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NUCLEIC ACIDS IN GENE DELIVERY AND GENE REGULATION

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“It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material.” [Concluding remark in the paper by Watson and Crick announcing discovery of the structure of DNA.]

— Francis Crick

In J.D. Watson and F.H.C. Crick, 'A Structure for Deoxyribose Nucleic Acid,' Letter in *Nature* (25 Apr 1953), **171**,

An experiment is a question which science poses to Nature, and a measurement is the recording of Nature's answer.

— Max Planck

Scientific Autobiography (1949), 110.

To my family

ABSTRACT

The concept of gene therapy, initially attributed to a technology that would allow the correction of inherited genetic disease, has evolved over the years. The realization of the immense technical hurdles to achieve genetic correction led to a broadening of the concept now including the transfer of genetic material whose expression will counteract, mitigate or revert a disease phenotype. This capacity to mitigate or revert the disease phenotype has also been achieved by gene expression regulation, at the level of the DNA or RNA, by the use of antisense or anti-gene technologies.
Therefore, at present, gene therapy encompasses not only the introduction and expression of therapeutic genes but also the regulation of gene expression itself which, when mediated by short oligonucleotides, gave rise to the concept of oligonucleotide-mediated gene therapy.

The evolution of gene therapy has been in straight connection with developments on gene delivery mechanisms and new nucleic-acid based chemistries, which have allowed progress in oligonucleotide based antisense / anti-gene methods.

Gene delivery mechanisms have relied mostly on viral-vehicles due to their known ability to carry and efficiently deliver their own genetic information to cells, a capacity perfected over the period of millions of years. However, production complexity and safety concerns have turned the attention to the possibility to use synthetically derived vehicles to achieve the same goal. These non-viral gene delivery methods, although regarded as safe, have not yet reached the efficiency of viral methods.

Increasing efficiency of non-viral vectors is a process involving mechanisms for protection and stabilization of the nucleic acid cargo (DNA, RNA, oligonucleotides) in extracellular biological fluids, as well as the intracellular release of the cargo. The cell itself presents several barriers for nucleic-acids cargo delivery such as the cellular membrane, endocytic vesicle release and the nuclear envelope.
In this thesis, in paper I, is presented a novel way to deal with nuclear membrane translocation when the nucleic-acid cargo needs to access the nuclear interior to exercise its action. The developed method uses the cells own nuclear import machinery through the use of a synthetic nucleic-acid that acts itself as the nuclear localization signal. In paper II, we tackle the barriers formed by the cell membrane and endosomal

vesicle release. In this paper a new type of cell-penetrating-peptide (CPP) was developed for delivery of splice-correction oligonucletides (single-stranded oligonucleotide). The CPP efficiently formed complexes with the oligonucleotides through non-covalent interactions, and these complexes were shown to have the capacity to efficiently be taken up by cells and be released from endocytic vesicles thus delivering the oligo to the intracellular environment.

We then turn to oligonucleotide-mediated gene regulation. In paper III we explore the use of oligonucleotides to correct an aberrant splicing of the *BTK* gene leading to loss of BTK protein production. The lack of BTK correlates with the absence of circulating B-lymphocytes thus causing the disease X-linked agammaglobulinemia.

In paper IV we explore a new LNA-based oligonucleotide capable of enhanced double stranded DNA targeting and duplex invasion in order to develop a new tool for the anti-gene field.

LIST OF PUBLICATIONS

This thesis is based on the following articles:

I. **Moreno, P.M.**, Wenska, M., Lundin, K.E., Wrange, O., Stromberg, R. and Smith, C.I. (2009) A synthetic snRNA m$_3$G-CAP enhances nuclear delivery of exogenous proteins and nucleic acids. *Nucleic Acids Res*, **37**, 1925-1935.

II. Ezzat, K., El Andaloussi, S., Zaghloul, E.M., Lehto, T., Lindberg, S., **Moreno, P.M.,** Viola, J.R., Magdy, T., Abdo, R., Guterstam, P. *et al.* PepFect 14, a novel cell-penetrating peptide for oligonucleotide delivery in solution and as solid formulation. *Nucleic Acids Res*. (in press)

III. **Moreno, P.M.,** Bestas, B., Blomberg, K.E., Berglöf, A., Guterstam, P., El Andaloussi, S., Lundin, K.E., Wengel, J., Smith, C.I.E. Correction of BTK splicing mutations in X-linked agammaglobulinema (XLA) by an exon-skipping strategy. (Manuscript)

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Other publications by the author not included in this thesis:

V. Lundin, K.E.*, Hasan, M.*, **Moreno, P.M.*,** Tornquist, E., Oprea, I., Svahn, M.G., Simonson, E.O. and Smith, C.I. (2005) Increased stability and specificity through combined hybridization of peptide nucleic acid (PNA) and locked nucleic acid (LNA) to supercoiled plasmids for PNA-anchored "Bioplex" formation. *Biomol Eng*, **22**, 185-192. (*shared first authorship)

VI. Viola, J.R., Leijonmarck, H., Simonson, O.E., Oprea, II, Frithiof, R., Purhonen, P., **Moreno, P.M.,** Lundin, K.E., Stromberg, R. and Smith, C.I. (2009) Fatty acid-spermine conjugates as DNA carriers for nonviral in vivo gene delivery. *Gene Ther*, **16**, 1429-1440.

VII. Oprea, II, Simonson, O.E., **Moreno, P.M.,** Viola, J.R., Lundin, K.E. and Smith, C.I. (2010) Temperature-assisted cyclic hybridization (TACH): an improved method for supercoiled DNA hybridization. *Mol Biotechnol*, **45**, 171-179.

VIII. Andaloussi, S.E., Lehto, T., Mager, I., Rosenthal-Aizman, K., Oprea, II, Simonson, O.E., Sork, H., Ezzat, K., Copolovici, D.M., Kurrikoff, K., Viola, J.R., Zaghloul, E.M., Sillard, R., Johansson, H.J., Said Hassane, F., Guterstam, P., Suhorutsenko, J., **Moreno, P.M.,** Oskolkov, N., Halldin, J., Tedebark, U., Metspalu, A., Lebleu, B., Lehtio, J., Smith, C.I., and Langel, U. Design of a peptide-based vector, PepFect6, for efficient delivery of siRNA in cell culture and systemically in vivo. *Nucleic Acids Res*, **39**, 3972-3987. (in press)

IX. Zaghloul, E.M., Madsen, A.S., **Moreno, P.M.,** Oprea, II, El-Andaloussi, S., Bestas, B., Gupta, P., Pedersen, E.B., Lundin, K.E., Wengel, J. *et*

al. Optimizing anti-gene oligonucleotide 'Zorro-LNA' for improved strand invasion into duplex DNA. *Nucleic Acids Res*, **39**, 1142-1154. (in press)

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LIST OF ABBREVIATIONS

Ago2	Argonaute 2
AON	Antisense Oligonucleotide
BMD	Becker Muscular Dystrophy
CBC	Cap Binding Complex
CNS	Central nervous system
CPP	Cell Penetrating Peptide
DMD	Duchenne Muscular Dystrophie
DOPE	1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine
DOTAP	N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride
DOTMA	N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride
DTS	DNA Targeting Sequence
ESE	Exonic Splicing Enhancer
ESS	Exonic Splicing Silencer
IBB	Importin-β Binding
ISE	Intronic Splicing Enhancer
ISS	Intronic Splicing Silencer
HIV	Human Immunodeficiency Virus
LNA	Locked Nucleic Acid
m_3G	2,2,7-trimethylguanosine
NES	Nuclear Export Signal
NLS	Nuclear Localization Signal
NPC	Nuclear Pore Complex
NTR	Nuclear Transport Receptor
Nups	Nucleoporins
ON	Oligonucleotide
PAMAM	Polyamidoamine
PEI	Polyethyleneimine
PLL	Poly-L-Lysine
PMO	Phosphorodiamidate Morpholino Oligomer
PNA	Peptide Nucleic Acid
pRNA	Promoter associated RNA
PS	Phosphorothyoate

