simplify the foreseeable clinical protocols for the treatment of chronic disorders with inflammatory components. Thus, until better control on the short-term and long-term immunogenicity of gene delivery systems can be obtained, gene therapy cannot be considered as a first priority for this class of disorders where it could ultimately exacerbate the outcome instead of bringing a therapeutical effect (113).

Finally, the immune system poses a problem also when the vectors themselves are clean of any proinflammatory properties. In fact, in some genetic loss-of-functions the resident gene is either deleted or totally non-functional. In this cases, the expression of the healthy gene product can lead to tissue rejection since it is detected as a 'foreign' antigen by the host immune system (23,69,114). For these cases, tolerisation strategies must be devised before considering gene transfer.

3.2. Safety considerations, from RCP to insertional mutagenesis

The immune reactions are not our sole hurdle in virally-assisted gene transfer. Some capsid proteins, while providing useful functions such as translocation and protection from degratative enzymes, are themselves toxic and can produce adverse reactions. In fact, most biologically assembled viral preparations contain a large excess (between 10 and 100 fold) of non functional viral particles. these particles can significantly contribute to the overall toxicity of the gene transfer procedure. Also we have mentioned that no biological recombinant viral preparation can be a priori guaranteed to be free of adventitious recombinants that have re-acquired viral genes sufficient for autonomous replication (the so-called RCPs, see 2.4.1). Even if the incidence of RCP can be reduced to less than one event in 10exp[14], this may pose serious constraints and cause severs costs augmentations to the industrial preparation of clinical materials.

The vectors that currently permit permanent transfer do not have ways of controlling the site of integration of the transgene. Thus, every cellular integration event is in principle an insertional mutagenesis event. At the somatic level, the large amount of insertional mutagenesis has the potential of generating pro-tumorigenic cells by activating protooncogenes. This relegates the use of integrating vectors for the treatment of life-threatening diseases, where the risk of generating a secondary tumor is still acceptable. The random mutagenesis generates another dilemma if the gene delivery vector transforms germ cells. In this case we would have a large number of aditional mutations that would be inherited to subsequent generations, and the potential benefit for the treated individual could become a strong disadvantage for his/her progeny. These dilemmas will be solved when we will be able to assemble vectors that can permanently deliver transgenes in specific chromosomal locations. Considering the current pace of progress, this goal should not be so far.

3.3. Pulling it all together

If a pragmatic reader had the patience to read all the good and bad news about the existing and prospected vectors, he/she may ask: but after all, which vector/delivery is suitable for my goal?

In the Table 1 we summarise the suitability of the currently available gene transfer methods (columns) for different types of treatment. the number of '+' signs indicates qualitatively the suitability of a given combination. We hope that this synopsis may help in the choice of the most appropriate combination.

TABLE 1

Table 1 Qualitative assessment of suitability of delivery vehicles

Application	*ADV*	*AAV*	*HSV*	*HIV*	*OLI*	*PEM*	*GUN*	*LIP*
Vaccination /prevention	++	(+)	(+)	(+)	-	-	+++	(+)
Acute treatment	++	-	-	-	++	-	-	+
Chronic treatment	-	++	++	+++	(+)	-	-	+
In vivo local delivery	++	++	++	++	++	+	++	+
In vivo systemic delivery	+	+	+	+	+	n	n	+
Ex vivo delivery	++	+	+	+++	++	++	+	++
Single administration	+++	+++	+++	+++	+	+	+	+
Repeated administration	(+)	+	(+)	++	+++	+	+++	+++
Treat loss-of-function	+++	+++	+++	+++	+	++	+	++
Treat gain-of-function	(+)	+	+	+	+++	-	-	+
Gene correction	-	-	-	-	+++	-	-	-

Symbols: Adv, Adenovirus vectors; Aav, adeno-associated virus vectors; Hsv, Herpes virus vectors; Hiv, lentiviral vectors; Oli, oligonucleotides; Pem, pressure or electroporation mediated delivery; Gun, biolistic or macroinjection; Lip, lipoplexes or polyplexes. Symbols for suitability: -, not suitable; (+) questionable; +, hardly suitable; ++ , offers several applications; +++, excellent choice; n, does not apply. Most of the indicated degrees of suitability are justified in this chapter.

3.4. Outlook: Will rudimentary vectorology with all its troubles survive the emerging challenge of stem cell therapy?

Stem cell research has been booming in the last months. Primordial cells for almost all the tissues, including the CNS, have been characterised and the major hope is that the ex vivo cultivation of those may permit tissue regeneration for various treatments (115,116). The most spectacular observation is certainly that some stem cells seem to be able to trans-determination, that is to give rise to differentiated cells that are different than the donor tissue (97,116,117). Many of the claims and reports in this field are not yet even in the peer-reviewed literature but have been propagated through press releases or news-agencies despatches. According to them, in a foreseeable future, it should be possible to explant bone marrow cells and later reconstruct muscle, nerves, bones, epidermis and other types of tissues from this original population. The mechanisms that govern the maintenance of the pluripotency and the commitment towards one or another lineage are still obscure, but they are so intensively studied that we can anticipate major breakthroughs within the next few years.

3.4.1 The worst and best case scenario

Being able to culture stem cells without losing their pluripotency and to then determine their commitment would pave the way to autologous organ reconstruction that could cure an immensely large number of degenerative disorders. If the disorder has a genetic component, the corresponding correction could be easily achieved with conventional gene transfection ex-vivo and corresponding selection of pre-characterised recombinant cell clones. This would render obsolete most of the efforts to obtain high efficiency gene delivery vectors. Those latter would only be required for those cases where a cell therapy is not indicated, such as in acute treatments or corrections of gain-of-function disorders.

Thus, taken at face value, cell therapy has all the hallmarks to become a superior procedure for the treatment of chronic conditions. The worst that could happen is when patents and human ambitions would transform the noble tendency towards a better therapy into a ferocious battle between gene therapists and cell therapists for the best slices of the health market.

The balance of the odds for gene or cell therapy could change drastically if gene correction procedures such as chimeroplasty (see 2.5.1) would confirm their efficacy or if hybrid vectors (see 2.3.1), artificial viruses (see 2.1.2) or coherently integrating vectors (see 1.3, 2.3.3, 3.1) would make the long-sought brakthrough. If any of those tools would become generally applicable, then gene transfer in vivo would certainly remain competitive, since it implies lower costs, shorter intervention time and probably also lower invasivity than cell therapy.