Ran	RAs-related Nuclear protein
RanGAP	Ran GTPase-activating protein
RanGEF	Ran Guanine-nucleotide-Exchange Factor
RCC1	Regulator of chromosome condensation 1
RISC	RNA-induced silencing complex
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SCID	Severe Combined Immunodeficiency
SCO	Splice Correcting Oligonucleotide
siRNA	Small interfering RNA
SMA	Spinal Muscular Atrophy
SMGA	Smooth Muscle Gamma Actin
SMN	Survival of Motor Neuron
snRNA	Small nuclear RNA
SPN1	Snurportin
SRF	Serum Response Factor
SSO	Splice Switching Oligonucleotide
STV	Streptavidin
TGS1	trimethylguanosine synthase 1
TGS	Trnscriptional Gene Silencing
T_m	Melting Temperature
TFO	Triplex Forming Oligonucleotide
UTR	Untranslated Region
WGA	Wheat Germ Agglutinin
7mG	7-monomethylguanosine

1 INTRODUCTION

The concept of gene therapy was originally attributed to the idea by which the transfer of DNA material into cells would serve as corrective measure for genetic diseases. This initial concept was soon found to be very technically demanding. Thus, another broader definition emerged considering gene therapy as a therapeutic technique by which a functional gene is inserted in the human patient cells to correct a genetic dysfunction or to give a new function to those cells (1). This concept can be even further expanded to include the specific regulation of gene expression mediated by DNA or RNA targeted short oligonucleotides, as in antisense or anti-gene therapy.

However, to achieve the full realization of the gene therapy concept, successful means to deliver the nucleic-acid payload, comprising both long double-stranded DNA genetic material or short single and double-stranded oligonucleotides, must be in place.

1.1 GENE THERAPY

Already in the 1960's the initial idea of gene therapy started to emerge in a pure speculative manner, with the first ethical debates emerging towards the end of that decade. At that time, some of the discussions around the gene therapy concept were brought forward in different publications. Some comments and reflections were present in statements such as the one by Joshua Lederberg on the "Biological future of man: "the ultimate application of molecular biology would be the direct control of nucleotide sequences in human chromosomes, coupled with recognition, selection, and integration of the desired genes" (2); and Edward Tatum:"…We even can be somewhat optimistic about the long-range possibility of therapy based on the isolation or design, synthesis, and introduction of new genes into defective cells of particular organs" (3). Until the end of that decade others expanded on the idea but gene therapy still remained mostly a thing of theoretical debate.

It was not until the late 1980's, after the invention of recombinant DNA technology (4) and the development of retroviral vectors, which were in common laboratory use by the mid 80's, that the first human trials for gene therapy were performed. Thus in 1989, Dr. Steven Rosenberg initiated the first RAC-approved gene therapy trial, which was actually a gene-marking study of tumor-infiltrating lymphocytes (TIL) (5). Effectively, the first therapeutic gene therapy trial started in 1990 and was conducted by Dr. R. Michael Blaese's group. Using a retroviral vector, the adenosine deaminase gene was transferred into T-cells as therapy against severe combined immunodeficiency (SCID). The protocol appeared to be safe for the patients, however the clinical benefits were not significant (6,7).

Since that time, and with the human genome coming full circle, several genes associated with disease have been identified which translated in many of those genes being cloned and made to express by different vectors for therapeutic purposes. This as concomitantly led to an increasing number of gene therapy trials using different vectors such as: recombinant adenovirus in cystic fibrosis patients (8); adeno-associated virus in hemophilia B patients (9); lentivirus for hemophilia patients (10), among others. During these times gene therapy has promised much but has nonetheless encountered several hurdles.

Perhaps the most dramatic realization of these setbacks comes from the case of an *ex-vivo* gene therapy trial for SCID-X1. In 2000, Alain Fischer reported the successful *ex-vivo* gene transfer of human gamma chain into CD34+ cells, using a retrovirus

vector, to treat infants affected by SCID-X1 (11). The reports on the achievement of full correction of disease and phenotype generated great enthusiasm which was later turned into great concern when 3 of the children developed T cell leukemia. This was attributed to an insertional mutagenesis near the LMO2 oncogene, which led to its overexpression (12,13).

The adverse effect exemplified here is reminiscent of the pathologic nature of the parental viruses used in this trial and points out some of the possible limitations when using modified viruses for gene transfer. As will be discussed later, this warranted the development of non-viral vectors with an inherent lower toxicity profile.

Throughout the time, gene therapy, initially intended for the treatment of monogenic disorders, widened its concept to embrace the treatment of other diseases with complex pathologies such as neurodegenerative diseases (eg. Parkinson's, Alzheimer's), infectious diseases (eg. HIV) and cancer. In parallel, new derivations for gene therapy came with the realization of the antisense technology, opening the field of antisense gene therapy, which will be discussed in more detail later in this thesis.

1.2 GENE DELIVERY

The success of gene therapy is strongly dependent of the successful development of efficient and safe delivery vectors. Two different lines of research have emerged, one focusing on the development of viral carriers and another focusing on synthetic delivery systems (non-viral carriers). As already stated, viral vectors were the first to be used in clinical trials. Viruses are highly efficient biological machines. They have evolved for million years perfecting their capacity to access the host cell and use the cells own machinery to aid in their process of replication and infection. So it came as no surprise that viruses, as efficient machines for delivery of exogenous genes achieving good expression levels, were initially the vectors of choice for gene therapy protocols. They are at present still the most widely used in human clinical trials worldwide (Fig.1).

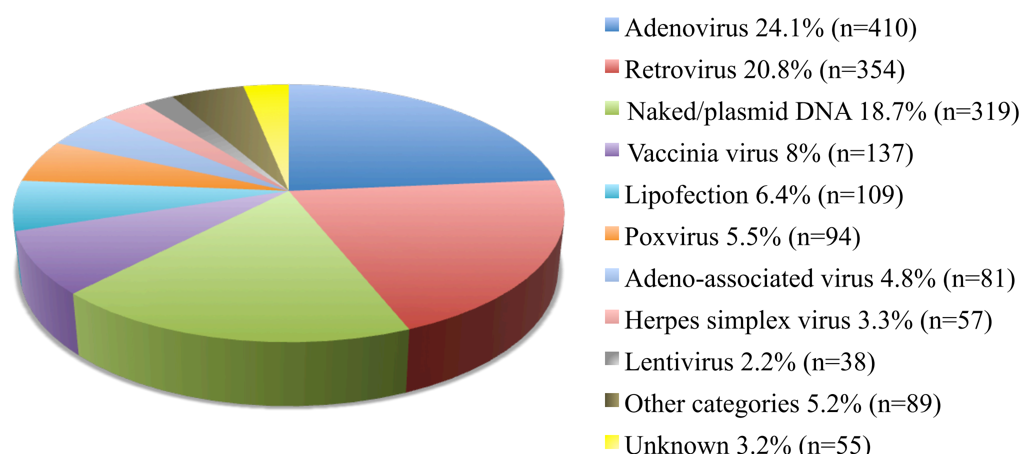

Figure 1: Type of vectors used in clinical trials worldwide. Information obtained from (14) with permission from Journal of Gene Medicine 2011 © John Wiley and Sons Limited.