3.4.2. A final homily for gene transfer

We have recapitulated the aims and efforts towards developing tools and methods for efficient gene transfer. When taken pessimistically, one could imagine that the few, but highly celebrated, therapeutic achievements are condemned to remain anecdotal and one wonders why should the scientists continue in this direction that has brought more frustrations than successes. From the point of view of fundamental research, the answer is refreshingly simple. While trying to solve the engineering problem of gene therapy, scientists have re-discovered and partially solved old neglected problems related to cell biology, virology, molecular transport and degradation, cell surface properties, etc. Furthermore, the pre-clinical efforts have produced vectors that are phenomenal tools for fundamental research. Gene transfer vectors are already considered for hit-and-run gene alteration procedures that will permit temporally and spatially controlled gene knock-out or knock-in in experimental animals, a situation which is laborious to achieve with conventional transgenesis. Furthermore, the gene transfer vectors open the way to the experimentation with primary cell cultures, that are notoriously refractory to biochemical gene transfer. This will permit the functional study of genes under semi- or fully-physiological conditions and a better understanding of the intricate interactions between gene products. Therefore, gene therapy has brought an immense flood of novel knowledge that will substantially accelerate the overall progress in experimental life sciences.

From the clinical/pragmatic point of view, the heroic efforts towards gene therapy must be regarded as necessary steps that have broken the ice and paved the way towards more efficacious molecular therapies. We should not forget that any technological progress, from the aeroplane to the computer, has started with prototypes that seem almost ridiculous when compared with the today's opportunities. But without these glorious steps we would still be devoid of such marvellous achievements and would still be gasping intellectual conjectures about their feasibility instead of enjoying their concrete advantages. So, let's keep going and be proud thereof!

Acknowledgements:

This work has been supported by the Canton of Fribourg and by the Swiss National Science Foundation, program NFP37 'somatic gene therapy' (www.unifr.ch/nfp37)

References

1. Paillard, F. 1997. Commentary: Bystander effects in enzyme/prodrug gene therapy. Hum Gene Ther. 8: 1733-1736.
2. Dilber, M.S., and Smith, C.I.E. 1997. Suicide genes and bystander killing: local and distant effects. Gene Ther. 4: 273-274.
3. Bennett, J., Zeng, Y., Bajwa, R., Klatt, L., Li, Y., and Maguire, A.M. 1998. Adenovirus-mediated delivery of rhodopsin-promoted bcl-2 results in a delay in photoreceptor cell death in the rd/rd mouse. Gene Ther. 5: 1156-1164.
4. Boyer, O., Zhao, J.C., Cohen, J.L., Depetris, D., Yagello, M., Lejeune, L., Bruel, S., Mattei, M.G., and Klatzmann, D. 1997. Position-dependent variegation of a CD4 minigene with targeted expression to mature CD4+ T cells. J Immunol. 159: 3383-3390.
5. de Geest, B., van, L.S., Lox, M., Collen, D., and 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.
6. Larochelle, N., Lochmüller, H., Zhao, J., Jani, A., Hallauer, P., Hastings, K., Massie, B., Prescott, S., Petrof, B., Karpati, G.*et al.* 1997. Efficient muscle-specific transgene expression after adenovirus-mediated gene transfer in mice using a 1.35 kb muscle creatine kinase promoter/enhancer. Gene Ther. 4: 465-472.

7. Grosveld, F. 1999. Activation by locus control regions? Curr Opin Genet Dev. 9: 152-157.
8. Grosveld, F., De, B.E., Dillon, N., Gribnau, J., Milot, E., Trimborn, T., Wijgerde, M., and Fraser, P. 1998. The dynamics of globin gene expression and gene therapy vectors. Ann N Y Acad Sci. 850: 18-27.
9. Fox, C., and Rine, J. 1996. Influences of the cell cycle on silencing. Cell. 8: 354-357.
10. Paillard, F. 1997. commentary: Promoter Attenuation in Gene Therapy: Causes and Remedies. Hum Gene Ther. 8: 2009-2010.
11. Ratcliff, F., Harrison, B., and Baulcombe, D. 1997. A Similarity Between Viral Defense and Gene Silencing in Plants. Science. 276: 1558-1560.
12. Emery, D.W., Chen, H., Li, Q., and Stamatoyannopoulos, G. 1998. Development of a condensed locus control region cassette and testing in retrovirus vectors for A gamma-globin. Blood Cells Mol Dis. 24: 322-339.
13. Inoue, T., Yamaza, H., Sakai, Y., Mizuno, S., Ohno, M., Hamasaki, N., and Fukumaki, Y. 1999. Position-independent human beta-globin gene expression mediated by a recombinant adeno-associated virus vector carrying the chicken beta-globin insulator. J Hum Genet. 44: 152-162.
14. Dupraz, P., Rinsch, C., Pralong, W.F., Rolland, E., Zufferey, R., Trono, D., and Thorens, B. 1999. Lentivirus-mediated Bcl-2 expression in beta-TC-tet cells improves resistance to hypoxia and cytokine-induced apoptosis while preserving in vitro and in vivo vontrol of insulin secretion. Gene Ther. 6: 1160-1169.
15. Rinsch, C., Regulier, E., Deglon, N., Dalle, B., Beuzard, Y., and Aebischer, P. 1997. A gene therapy approach to regulated delivery of erythropoietin as a function of oxygen tension [see comments]. Hum Gene Ther. 8: 1881-1889.
16. Simpson, A.M., Marshall, G.M., Tuch, B.E., Maxwell, L., Szymanska, B., Tu, J., Beynon, S., Swan, M.A., and Camacho, M. 1997. Gene therapy of diabetes: glucose-stimulated insulin secretion in a human hepatoma cell line (HEP G2ins/g). Gene Ther. 4: 1202-1215.
17. No, D., Yao, T.P., and Evans, R.M. 1996. Ecdysone-inducible gene expression in mammalian cells and transgenic mice. Proc Natl Acad Sci U S A. 93: 3346-3351.
18. Burcin, M.M., Schiedner, G., Kochanek, S., Tsai, S.Y., and O'Malley, B.W. 1999. Adenovirus-mediated regulable target gene expression in vivo. Proc Natl Acad Sci U S A. 96: 355-360.
19. Miller, N., and Whelan, J. 1997. Progress in Transcriptionally Targeted and Regulatable Vectors for Genetic Therapy. Hum Gene Ther. 8: 803-815.
20. McInerney, E.M., and Katzenellenbogen, B.S. 1996. Different regions in activation function-1 of the human estrogen receptor required for antiestrogen- and estradiol-dependent transcription activation. J Biol Chem. 271: 24172-24178.
21. Pollock, R., and Rivera, V.M. 1999. Regulation of gene expression with synthetic dimerizers. Methods Enzymol. 306: 263-281.
22. Bujard, H. 1999. Controlling genes with tetracyclines. J Gene Med. 1: 372-374.
23. Kay, M.A., Manno, C.S., Ragni, M.V., Larson, P.J., Couto, L.B., McClelland, A., Glader, B., Chew, A.J., Tai, S.J., Herzog, R.W.*et al.* 2000. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with

an AAV vector [see comments]. Nat Genet. 24: 257-261.