The most used viral systems include biologically engineered retrovirus, lentivirus, adenovirus, adeno-associated virus, and herpes simplex virus (HSV-1) (Fig.1). Despite wide use they still possess important limitations, perhaps the most significant being the possibility of acute immune responses as a reaction to the viral proteins (especially occurring with repeated administrations), the possibility of generating replication-competent viruses by uncontrolled recombination with homologous sequences in helper cell lines, and insertional mutagenesis, which, if occurring close to oncogenes, may lead to their activation (15,16).

One can say that a non-viral approach for gene transfer was already being projected by papers in the 1970's showing specific introduction of genes into mammalian cells by chromosome-mediated gene transfer (17) and by purified DNA-mediated gene transfer (18). A facilitated delivery of purified DNA into mammalian cells was also present in a study by Graham and van der EB in 1973, demonstrating the transfer of viral DNA into cells by a process of co-precipitation with calcium phosphate (19). Perhaps the most seminal paper on "naked" DNA gene transfer came later from Wolff and colleagues in 1990 showing expression of reporter genes after direct injection into the mouse skeletal muscle (20).

Development of non-viral vectors has been supported by their recognized ease of use, the suitability for high-scale production under GMP manufacturing, lack of specific immune responses and a large DNA packing capacity.

The three main non-viral systems to date are the simple "naked" DNA, cationic lipids and cationic polymers.

1.2.1 Naked DNA delivery

Regarding naked DNA delivery it has been already referred that intramuscular injections are quite successful in achieving transgene expression, however, the same is in general not true when we talk about intravascular injections. This notion has been however somewhat contradicted by work from Jon Wolff's lab and Dexi Liu's lab demonstrating efficient gene expression in hepatocytes following the injection of large volumes of plasmid DNA via the mouse hepatic vein and tail vein (21,22). These hydrodynamic injection techniques, hence designated due to the relatively big volumes of solution injected over a very short period of time, have latter been applied for transgene delivery in the kidneys following injection into the renal vein (23), into the limb muscle via intra-arterial injections, and to a smaller percentage (10-30%) of tumor cells in the brain after injection into the common carotid artery (24). While efficiencies have been good the complex surgical nature of these procedures would limit their application in clinical settings. This notion has however been disputed by the hydrodynamic limb vein (HLV) delivery method where the nucleic acid solution is injected into a limb vein while the blood flow in and out of the limb is blocked by a tourniquet (25). The significant levels of gene expression, the possibility to target a significant muscle mass both to be used as a site of expression of different therapeutic proteins or for the actual treatment of muscle diseases, as well as the lack of procedure associated toxicity, has prompted the HLV method as a candidate for clinical applications (26,27).

1.2.2 Lipoplexes

The second most characterized method is the use of cationic lipids for complexation with the negatively charged DNA molecules, thereby creating the lipoplexes. The first lipoplexes were described in 1987 by Felgner *et al* (28) and they were composed of the cationic lipid DOTMA. Since then, progress in lipoplex delivery has presented us with the synthesis of new cationic lipids such as eg. DOTAP and DOSPA (29). The mechanism of uptake of lipoplexes is thought to be primarily through endocytosis (30), as such, the currently used lipoplexes are prepared with neutral or fusogenic helper-lipids such as DOPE to improve escape from endosomes (31). *In-vivo*, efficiency of lipoplexes as, nonetheless, been limited by problems such as aggregation in blood, non-specific binding to serum proteins, and low accessibility to the nucleus of non-dividing cells (32). One should say though that strategies to overcome some of these problems

have been investigated such as the use of PEG as a shielding agent to avoid aggregation in blood (33).

1.2.3 Polyplexes

The third most well characterized method involves the use of cationic polymers, which by charge interaction are able to condense DNA into complexes designated by polyplexes. This concept was first presented at the same time as lipoplexes, in 1987, by Wu and Wu (34). The cationic polymer used at that time was poly-L-Lysine (PLL). PLL, together with polyethyleneimine (PEI) and polyamidoamine (PAMAM) are the most studied polyplexes. Common to all cationic polymers is their lack of an hydrophobic domain, which precludes them from any direct interactions with the endosomal membrane leading to fusion or destabilization of the endosomal membrane, as is the case with cationic lipids. In the instance of PLL this leads to extremely inefficient transfections unless used in conjunction with endosome-lytic agents (eg. inactivated adenovirus, (35)). In contrast, PEI, one of the most promising polymers capable of achieving remarkable transfection efficiencies, owes its effectiveness exactly to the fact that it is capable of promoting its own endosomal escape due to its intrinsic endosome-bufferig capacity, often referred to as the proton-sponge effect (36). As lipoplexes, polyplexes suffer from non-specific interactions with serum proteins, forming aggregates that are rapidly cleared from circulation. Again, PEG has been used to shield polyplexes from these non-specific interactions as a way to prolong its circulation in blood (37). Other modifications of polyplexes, and also lipoplexes, have been the conjugation of cell specific ligands to take advantage of receptor mediated uptake mechanisms, thereby achieving cell/organ specific delivery (38-41).

1.2.4 CPPs

The first cell penetrating peptide (CPP) was reported in 1994, when it was observed that a short 16 amino acid long fragment corresponding to the third helix of the Antennapedia homeodomain had the capacity to translocate through biological membranes (42). This was in line and followed earlier reports from 1988 on the capacity of a full-length HIV protein, Tat, to be taken up by cells in culture (43,44).

A number of peptides with the above described capacity has emerged and, while the exact definition of CPP is still debatable they all share some basic features: i) usually less than 30 amino-acids long; ii) polybasic and/or amphipatic; iii) capacity to translocate across cell membranes and to carry molecules along in this process.

Table 1: Examples of some CPP sequences

CPP	SEQUENCE	REF
Penetratin	RQIKIWFQNRRMKWKK	(42)
Tat	GRKKRRQRRRPPQ	(45)
M918	MVTVLFRRLRIRRACGPPRVRV-NH_2	(46)
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL-NH_2	(47)
TP10	AGYLLGKINLKALAALAKKIL-NH_2	(48)
MAP	KLALKLALKALKAALKLA-NH_2	(49)
R9	H-RRRRRRRRR-NH_2	(50)
Pep-1	KETWWETWWTEWSQPKKKRKV	(51)
MPG	GALFLGWLGAAGSTMGAPKKKRKV	(52)
$(RXR)_4$	H-RXRRXRRXRRXR-OH	(53)

X=6-aminohexanoic acids

Different cargoes have been shown to be able to be transported into cells by CPPs such as peptides (54), proteins (55), drugs (56), plasmid DNA (57), liposomes (58), nanoparticles (59), oligonucleotides (60,61) and siRNA (62). For the purpose of this thesis we will concentrate mainly on delivery of nucleic acid-based cargoes.

There are basically two strategies for CPP-mediated cargo transport into cells, namely, a covalent strategy where the CPP is physically linked to its cargo, or a non-covalent strategy where the CPP interacts with the cargo mainly through electrostatic interactions.

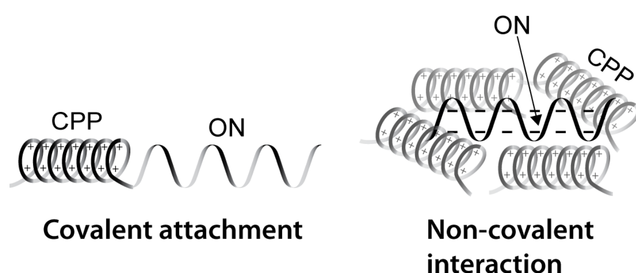

Figure 1. The two strategies for CPP-mediated transport of its cargo (in this case oligonucleotides, which can be of different chemistries): covalent attachment and non-covalent through electrostatic interactions.

Covalent linkages between CPP and oligonucleotides have been especially useful when the oligonucleotide backbone renders them uncharged, such as in the case of peptide nucleic acid (PNA) or morpholino (PMO) oligomers. These conjugates have been especially useful and have shown promising results in the inhibition of virus

replication in cell culture and animal models (63), but perhaps the most striking results have been achieved in a splice switching strategy to achieve exon skipping of the mutated dystrophin gene in Duchenne's muscular dystrophy (DMD). The DMD splicing case will be discussed in more detail in a later section of this thesis. One can however say that in comparison to the *in-vivo* administration of naked PMOs, which have resulted in low splice switching levels, CPP-PMO conjugates have shown a dramatically improved efficiency of splice switching in a DMD mouse model, with restoration of dystrophin protein to more than 50% of normal levels in all peripheral muscles and evidence of restored dystrophin protein in the cardiac muscle, which was observed for the first time (64-66).

The non-covalent strategy comes from the simple mixing of the peptide and plasmid DNA or oligonucleotide in solution. Normally an efficient complexation requires an excess of peptide in relation to the negatively charged nucleic-acid in question. CPPs used in a non-covalent strategy are exemplified by the use of MPG (a fusion peptide between a hydrophobic segment of HIV-1 gp41 and nuclear localization sequence SV40) to form complexes with siRNA, which have been used *in-vitro* in several cell lines (67,68) and *in-vivo* for targeting OCT-4 in mouse blastocytes (69) or Cyclin B1, an essential cell cycle protein, in tumour model mice (70). Polyarginines have also been used, achieving very interesting results mostly when conjugated to cholesteryl (Chol-R9) for the *in-vivo* delivery of siRNA against VEGF (71), conjugated to a small peptide derived from rabies virus glycoprotein (RVG-R9) for siRNA delivery to the CNS by crossing the blood-brain-barrier (72), or even conjugated to CD7-specific single-chain antibody (scFvCD7-R9) for T cell-specific siRNA delivery (73). Other examples for siRNA delivery are penetratin-derived peptides (74), Tat peptide associated with an RNA binding motif (75), and transportan derived peptides (62). CPPs can also be used in a co-incubation strategy with oligonucleotides and cationic lipids enhancing the intracellular delivery in comparison to lipoplexes only (76).

1.2.4.1 CPP – Mechanism of uptake

The mechanism of cellular uptake of CPP and its cargo (either covalently or non-covalently attached) remains ambiguous. The discussion as led to the idea that both the peptide (CPP) and cargo properties can have an influence on the mechanism of uptake. Variation on the context of the experimental conditions can also play a fundamental role (77).

In any case, there are two main pathways indicated as responsible for the uptake, one being the direct translocation through the plasma membrane and the other the endocytic pathway. Direct translocation would involve destabilization of the plasma membrane by direct interaction of the peptides with the lipid bilayer initially at its surface. Biophysical methods have proposed different models such as the barrel-stave, inverted micelle, and carpet model as types of structures that could allow penetration of the peptides into the intracellular milieu (78-80). A study with arginine rich peptides employed genetically engineered cells with different endocytic pathways blocked, as well as different temperatures to study their translocation. It was concluded that it was not dependent on any endocytic or pinocytic pathways (81). The study however did not employ any cargo-peptide conjugates, which have been recognized to strongly affect the mechanism of uptake (82).

Concerning the process of endocytosis, which includes phagocytosis and pinocytosis, it is a regulated process by which the cell takes up fluids and extracellular molecules. Phagocytosis is associated to specialized cells such as macrophages and neutrophiles, and is a process used to engulf big particles in an actin-dependent manner (83). Pinocytosis occurs in all cell types and 4 distinct mechanisms are associated to it: macropinocytosis, clathrin-mediated, caveloae-mediated, clathrin/caveolae-independent (84,85). CPPs can in principle use not only a single mechanism of endocytosis but in fact take advantage of several mechanisms in parallel as suggested by a study on the uptake mechanism of antennapedia/penetratin peptide, nona-arginine (R9), and Tat, showing that not a single endocytic mechanism could be resolved, but instead macropinocytosis, clathrin and caveloae-mediated endocytosis were occurring in parallel (86).

Since cargo can affect not only the route of delivery but also the extent of delivery, every CPP-cargo complex should be investigated individually and through the use of several methods in parallel given that there is no single method capable of providing a clear-cut view on the exact mechanisms of uptake. Hence a combination of methods should be used, perhaps paying special attention to quantitative uptake by spectrofluorometry and confocal microscopy (87). In any case the most decisive method when it comes to efficacy evaluation is a functional biological assay. One typical *in-vitro* biological assay, recurrently used when assessing the bioavailability of CPP-oligonucleotide complexes, employs the HeLa/Luc705 cell line in a Luciferase splice correction assay. The cargo molecule is thus a steric block oligonucleotide capable of redirecting a pre-mRNA mis-splice occurring in a stably expressed

luciferase gene due to the introduction of a mutant β-globin intron in the middle of the Luc gene (88).

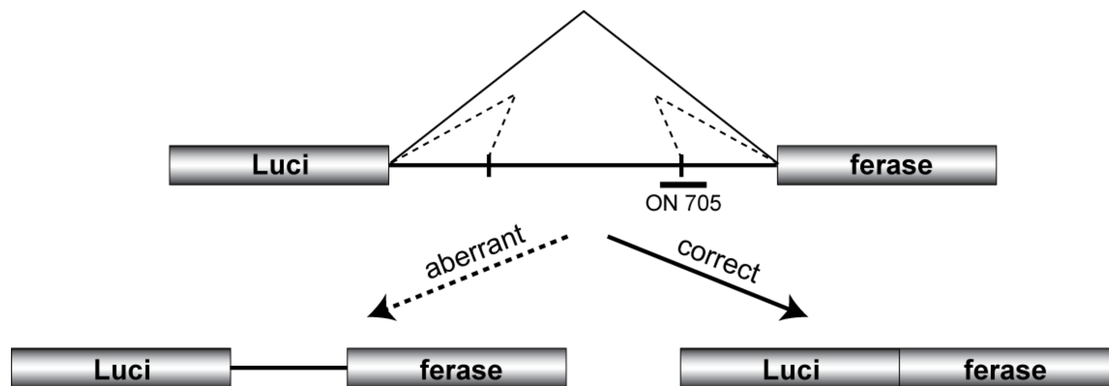

Figure 2. Reporter system for splice switching. A HeLa stable cell line stably expresses a Luciferase gene interrupted in the middle by a mutated β-globin intron 2. The mutation at position 705 produces a 5'-SS and activates a cryptic 3'-SS leading to the production of an aberrantly spliced mRNA unless the splice site is masked by a splice correcting oligo (ON 705) (88).

1.2.5 Intracellular barriers – The nuclear envelope

Nuclear import of therapeutic molecules is of great importance for the area of gene delivery. This is especially true in non-viral vector development since these transport systems need to mimic most of the virus strategies used to overcome several cellular barriers, of which the nuclear membrane is the ultimate one.