24. Ulmer, J., and Liu, M. 1999. Delivery systems and ajuvants for DNA vaccines. In The Development of Human Gene Therapy. T. Friedmann, editor. Cold Spring Harbor: CSH Laboratory Press, NY. 309-330.
25. Restifo, N.P., Ying, H., Hwang, L., and Leitner, W.W. 2000. The promise of nucleic acid vaccines. Gene Ther. 7: 89-92.
26. Baumgartner, I., Pieczek, A., Manor, O., Blair, R., Kearney, M., Walsh, K., and Isner, J.M. 1998. Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia [see comments]. Circulation. 97: 1114-1123.
27. Dash, P.R., Read, M.L., Barrett, L.B., Wolfert, M.A., and Seymour, L.W. 1999. Factors affecting blood clearance and in vivo distribution of polyelectrolyte complexes for gene delivery. Gene Ther. 6: 643-650.
28. Lunsford, L., McKeever, U., Eckstein, V., and Hedley, M.L. 1999. Tissue distribution and persistence in mice of plasmid DNA encapsulated in a PLGA-based microsphere delivery vehicle [In Process Citation]. J Drug Target. 8: 39-50.
29. Reynolds, P., Dmitriev, I., and Curiel, D. 1999. Insertion of an RGD motif into the HI loop of adenovirus fiber protein alters the distribution of transgene expression of the systemically administered vector. Gene Ther. 6: 1336-1339.
30. Thierry, A.R., Rabinovich, P., Peng, B., Mahan, L.C., Bryant, J.L., and Gallo, R.C. 1997. Characterization of liposome-mediated gene delivery: expression, stability and pharmacokinetics of plasmid DNA. Gene Ther. 4: 226-237.
31. Ye, X., Jerebtsova, M., and Ray, P.E. 2000. Liver bypass significantly increases the transduction efficiency of recombinant adenoviral vectors in the lung, intestine, and kidney. Hum Gene Ther. 11: 621-627.
32. Schagen, F.H.E., Rademaker, H.J., Fallaux, F.J., and Hoeben, R.C. 2000. Insertion vectors for gene therapy. Gene Ther. 7: 271-272.
33. Dyer, M.R., and Herrling, P.L. 2000. Progress and potential for gene-based medicines. Mol Ther. 1: 213.
34. Ascenzioni, F., Donini, P., and Lipps, H.J. 1997. Mammalian artificial chromosomes--vectors for somatic gene therapy. Cancer Lett. 118: 135-142.
35. Hitt, M., Parks, R.J., and Graham, F.L. 1999. Structure and genetic organisation of adenovirus vectors. In The Development of Human Gene Therapy. T. Friedmann, editor. Cold Spring Harbor, NY: CSH Laboratory Press. 61-86.
36. Costantini, L.C., Bakowska, J.C., Breakfield, X.O., and Isacson, O. 2000. Gene therapy in the CNS. Gene Ther. 7: 93-109.
37. Wolff, J. 1999. Naked DNA gene transfer in mammalian cells. In The Development of Human Gene Therapy. T. Friedmann, editor. Cold Spring Harbor: CSH Laboratory Press, NY. 279-307.
38. Beardsley, T. 2000. Working under pressure. Sci Am. 282: 34.
39. von der Leyen, H.E., Braun-Dullaeus, R., Mann, M.J., Zhang, L., Hiebauer, J., and Dzau, V.J. 1999. A Pressure-mediated nonviral method for efficient arterial gene and oligonucleotide transfer. Hum Gene Ther. 10: 2355-2364.
40. Wells, J.M., Li, L.H., Sen, A., Hahreis, G.P., and Hui, S.W. 2000. Electroporation enhanced gene

delivery in mammary tumours. Gene Ther. 7: 541-547.

41. Yanez, R.J., and Porter, A.C.G. 1998. Therapeutic gene targeting. Gene Ther. 5: 149-159.
42. Mahvi, D. 1997. Phase I/IB Study of Immunization with Autologous Tumor Cells Transfected with the GM-CSF Gene by Particle-Mediated Transfer in Patients with Melanoma or Sarcoma. Hum Gene Ther. 8: 875-891.
43. Rakhmilevich, A., Janssen, K., Turner, J., Culp, J., and Yang, N.S. 1997. Cytokine gene therapy of cancer using gene gun technology: superior antitumor activity of interleukin-12. Hum Gene Ther. 8: 1303-1311.
44. Waelti, E., and Glück, R. 1998. Delivery to cancer cells of antisense L-myc oligonucleotides incorporated in fusogenic, cationic-lipid-reconstituted influenza-virus envelopes (cationic virosomes). Int J Cancer. 77: 1-6.
45. Saeki, Y., Matsumoto, N., Nakano, Y., Mori, M., Awai, K., and Kaneda, Y. 1997. Development and characterization of cationic liposomes conjugated with HVJ (Sendai Virus) : reciprocal effect of cationic lipid for In Vitro and In Vivo gene tranfer. Hum Gene Ther. 8: 2133-2141.
46. Schoen, P., Chonn, A., Cullis, P.R., Wilschut, J., and Scherrer, P. 1999. Gene transfer mediated by fusion protein hemagglutinin reconstituted in cationic lipid vesicles. Gene Ther. 6: 823-832.
47. Wolff, J.A. 1997. Naked DNA transport and expression in mammalian cells. Neuromuscul Disord. 7: 314-318.
48. Branden, L.J., Mohamed, A.J., and Smith, C.I. 1999. A peptide nucleic acid-nuclear localization signal fusion that mediates nuclear transport of DNA. Nat Biotechnol. 17: 784-787.
49. Neves, C., Byk, G., Scherman, D., and Wils, P. 1999. Coupling of a targeting peptide to plasmid DNA by covalent triple helix formation. FEBS letters. 453: 41-45.
50. Budker, V., T., B., G., Z., Subbotin, V., Loomis, A., and Wolff, J. 2000. Hypothesis; naked plasmid DNA is taken up by cells in vivo by a receptor-mediated process. Jour Gene Med. 2: 76-88.
51. Felgner, P., Zelphati, O., and Liang, X. 1999. Advances in synthetic gene delivery system technology. In The Development of Human Gene Therapy. T. Friedmann, editor. Cold Spring Harbor, NY: CSH Laboratory Press. 241-260.
52. Cotten, M., and Wagner, E. 1999. Receptor-mediated gene delivery strategies. In The Development of Human Gene Therapy. T. Friedmann, editor. Cold Spring Harbor, NY: CSH Laboratory Press. 261-277.
53. Shea, L.D., Smiley, E., Bonadio, J., and Mooney, D.J. 1999. DNA delivery from polymer matrices for tissue engineering [see comments] [published erratum appears in Nat Biotechnol 1999 Aug;17 (8) :817]. Nat Biotechnol. 17: 551-554.
54. Roy, K., Mao, H.Q., Huang, S.K., and Leong, K.W. 1999. Oral gene delivery with chitosan--DNA nanoparticles generates immunologic protection in a murine model of peanut allergy [see comments]. Nat Med. 5: 387-391.
55. Mathiowitz, E., Jacob, J.S., Jong, Y.S., Carino, G.P., Chickering, D.E., Chaturvedi, P., Santos, C.A., Vijayaraghavan, K., Montgomery, S., Bassett, M.*et al.* 1997. Biologically erodable microspheres as potential oral drug delivery systems. Nature. 386: 410-414.

56. Hedley, M.L., Curley, J., and Urban, R. 1998. Microspheres containing plasmid-encoded antigens elicit cytotoxic T-cell responses. Nat Med. 4: 365-368.
57. Lewis, M.E. 1999. Crossing the blood-brain barrier to central nervous system gene therapy. Clin Genet. 56: 10-13.
58. Giancotti, F.G., and Ruoslahti, E. 1999. Integrin signaling. Science. 285: 1028-1032.
59. Folkman, J. 1999. Angiogenic zip code. Nature Biotech. 17: 749.
60. Hartmann, G., and Krieg, A.M. 1999. CPG DNA and LPS induce distinct patterns of activation in human monocytes. Gene Ther. 6: 893-903.
61. Krieg, A.M. 2000. Minding the Cs and the Gs. Mol Ther. 1: 209.
62. McLachlan, G., Stevenson, B.J., Davidson, D.J., and Porteous, D.J. 2000. Bacterial DNA is implicated in the inflammatory response to delivery of DNA/DOTAP to mouse lungs. Gene Ther. 7: 384-392.
63. Paillard, F. 1999. CpG: The double-edged sword. Hum Gene Ther. 10: 2089-2090.
64. Luft, F.C. 1999. Can VP22 resurrect gene therapy? J Mol Med. 77: 575-576.
65. Elliott, G., and O'Hare, P. 1999. Intercellular trafficking of VP22-GFP fusion proteins. Gene Ther. 6: 149-151.
66. Wivel, N.A., Gao, G.-P., and Wilson, J.M. 1999. Adenovirus vectors. In The Development of Human Gene Therapy. T. Friedmann, editor. Cold Spring Harbor, NY: CSH Laboratory Press. 87-110.
67. Amalfitano, A. 1999. Next-generation adenoviral vectors: new and improved. Gene Ther. 6: 1643-1645.
68. Wickham, T.J. 2000. Targeting adenovirus. Gene Ther. 7: 110-114.
69. Gallo-Penn, A.M., Shirley, P.S., Andrews, J.L., Kayda, D.B., Pinkstaff, A.M., Kaloss, M., Tinlin, S., Cameron, C., Notley, C., Hough, C.*et al.* 1999. In vivo evaluation of an adenoviral vector encoding canine factor VIII: high-level, sustained expression in hemophiliac mice. Hum Gene Ther. 10: 1791-1802.
70. Sandig, V., Youil, R., Bett, A.J., Franlin, L.L., Oshima, M., Maione, D., Wang, F., Metzker, M.L., Savino, R., and Caskey, T. 2000. Optimization of the helper-dependent adenovirus system for production and potency in vivo. Proc Natl Acad Sci. 97: 1002-1007.
71. Heise, C.C., Williams, A.M., Xue, S., Propst, M., and Kirn, D.H. 1999. Intravenous administration of ONYX-015, a selectively replicating adenovirus, induces antitumoral efficacy. Cancer Res. 59: 2623-2628.
72. Rogulski, K.R., Wing, M.S., Paielli, D.L., Gilbert, J.D., Kim, J.H., and Freytag, S.O. 2000. Double suicide gene therapy augments the antitumor activity of a replication-competent lytic adenovirus through enhanced cytotoxicity and radiosensitization. Hum Gene Ther. 11: 67-76.
73. Reynolds, P.N., and Curiel, D.T. 1999. Strategies to adapt adenoviral vectors to gene therapy applications: targeting and integration. In The Development of Human Gene Therapy. T. Friedmann, editor. Cold Spring Harbor, NY: CSH Laboratory Press. 111-130.
74. Carew, J.F., Kooby, D.A., Halterman, M.W., Federoff, H.J., and Fong, Y. 1999. Selective infection and cytolysis of human head and neck squamous cell carcinoma with sparing of normal mucosa my a cytotoxic herpes simplex virus type 1 (G207). Hum Gene Ther. 10: 1599-1606.
75. Chase, M., Chung, R., and Chiocca, E.A. 1998. An oncolytic viral mutant

that delivers the CYP2B1 transgene and augments cyclophosphamide chemotherapy. Nature Biotechnology. 16: 444-448.