The nuclear membrane, also named nuclear envelope, is bilayer in nature and acts as a molecular sieve tightly regulating the traffic of molecules in and out of the nucleus. The high level of control in the transport of molecules from and to the nucleus is of uttermost importance to eukaryotic cells allowing them to achieve a strict regulation of gene expression by controlling the entry of key signalling molecules to the nucleus as well as being critical for a correct cell division (89,90). The "door" through which the nucleo-cytoplasmic transport occurs is a large multiprotein channel that spans the double-lipid bilayer of the nuclear envelope and is designated nuclear pore complex (NPC). Studies in the vertebrate *Xenopus laevis* have revealed a molecular mass of around 112 MDa (91) whereas in the invertebrate *Saccharomyces cerevisiae* it was determined to be around 66 MDa (92). There are approximately 2000-4000 NPC in a nuclear envelope, each consisting of multiple copies of around 30 different proteins

known as nucleoporins (Nups) (89,93). This doorway (the NPC) acts as a diffusion channel, which, with a diameter of around 90-100Å, allows the passage of macromolecules of roughly 40KDa while larger cargoes with diameters up to 390Å require an active transport mediated by specific import/export receptors (94,95). The selectivity of the pore is achieved by the presence of natively unfolded Nup modules containing numerous FG repeats, each unit containing a hydrophobic cluster, typically of the sequence FG (phenylalanine-glycine), GLFG (L, leucine), or FxFG (x, any), embedded into a more hydrophilic spacer sequence (96). While still under debate, it is considered that FG repeats can form a very selective hydrogel with capacity to interact with nuclear transport receptor proteins (NTRs) whilst still remaining firm barriers towards inert macromolecules lacking any transport capabilities, and even possessing "self-healing" properties that allows it to immediately reseal behind a translocating molecule (97,98).

The transport mechanism itself has as minimum requirements the presence of a transport signal and a shuttling receptor capable of recognizing that signal. Basically, proteins to be transported in or out of the nucleus need to have present a specific amino-acid sequence designated nuclear localization signal (NLS) or nuclear export signal (NES), respectively.

This thesis will focus on the nuclear import mechanism.

The classic mechanism of nuclear import is mediated by NLS peptides that can contain one or two clusters of basic residues. The prototypical monopartite NLS, described in the mid 80's, is exemplified by the Simian Virus 40 (SV40) large T antigen NLS which contains a cluster of 4-5 consecutive positively charged amino-acids (99,100). The prototypical bipartite NLS comes from the example of nucleoplasmin which has a second, generally smaller, cluster located 10-12 residues downstream of the first (101). A transport adapter, importin-α, directly recognizes these signals, through its two specific lysine-binding pockets located at the surface (102,103). Additionally, importin-α contains at the N-terminus a domain for direct binding to the carrier protein importin-β (also named karyopherin β1) (104). This domain, the importin-β binding domain (IBB), contains a cluster of basic amino-acids, and is normally bound to the NLS binding pockets of importin-α directly competing with the NLSs (105). This points to the idea that NLS binding to importin-α is facilitated when it is already in complex with importin-β due to the delocalization of the IBB domain out of the NLS binding pockets. This suggests in turn that in the cell, NLS containing proteins bind primarily to

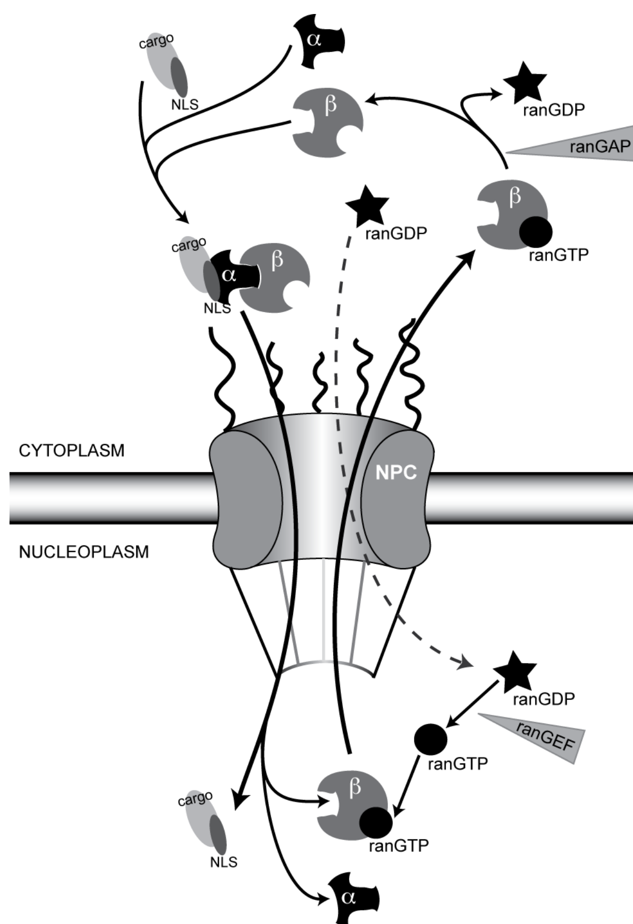

Figure 3. Mechanism of nuclear import. An NLS bearing cargo is recognized by the Imp-α/β complex. The cargo-Imp-α/β complex is translocated thorugh the nuclear pore (NPC). Once in the nucleoplasms it is disassembled by the binding of RanGTP to Imp-β. Imp-α and Imp-β are transported back to cytoplasm where RanGTP hydrolysis by RanGAP allows the importins to be free again to restart the process.

the importin-α/β complex. The cargo-importin-α/β is then able to translocate through the nuclear pore into the nucleus via direct interactions of the importin-β with the NPC nucleoporins. Directionality of transport is assured by an energy dependent process that maintains high concentration levels of RanGTP in the nucleus, responsible for inducing the dissociation of the cargo-import complex (106). The mechanism is as follows: once the cargo-importin α/β complex is imported into the nucleus, RanGTP binds to importin-β leading to the release of the cargo and dissociation of the whole complex. Importin-β complexed with RanGTP is then recycled to the cytoplasm, whereas importin-α is exported complexed with the β-karyopherin CAS and RanGTP. The importins-RanGTP complexes are dissociated in the cytoplasm after the hydrolysis of RanGTP to RanGDP, which is facilitated by RanGAP at the cytoplasmic face of the NPC. RanGDP is then imported back to the nucleus to be phosphorylated again to RanGTP by the guanine exchange factor (RanGEF) RCC1. Thus, the concerted action of RanGAP (GTPase-activating protein), which is found predominantly in the cytoplasm, and RanGEF (guanine-nucleotide-exchange factor), which is found predominantly in the nucleus, generates the necessary asymmetrical distribution, with RanGDP located in the cytoplasm and RanGTP within the nucleus (106).

Not all NLS bind to importin-α as the first step. Instead they can bind directly to importin-β, bind to transportin (karyopherin β2) or bind to different import adaptor proteins like snurportin (SPN1). Table 2 presents a list with NLS sequence examples and carrier proteins used for their nuclear transport.

Table 2: Examples of Nuclear localization signal (NLS) sequences and import factors involved in the nuclear translocation

CARGO	Peptide sequence motifs / Domains	Transport Factors
SV40 large T-antigen	PKKKRKV	Imp α/ Imp β
Myc	PAAKRKVLD	Imp α / Imp β
HIV-1 Rev	RQARRNRRNRRRRWR	Imp β
HIV TAT	GRKKRRQRRRPPQC	Imp β
Nucleoplasmin	**KR**PAATKKAGQA**KKKK**LDK	Imp α / Imp β
hnRNPA 1 (M9 NLS)	GNQSSNFGPMKGGNFGGRSSGPYG GGGQYFAKPRNQGGY	Transportin
U snRNP	m_3G-CAP and Sm core proteins	Snurportin / Imp β

1.2.5.1 Nuclear Import of Small Nuclear RNAs

Of relevance to the work presented in this thesis is a more detailed view on the nuclear import of snRNAs, specifically, uridine rich small nuclear RNAs (U-snRNAs). U-snRNAs are a major component of the spliceosomal U-snRNPs undergoing a complex biogenesis process both in the nucleus and cytoplasm. U snRNAs U1, U2, U4 and U5 are synthesized in the nucleus by RNA polymerase II where they acquire a 7'-monomethylated (7mG) cap structure at their 5'-end. This 7mG-cap participates in the snRNA export to the cytosol by action of a nuclear cap-binding complex (CBC) (107). Once in the