76. Martiniello-Wilks, R., Garcia-Aragon, J., Daja, M., Russell, P., Both, G., Molloy, P., Lockett, L., and Russell, P. 1998. In Vivo gene therapy for prostate cancer: preclinical evaluation of two different enzyme-directed prodrug therapy systems delivered by identical adenovirus vectors. Hum Gene Ther. 9: 1617-1626.
77. Morris, J.C., and Wildner, O. 2000. Therapy of head and neck squamous cell carcinoma with an oncolytic adenovirus expressing HSV-tk. Molecular Therapy. 1: 56-62.
78. Hirata, R.K., and Russell, D.W. 2000. Design and packaging of adeno-associated virus gene targeting vectors [In Process Citation]. J Virol. 74: 4612-4620.
79. Fu, Y., Wang, Y., and Evans, S. 1998. Viral sequences enable efficient and tissue-specific expression of transgenes in Xenopus. Nature Biotechnology. 16: 253-257.
80. Chao, H., Samulski, R.J., Bellinger, D.A., Monahan, P.E.m.N.T.C., and Walsh, C.E. 1999. Persistent expression of canine factor IX in hemophilia B canines. Gene Ther. 6: 1695-1704.
81. Monahan, P.E., Samulski, R.J., Tazelaar, J., Xiao, X., Nichols, T.C., Bellinger, D.A., Read, M.S., and Walsh, C.E. 1998. Direct intramuscular injection with recombinant AAV vectors results in sustained expression in a dog model of hemophilia. Gene Ther. 5: 40-49.
82. Stephenson, J. 2000. Gene therapy trials show clinical efficacy. JAMA. 283: 589-590.
83. Wang, D., Fischer, H., Zhang, L., Fan, P., Ding, R.X., and Dong, J. 1999. Efficient CFTR expression from AAV vectors packaged with promoters - the second generation. Gene Ther. 6: 667-675.
84. Fraefel, C., Jacoby, D.R., Lage, C., Hilderbrand, H., Chou, J.Y., Alt, F.W., Breakefield, X.O., and Majzoub, J.A. 1997. Gene transfer into hepatocytes mediated by helper virus-free HSV/AAV hybrid vectors. Mol Med. 3: 813-825.
85. Watkins, S.J., Mesyanzhinov, V.V., Kurochkina, L.P., and Hawkins, R.E. 1997. The ‘adenobody’ approach to viral targeting: specific and enhanced adenoviral gene delivery. Gene Ther. 4: 1004-1012.
86. Paillard, F. 1999. Dressing up adenoviruses to modify their tropism. Hum Gene Ther. 10: 2575-2576.
87. Brenner, M. 2000. Reports of adenovector 'death' are greatly exaggerated. mol ther. 1: 205.
88. Friedmann, T. 2000. Principles for human gene therapy. Science. 287: 2163-2165.
89. Marshall, E. 1999. Gene therapy death prompts review of adenovirus vector. Science. 286: 22442245.
90. Rosenberg, L.E., and Schechter, A.N. 2000. Gene therapist: heal thyself. Science. 287: 1751.
91. Steele, F. 2000. Painful lessons. Mol Ther. 1: iii.
92. Wang, L., Nichols, T.C., Read, M.S., Bellinger, D.A., and Verma, I.M. 2000. Sustained expression of therapeutic levels of factor IX in hemophilia B dogs by AAV-mediated gene therapy in liver. Mol Ther. 1: 154-158.
93. Yee, J.-K. 1999. Retroviral vectors. In The Development of Human Gene Therapy. T. Friedmann, editor. Cold Spring Harbor: CSH Laboratory Press, NY. 21-45.
94. Naldini, L., and Verma, I. 1999. Lentiviral vectors. In The Development of Human Gene Therapy. T. Friedmann, editor. Cold Spring Harbor, NY: CSH Laboratory Press. 47-60.

95. Dull, T., Zufferey, R., Kelly, M., Mandel, R.J., Nguyen, M., Trono, D., and Naldini, L. 1998. A third-generation lentivirus vector with a conditional packaging system. J Virol. 72: 8463-8471.
96. Culver, K.W., Anderson, W.F., and Blaese, R.M. 1991. Lymphocyte gene therapy. Hum Gene Ther. 2: 107-109.
97. Bordignon, C., Carlo-Stella, C., Colombo, M.P., De, V.A., Lanata, L., Lemoli, R.M., Locatelli, F., Olivieri, A., Rondelli, D., Zanon, P.*et al.* 1999. Cell therapy: achievements and perspectives. Haematologica. 84: 1110-1149.
98. Cavazzana-Calvo, M., Hacein-Bey, S., de, S.B.G., Gross, F., Yvon, E., Nusbaum, P., Selz, F., Hue, C., Certain, S., Casanova, J.L.*et al.* 2000. Gene therapy of human severe combined immunodeficiency (SCID) -X1 disease [see comments]. Science. 288: 669-672.
99. Galderisi, U., Cascino, A., and Giordano, A. 1999. Antisense oligonucleotides as therapeutic agents. J Cell Physiol. 181: 251-257.
100. Kruger, M., Beger, C., and Wong-Staal, F. 1999. Use of ribozymes to inhibit gene expression. Methods Enzymol. 306: 207-225.
101. Kukreti, S., Sun, J.S., Loakes, D., Brown, D., Nguyen, C.H., Bisagni, E., Garestier, T., and Helene, C. 1998. Triple helices formed at oligopyrimidine . oligopurine sequences with base pair inversions: effect of a triplex-specific ligand on stability and selectivity. Nucl Acids Res. 26: 2179-2183.
102. Braun, S., Chen, K., Battiwalla, M., and Cornetta, K. 1997. Gene therapy strategies for leukemia. Mol Med Today. 3: 39-46.
103. Faruqi, A., Egholm, M., and Glazer, P. 1998. Peptide nucleic acid-targeted mutagenesis of a chromosomal gene in mouse cells. Proc Natl Acad Sci. 95: 1398-1403.
104. Culver, K.W., Hsieh, W.T., Huyen, Y., Chen, V., Liu, J., Khripine, Y., and Khorlin, A. 1999. Correction of chromosomal point mutations in human cells with bifunctional oligonucleotides. Nature Biotech. 17: 989-993.
105. Uhlmann, E. 1998. Peptide nucleic acids (PNA) and PNA-DNA chimeras: from high binding affinity towards biological function. BiolChem. 379: 1045-1052.
106. Nielsen, P.E. 1999. Peptide nucleic acids as therapeutic agents. Curr Opin Struct Biol. 9: 353-357.
107. Chinnery, P.F., Taylor, R.W., Diekert, K., Lill, R., Turnbull, D.M., and Lightowlers, R.N. 1999. Peptide nucleic acid delivery to human mitochondria [see comments]. Gene Ther. 6: 1919-1928.
108. Dr, K. 1998. Chimeraplasty: a gene modification technology employing oligonucleotides. Hum Gene Ther. 9: 2435-2437.
109. Kren, B.T., Parashar, B., Bandyopadhyay, P., Chowdhury, N.R., Chowdhury, J.R., and Steer, C.J. 1999. Correction of the UDP-glucuronosyltransferase gene defect in the gunn rat model of crigler-najjar syndrome type I with a chimeric oligonucleotide. Proc Natl Acad Sci U S A. 96: 10349-10354.
110. Gura, T. 1999. Repairing the genome’s spelling mistakes. Science. 285: 316-318.
111. Blaese, R.M. 1999. Optimism regarding the use of RNA/DNA hybrids to repair genes at high efficiency. J Gene Med. 1: 144-147.
112. Kren, B., Bandyopadhyay, P., and Steer, C. 1998. In vivo site-directed mutagenesis of the factor IX gene by chimeric RNA/DNA oligonucleotides. Nature Medicine. 4: 285-290.

113. Deng, G.M., Nilsson, I.M., Verdrengh, M., Collins, L.V., and Tarkowski, A. 1999. Intra-articularly localized bacterial DNA containing CpG motifs induces arthritis. Nat Med. 5: 702-705.
114. Mannucci, P.M., Zanetti, A.R., Gringeri, A., Tanzi, E., Morfini, M., Messori, A., Tirindelli, M.C., de, B.R., Ciavarella, N., and Colombo, M. 1989. Long-term immunogenicity of a plasma-derived hepatitis B vaccine in HIV seropositive and HIV seronegative hemophiliacs. Arch Intern Med. 149: 1333-1337.
115. Asahara, T., Kalka, C., and Isner, J.M. 2000. Stem cell therapy and gene transfer for regeneration. Gene Ther. 7: 451-457.
116. Weissmann, I. 2000. Translating stem and progenitor cell biology to the clinic: barriers and opportunities. Science. 287: 1442-1446.
117. Brenner, M. 1999. Gene transfer and stem cell transplantation. In The Development of Human Gene Therapy. T. Friedmann, editor. Cold Spring Harbor, NY: CSH Laboratory Press. 459-476.

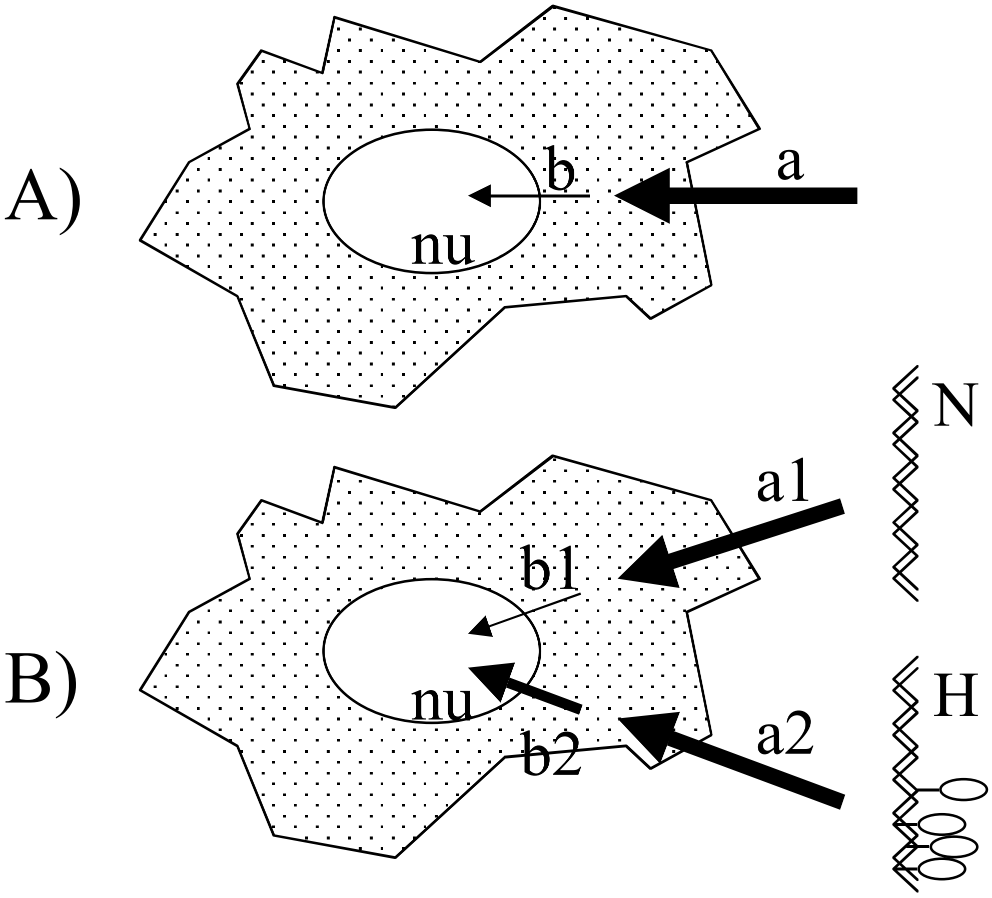


FIGURE 1, The two barriers for gene transfer and the principle of SMGD
A. In gene transfer the genetic material must pass two barriers: the cell membrane (a) and the nuclear envelope (b). Only a small proportion of the transferred nucleic acids undergoes nuclear transfer (compare thin arrow under b and thick arrow under a).
B. The concept of steroid mediated gene delivery. Conventional DNA (N), even if abundantly transfected (thick arrow marked a1) is only poorly translocated into the nucleus (thin arrow marked b1). Ligand-decorated DNA (marked H, where ovals with bar represent covalently linked ligands) is equally well transfected (a2) but better transported to the nucleus (b2) by the nuclear receptor that binds to the cognate ligand. This approach permits the selective facilitation of nuclear uptake of transgenes. In our laboratory we have proved this concept with model systems involving the glucocorticoid receptor (Ceppi et al. in preparation).