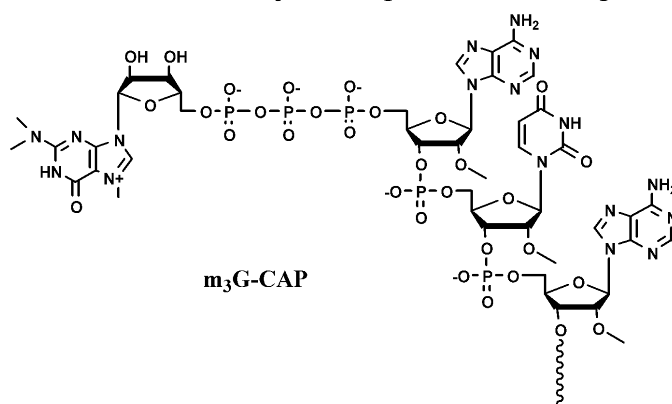

Figure 4. 2,2,7-trimethylguanosine cap structure (m_3G-CAP).

cytoplasm the snRNAs associate with the survival of motor neurons (SMN) complex formed by the SMN proteins and at least six distinct Gemin proteins (108). The assembly of this complex on the snRNA promotes the subsequent maturation events which ultimately lead to the recruitment of a hypermethylase (TGS1) that catalyzes the transfer of two additional methyl groups to the 7mG-cap converting it to the 2,2,7-trimethylguanosine (m_3G) cap (109,110). The m_3G-cap acts as an independent NLS signal, which together with a less defined NLS signal present in SMN associated proteins (111) results in re-import to the nucleus. The m_3G-cap structure is specifically recognized by the snurportin protein import adaptor (SPN1) (112,113) which then binds to importin-β (114). Analysis of an m_3G-cap analogue (m_3GpppG) bound to the C-terminal domain of SPN1 shed some light on the structural basis for the import of the m_3G-cap while 7mG-cap is excluded. It was reported that the two extra methyl groups on the guanosine allow Van der Waal contacts with Tryp residues in the SPN1 binding pocket. More importantly was the fact that binding of the m_3G-cap to SPN1 requires hydrophobic interactions that do not allow any water molecules to be present. The m_3G-cap having a smaller hydration shell than 7mG, due to the dimethylated N2, has a big energetic advantage (113). In the same study, crystallization with an extended m_3G-cap containing 3 additional methylated residues similar to naturally occurring snRNA ($m_3GpppA_mU_mA_m$) was not possible, however determination of dissociation constants led to the observation that the extended cap had around 5x more binding affinity to SPN1 than the dinucleotide cap leaving open the question on the structural role of these extra 2'-*O*-methylated residues on the overall binding to SPN1.

It is of interest to add that this complex is thought to mediate an energy-independent nuclear translocation of snRNPs (115,116).

The m_3G-cap remains, so far, the only fully characterized nucleic acid structure capable of acting independently as an NLS signal.

1.2.5.2 Nuclear Delivery of exogenous nucleic acids

The efficiency of gene delivery is strongly connected to the capacity of the exogenous nucleic acid material to be translocated across the nuclear envelope. During common *in-vitro* laboratory procedures for cell transfection the cells are usually actively dividing, meaning that the nuclear envelope is broken down in each mitosis phase and reassembled after M phase completion. This process translates into an easy access of any nucleic acid (eg. plasmid DNA) to the nucleus during the M phase and concomitantly high transfection efficiencies. Such is not the case in primary cells, or

terminally differentiated cells, and therefore the nuclear membrane barrier strongly affects *in-vivo* gene delivery efficiencies (unless the target cells are actively dividing cancer cells).

It is therefore with no surprise that some of the efforts in the development of non-viral vectors have been directed towards improvements in nuclear targeting. Most of the approaches to tackle this problem have concentrated on hijacking the intracellular mechanisms and proteins involved in nuclear import.

The first evidence that DNA could indeed translocate through the nuclear pores came from a study by Wolff and co-workers where they microinjected plasmid DNA into the cytosol of postmitotic primary rat myotubes. Plasmid DNA was indeed found in the nuclei of the cells, however it is also a fact that large amounts of DNA were microinjected, most of it was residing at the site of injection and the process was extremely dependent on the injection site being close to the nuclear envelope. Nevertheless, the process was found to be energy dependent and inhibited by wheat germ agglutinin (WGA, a blocker of transport through the nuclear pores) indicating that the transport process was closely following the entry route of karyophilic proteins (117).

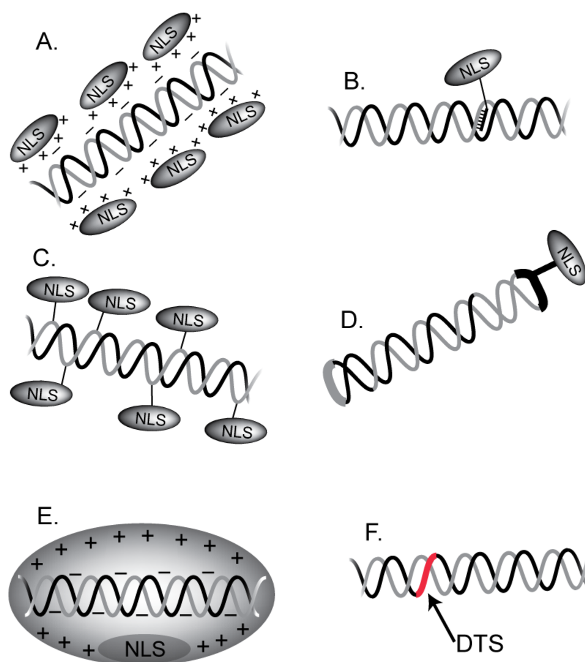

Figure 5. Examples of methods to enhance nuclear import of DNA. **A)** Non-covalent electrostatic association with NLS peptides; **B)** Sequence-specific association through NLS-conjugated PNA-anchors;
C) Covalent coupling of NLS through chemical or photoactive groups; **D)** NLS sequence ligated to hairpin sequences on the ends of linear DNA sequences **E)** Ionic association with karyophilic proteins;
F) DNA bearing DNA-Targeting-Sequences recognized in the cytoplasm by transcription factors that will mediate nuclear import through import protein interactions.

Several strategies have thus tried to use the entry route of karyophilic proteins to increase nuclear import of DNA. Most have relied on the import mechanics of NLS signals and can be divided into different categories: I) Non-covalent association of NLS to DNA; II) Covalent attachment of NLS to DNA; III) Association of DNA with proteins with nuclear import capacity.

Non-covalent association

Some strategies have relied on the electrostatic association of the common SV40 NLS peptide to plasmid DNA for uptake and nuclear localization in zebrafish (118) or even tetrameric versions of the same NLS separated by glycine units with capacity to complex plasmid DNA creating polyplexes that efficiently transfected various cell lines such as 16HBE14o-, HeLa S6, and Cos7 cells (119). Another approach used the M9 NLS peptide conjugated to a scrambled version of the SV40 NLS which was employed in this case has a simple cationic domain to allow interaction of the peptide conjugate to the DNA. The DNA-peptide complex was used together with lipofection to transfect highly confluent bovine aortic endothelial cells (BAECs) (120).

Instead of using the NLS peptides as the condensing/complexing agents themselves, other strategies have used condensing components like polylysine conjugated to the SV40 NLS peptide (121).

Another alternative to the unspecific electrostatic association is the use of sequence-specific DNA analog anchors conjugated with NLS peptides. In one example, peptide nucleic acid (PNA) clamps were conjugated to SV40 NLS peptide and associated to plasmid DNA via sequence specific hybridization. These complexes were transfected into cells by the use of PEI and an increase in expression was seen over naked (unhybridized) DNA (122). A sequence specific association was also achieved by the use of triplex forming oligonucleotides (TFOs) that were ligated in a subsequent step to a hairpin oligo conjugated to an NLS peptide. These padlock conjugates were then transfected via lipofection but no increase of expression was observed (123).