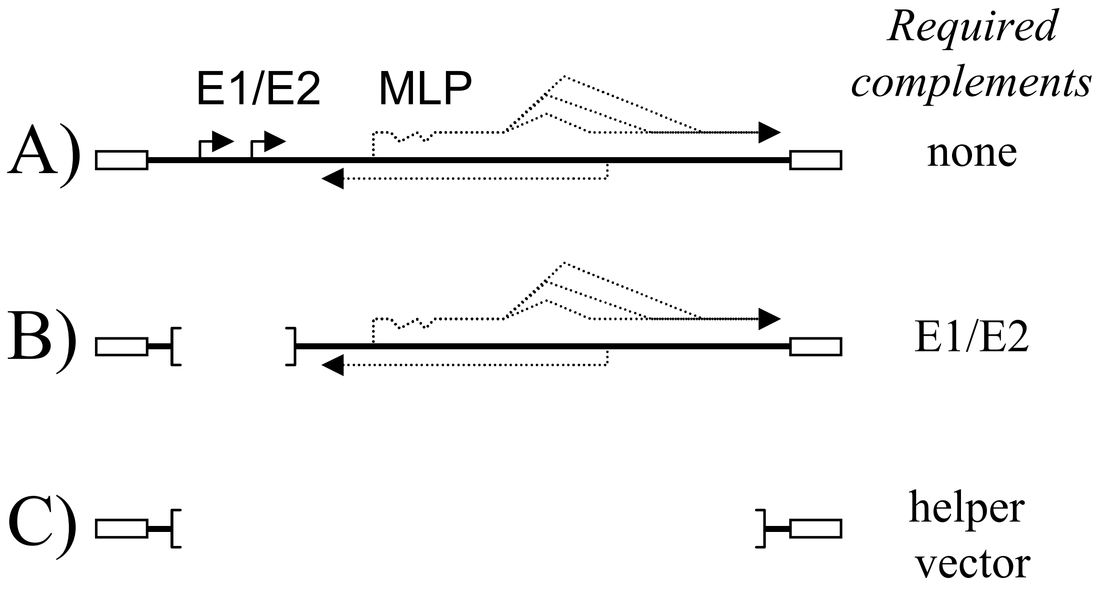


FIGURE 2: Genomes of wild type and recombinant adenovectors
A. The genome of wild type adenoviruses see text for references) is a linear 36 kb dsDNA flanked by inverted terminal repeats (hatched boxes) that are necessary for DNA replication and packaging. The indispensable early functions are E1 and E2 that are encoded by a 25% left portion of the genome (see E1 and E2). The late functions (capsid protein) are encoded by the remaining part and are mostly transcribed through the major late promoter (MLP) that gives rise to alternatively spliced mRNAs (broken dotted line), The late portion of the genome encodes in the opposite direction the viral DNA polymerase (dotted arrow pointing to left).
B. Replication-deficient adenovectors of the first generation are deleted in the early region and can be grown in packaging cells that provide the E1 and E2 functions. The deletion allows the accommodation of up to 8 kb of foreign DNA.
C. 'gutless' or 'high-capacity' adenovectors retain only the ITR region and can accommodate up to 32 kb of foreign DNA. These recombinant genomes can only be packaged in presence of a helper vector (see text).

4. APPENDIX

4.1. SUMMARY OF PROPERTIES OF SELECTED STEROID DERIVATIVES

Name	Hor	Spacer[1]	DNA binder	K_D(nM Dex)	NTI[2]	EOT[3]
CR6Et	Cort	6	EtBr	570	+	+
DR23Et	Dex	23	EtBr	2100	-	nd
DR4Sn	Dex	6	Spermine	370	-	nd
DR8Sn	Dex	8	Spermine	17	+	+
DR13Sn	Dex	13	Spermine	390	-	nd
DR9NP	Dex	9	Psoralen	100	++	+++
DR13NP	Dex	13	Psoralen	20’000	-	nd
Dex	Dex	no	no	3.7	+++	-

Abbreviations: Hor, hormone; Cort, cortisol; Dex, dexamethasone; EtBr, ethidium bromide; KD, thermodynamic equilibrium dissociation constant (measured by scatchard analysis of competition curves); NTI, nuclear translocation inducer (see Fig.1 of Paper1, chapter 3.1.); EOT, enhancement of transfection (see Fig. 4 of Paper1, chapter 3.1.).
1. Spacer length is comprising all the atoms separating the hormone moiety and the DNA binder function.
2. Qualifiers for NTI: -, no significant nuclear translocation (NT) with concentrations above 500 nM; +, more than 75% NT by 500 nM; ++, more than 75% NT by 50 nM; +++, more than 75% NT by 5nM.
3. Qualifiers for EOT: nd, not determined; +, 1.5-2 fold increase but without possible internal transfection reference; ++, 2-4 enhancement without possible internal reference; +++ 2-4 enhancement measured with internal reference.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ :
C07J 43/00, 41/00, C12N 15/87

A1

(11) International Publication Number: **WO 00/11018**

(43) International Publication Date: 2 March 2000 (02.03.00)

(21) International Application Number: PCT/CH99/00384

(22) International Filing Date: 19 August 1999 (19.08.99)

(30) Priority Data:
PCT/IB98/01306 21 August 1998 (21.08.98) IB

(71)(72) Applicants and Inventors: FREY, Felix [CH/CH]; University of Berne, Dept. of Internal Medicine, Freiburgstrasse, CH–3010 Bern (CH). RUSCONI, Sandro [CH/CH]; University of Fribourg, Biochemistry, Pérolles, CH–1700 Fribourg (CH).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: FREY, Brigitte [CH/CH]; University of Berne, Dept. of Internal Medicine, Freiburgstrasse, CH–3010 Bern (CH). WEHRLI, Hans–Ueli [CH/CH]; University of Berne, Dept. of Internal Medicine, Freiburgstrasse, CH–3010 Bern (CH).

(74) Common Representative: FREY, Felix; University of Berne, Dept. of Internal Medicine, Division of Nephrology, Freiburgstrasse, CH–3010 Bern (CH).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: CONJUGATES OF DNA INTERACTING GROUPS WITH STEROID HORMONES FOR USE AS NUCLEIC ACID TRANSFECTION AGENTS

(57) Abstract

The present invention relates to novel compounds comprising a steroid hormone linked to a DNA–interacting molecule that target nucleic acids to the cell nucleus. Further, the invention relates to a method for introducing nucleic acids into the nucleus of cells with the help of such compounds. Pharmaceutical preparations containing such compounds and the use of such compounds for gene therapy are also provided.