Covalent strategies

Direct conjugation of NLS peptides to DNA could offer a more stable alternative to the non-covalent strategies when the plasmid is *en-route* through the cytoplasm towards the nucleus.

Some approaches involved direct conjugation through photoactivation of a p-azido-tetrafluoro-benzyl modified NLS peptides to plasmid DNA while studying optimal spacer lengths between NLS to the anionic DNA backbone. The results in terms of nuclear uptake were however far from satisfactory although association with importins was seen in *in-vitro* tests (124,125). The observation that importin was binding but no functional effect in cells were seen can correlate to the fact that the unspecific covalent attachment of NLS peptides to plasmid DNA could render them transcriptionally

inactive but also to the difficulty of DNA to efficiently overcome the crowded cytoplasm and travel towards the nuclear periphery (126).

On the other hand, a correlation between NLS mediated DNA nuclear import and size has been made. A 900bp GFP-expression cassette was covalently labeled with biotin through the use of a PCR method with biotinylated-primers and subsequently incubated with NLS conjugated streptavidin. Microinjections into living cells resulted in increased uptake and expression of the GFP cassette in the nucleus (127).

A direct conjugation of NLS peptides to oligo primers has also been used in a PCR approach to label a Luciferase expressing plasmid. In this case, both electroporation and a cationic polymer based delivery system were used for transfection but no significant enhancement of gene expression was seen when using the NLS-conjugated plasmid (128).

Another method for specific association of NLS to plasmid DNA has been through the use of TFO involving triple helix formation and subsequent covalent bonding by photoactivation. Delivery to NIH3T3 by lipoplex formation was used and the modified plasmids were expressed indicating no loss of transcription activity. On the other hand there was no increase of gene expression when using the NLS modified plasmids (129).

Association with proteins with import capabilities

Association of DNA with viral proteins has also been investigated. One such case is exemplified by the complexation of DNA with the major core protein VII of adenovirus type 2 (Ad2). Adding the complexes directly to the cell medium resulted in rapid (2-4h) transport into the cell and the nuclei of about 40% of the cells (130). However, it has been recognized that there might have been an overestimation of the result due to plasmid residence at the nuclear envelope and not exactly inside the nucleus. Another example comes from the use of HIV-1 integrase (IN) enzyme for complexation and nuclear localization of DNA in a process mediated by the importin α/β complex. The IN alone was rapidly accumulating in cell nuclei and when in complex with DNA it maintained the ability to interact with importin α/β. A critical basic region ($K^{186}RK$) mediating the nuclear translocation was identified. The studies were however only done in an *in-vitro* system using semi-intact cells (131).

The use of histones has also been investigated in a way certainly inspired by their natural ability to condense DNA as one of their functions in the nucleosome particles. The 4 core histones H2A, H2B, H3 and H4, are transported from the cytoplasm to the nucleus by an NLS-dependent process (132,133). The linker histone H1 although not

having a clear sequence resembling classical NLS has a high density of basic amino-acids thought to be responsible for the nuclear localization activity (134) and has additionally been shown to interact with an importin beta/importin 7 complex (135).

Several of the histones have been used to complex and mediate transfection of DNA into several cell lines and primary cells in a process coined histonefection (136). In one example, a truncated H1.4 construct encompassing 387 nucleotides at the 3′-end was used to efficiently deliver DNA and siRNA to different cell lines in a process that seemed to be dependent on exogenous $CaCl_2$ addition (137). One inherent problem of using histonefection is the fact that an excess of extracellular histones could interfere with endogenous histones and normal DNA replication and transcription (136).

A strategy that has been showing some potential is making use of the association of transcription factors to the DNA by specific interaction with special sequences in the DNA designated DNA-targeting-sequences (DTS) (138). Work by Dean *et al* showed that DNA could be imported into the nucleus in a sequence specific way. Initially, microinjection of the SV40 5.2Kb genome into the cytoplasm of growth-arrested cells resulted in localization into the nucleus in 6-8h, detected by *in-situ* hybridization. In contrast, two other plasmids containing only bacterial and bacteriophage sequences remained in the cytosol (pBR322 and pBluescript). The origin of replication and early and late promoter sequences were then identified in the SV40 DNA that allowed nuclear import, moreover, this process was found to be dependent on active transcription (139). In a follow-up study they found that cloning of the SV40 enhancer sequence, containing the 72bp enhancer repeats, in several plasmids, was the minimum condition necessary for efficient nuclear import (140). The working model for this transport is based on the association of transcription factors in the cytosol (where they are expressed) to specific binding sequences in plasmid DNA. Since transcription factors bear NLS signals, they would carry with them the DNA to which they are bound during the nuclear import process (138).

Other sequences have been proposed to mediate DNA nuclear import. Mesika *et al* demonstrated that DNA vectors containing 5x NF-kβ binding sites had a 12-fold increase in nuclear entry in comparison to plasmids lacking the binding sites. This increase could be further enhanced by stimulation with an NF-kβ activator (TNF-α) (141).

Of added interest is the fact that the DTS mediated nuclear import can be used to achieve a cell specific import and thus gene expression. Such is the case reported by the use of the smooth muscle gamma actin (SMGA) promoter. A 176 bp portion of the

smooth muscle g-actin (SMGA) promoter was shown to be sufficient condition for the nuclear import of DNA specifically in smooth muscle cells. Binding sites for serum response factor (SRF) and NKX3-1/3-2 within this DTS were required for import (142). Both the SV40 and SMGA DTSs have been used *in-vivo*. Plasmids containing the SV40 DTS element downstream of the polyA site were electroporated into the intact mesenteric vasculature of the rat. DTS containing plasmids gave 10- and 40-fold higher Luciferase expression levels at 2 and 3 days post-transfection, respectively (143). The same method has been used together with plasmids containing the SMGS DTS. GFP gene expression was found to be cell specific, restricted to SMαA-expressing smooth muscle cells in the vessel wall, whereas none of the cells in the adventitia showed GFP expression (144).

At the moment, however, there seems to be no common features between promoters allowing to identify, beforehand, promoter or promoter regions that can support nuclear import of plasmid DNA.

1.3 NUCLEIC ACIDS FOR GENE MODULATION

The concept of oligonucleotide (ON) gene therapy basically started with the birth of the antisense field when Zamecnik and Stephenson, in 1978, reported on the inhibition of virus replication in cell culture by a DNA oligonucleotide complementary to the target RNA (145).

The idea on the use of oligonucleotides for therapeutic purposes hence emerged, backed up by the development, around the same time, of a method for ON solid phase synthesis (146).