CURRICULUM VITAE

PERSONALS

First name	MAURIZIO
Last name	CEPPI
Address	Route Neuve 26, 1700 FRIBOURG (Switzerland) Tel: +41 78 656 92 88, E-mail: maurizio@mceppi.ch
Date of birth	5th of December 1971
Nationality	Swiss
Civil status	Unmarried

EDUCATION

1997-2000	UNIVERSITY OF FRIBOURG (Switzerland) **PhD in Natural Sciences** *Specialization:* Cell Biology, Molecular Genetics, Gene Therapy. *Thesis title:* "Nuclear receptor as vehicles for gene transfer".
1990-1995	SWISS FEDERAL INSTITUTE OF TECHNOLOGY OF ZUERICH (ETHZ - Switzerland) **MSc in Natural Sciences** *Specialization:* Biotechnology, Molecular Biology, Microbiology, Immunology, Molecular Genetics, Pharmacology. *Thesis subject:* "Gene-specific DNA-Repair in yeast".

PROFESSIONAL EXPERIENCE

1997-2000	UNIVETSITY OF FRIBOURG (Switzerland) Development of a new gene transfer technology for somatic gene therapy treatments (patented worldwide). Supervision of students, co-management of laboratory trainings. Designed and maintained the website of the Swiss National Somatic Gene Therapy Program NFP37.
1998-2000	Established and co-managed in Fribourg (Switzerland) FRI-NET, CEPPI & BANFI (www.fri-net.ch), a company specialized in the creation, the design and the administration of websites for small businesses.
1996	AMS BIOTECHNOLOGY LTD., Lugano (Switzerland) Technical Sales Support, Computer Systems and Database Management.
1995	CROSS CANCER INSTITUTE, Edmonton (Canada) Traineeship within the Molecular Oncology Program.

SCIENTIFIC CONTRIBUTIONS

PUBLICATIONS

Ceppi, M. et al., " Selective enhancement of gene transfer by Steroid Mediated Gene Delivery (SMGD)" (submitted to *Nature Biotechnology*);
Rusconi, S. & Ceppi, M., "Vectors for gene delivery", book chapter in
Gene Therapy for Rheumatoid Arthritis, S.Gay, 2001, Humana Press (in press).

AWARDS

2000, first prize at the annual scientific contest organized by ASIRB (Swiss Italian Association for Biomedical Research) in collaboration with ROCHE DIAGNOSTIC LTD. as a reward for the scientific achievements obtained during the PhD studies.

PATENTS

Technique co-developed during PhD studies is protected internationally by a patent published under the PCT's number: WO 00/11018;
Title: "Conjugates of DNA interacting groups with steroid hormones for use as nucleic acid transfection agents".

INTERNATIONAL MEETINGS

2000, 1999, 1998, 1997, NFP37 Swiss National Somatic Gene Therapy Program, annual meetings, Fribourg (Switzerland).
1999, American Society for Gene Therapy, 2nd annual meeting,
Washington DC (USA).
1999, "Gene and oligonucleotide delivery of therapeutics and vaccines.",
workshop organized by the Department of Pharmacy (ETHZ),
Monte Verità – Ascona (Switzerland).
1998, "How does plasmid DNA enter the mammalian nuclear pore.",
workshop organized by the company TRANSGENE, Strasbourg (France).
1998, "Import – Export: viruses and microbes meet the nuclear pore.",
EMBO workshop, Leysin (Switzerland).

ADDITIONAL COURSES

1999	BIOINFORMATICS, GENOMICS "Introduction to sequence analysis", organized by the European Molecular Biology Network, EPFL (Lausanne, Switzerland).
1999	BUSINESS "Ergänzungskurs in Unternehmensgründung", organized by the University of Fribourg.
2000	INTELLECTUAL PROPERTY "Ideen schützen und nützen", organized by the Swiss Federal Institute of Intellectual Property and the University of Fribourg.

TECHNICAL SKILLS

Culture, transfection and microinjection of standard mammalian cell lines;
construction of expression vectors and recombinant adenoviruses;
confocal and fluorescent microscopy, 3-Dimensional image analysis;
Southern Blot, Northern Blot, Western Blot, RT-PCR, RNase protection assay,
culture and genetic manipulation of yeast cells; Bohr assay (T4EndoV test).

COMPUTING SKILLS

GENERAL
MS-Office: Word, Excel, Power Point, Access.
Graphics: Adobe Photoshop, Macromedia Freehand, CorelDraw.
Web design: HTML-language, Macromedia Dreamweaver & Flash, MS-FrontPage.

BIOINFORMATICS
GCG's Wisconsin Package, EXPASY proteomics web server, EMBNET web server.

LANGUAGE SKILLS

ITALIAN	Mother tongue.
ENGLISH	Fluent both oral and written.
GERMAN	Fluent both oral and written.
FRENCH	Fluent both oral and written.
SPANISH	Fair both oral and written.

REFERENCES

Prof. Sandro RUSCONI, Institute of Biochemistry, University of Fribourg,
Switzerland, Tel: +41 +26 300 86 56, E-mail: sandro.rusconi@unifr.ch

Prof. Fritz THOMA, Institute of Cell Biology, ETH-Zürich,
Switzerland, Tel: +41 +1 633 33 23, E-mail: fritz.thoma@cell.biol.ethz.ch

Dr. Alex SIM, AMS Biotechnology (Europe Ltd.), Oxfordshire,
United Kingdom, Tel: +44 +1235 82 82 00, E-mail: amsbio@dial.pipex.com

Dr. Roseline GODBOUT, Cross Cancer Insitute, Edmonton,
Canada, Tel: 001 780 432 89 01, E-mail: roseline@cancerboard.ab.ca

INTEREST

SPORT	Hiking, trekking, squash, tennis.
ART	Latin dance, painting.
OTHERS	Ecology, foreign politics, eastern philosophy.

ARMY

RANK	Private.
INCORPORATION	Cp Info FA II/8 (air-raid protection).

Declaration d'originalité

Je déclare sur mon honneur que j'ai accompli ma thèse de doctorat seul et sans aide extérieure non autorisée.

Maurizio Ceppi

Maurizio Ceppi