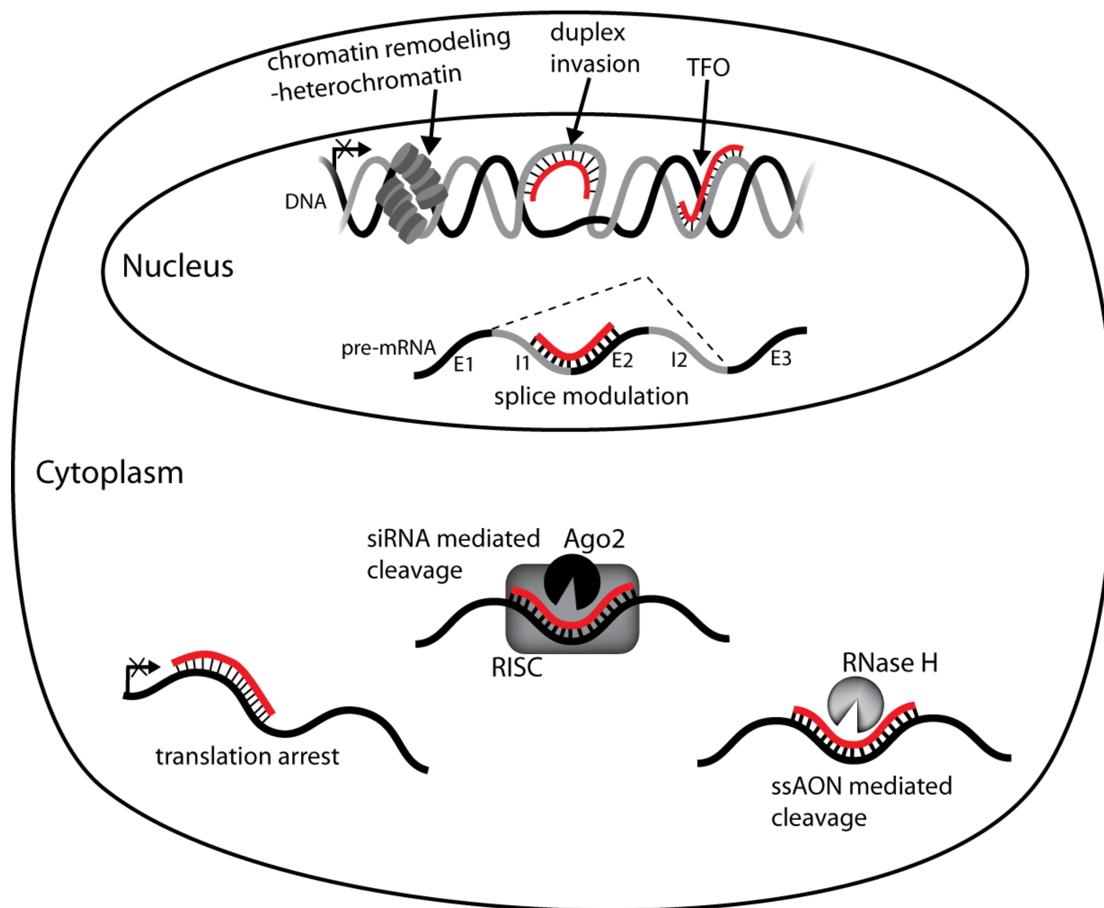

Figure 6. Antisense and anti-gene mechanisms overview. In the cytoplasm ssAONs can mediate translation arrest by steric blocking; siRNA mediates mRNA cleavage by recruiting the RISC complex and Argonaute 2 which has hydrolyzing activity; ssAONs forming a DNA-RNA hybrid duplex recruit RNase H which then cleaves the mRNA. In the nucleus ssAONs can invade the duplex DNA and arrest transcription initiation or elongation; TFOs binding to the major groove of dsDNA can create a steric block which can arrest transcrition initiation or elongation; siRNAs directed to promoter regions can activate epigenetic remodeling complexes inducing heterochromatin formation and silencing of gene expression.

The antisense concept was based on the notion of hybridization of an ON with RNA for blocking its function. The therapeutic ON field continued to advance and besides the classical antisense concept, other technologies have been developed using nucleic-acids for designing RNA-cleaving Ribozymes, DNAzymes and siRNA, all with capacity to suppress RNA translation. However, also ONs with DNA as their target have been designed for suppression of transcription, thereby achieving gene down-regulation.

1.3.1 Classical Antisense

Classical antisense oligonucleotides (AON) can operate using basically two mechanisms. The first mechanism consists on the simple steric block of ribosomal activity on mRNA thereby inhibiting translation. Steric block is also used for inhibition of splicing (or splice redirection) which will be covered in its own section in this thesis.

Translation inhibition by steric block has been achieved by targeting ONs to different regions in the mRNA. Thus, the 5'-UTR (147-149), translation initiation sites (150) and internal ribosome entry sites (151) have all been targeted by steric block oligos.

The majority of these early-days translation inhibition antisense oligos were used in cell-culture systems, and although regarded as not extremely efficient the development of new nucleic acid analogues provided new tools to increase this mechanism efficacy. Perhaps the most striking examples come from the use of morpholino oligomers (PMO), which will be discussed later.

The second mechanism, in contrast to the occupancy-mediated antisense just described, is based on the degradation of the target RNA. Hybridization of the AON to the target RNA creates an RNA-DNA hybrid that is specifically recognized by the enzyme RNase H which promotes the cleavage of the RNA strand. There are two classes of RNase H enzymes identified in mammalian cells differing in their cofactor requirements and activity (152-154). It is however recognized that RNase H1 is the enzyme playing the dominant role in the AON activity (155). RNase H2 is thought to be unavailable to participate in the hydrolysis of RNA, in AON-derived RNA-DNA hybrids,, due to the fact that it seems to be associated with chromatin, where it participates in DNA synthesis processes (156).

Activity of RNase H1 is very much dependent on the specific structure adopted by the RNA-DNA hybrid. An RNA-DNA heteroduplex adopts an eastern *O*-4'-*endo* sugar pucker, resulting in a helical conformation in which the RNA strand adopts A-form geometry and the DNA strand shares both the A- and B-form helical conformations. The adoption of this *O*-4'-*endo* conformation by the DNA partially narrows the major groove which is an essential condition for the tight binding of RNase H1 to the heteroduplex and catalytic activity (157). These specific structural requirements are the reason why RNase H can distinguish RNA-DNA from RNA-RNA or DNA-DNA homoduplexes but also lead to a fundamental limitation in AON activity. In fact, new generation of AONs, relying on nucleic acid analogues that confer AONs with a

structure that diverges from the DNA type, are not active for RNase H cleavage. The nucleotide features for an active AON were thus determined to be: i) a conformationally flexible sugar producing an *O*-4'-*endo* pucker when hybridized to RNA, ii) no sterically bulky 2'-substituents, and ii) a conformationally rigid phosphate backbone (158). This would preclude the use of many interesting new nucleotide analogues such as MOE (2'-methoxyethyl), PNA and LNA, however, a new AON design was developed where the analogues are only introduced as blocks at the 5'- and 3'-ends while the central region of the AON maintains DNA or DNA-like nucleotides. This created the concept of gap-mers which can still harness the positive properties (such as nuclease resistance and better duplex binding affinities) of the new nucleic acid analogues and support RNase H activity at the same time (159,160). More detail on some of these properties will be given further in the thesis.

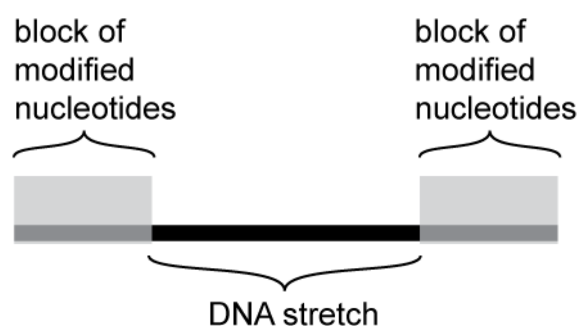

Figure 7. Schematic representation of a gap-mer for inducing RNase H mRNA degradation.

The first (and so far only) approved antisense drug, Vitravene (formivisen sodium) (161), is a fully phosphorothioate DNA-AON using the RNase H mechanism for the treatment of cytomegalovirus (CMV) retinitis in patients with AIDS .

1.3.2 siRNA

Another strategy for suppressing gene expression involves the use of yet another cellular enzyme to cleave the target RNA in a process that is initiated or mediated by dsRNA. This mechanism was recognized by Fire and Mello in 1998 when they injected a dsRNA into *C. elegans* which led to the sequence specific down-regulation of a target gene harboring the same sequence as the dsRNA (162). This marked the discovery of the RNA interference mechanism, for which the two investigators were awarded in 2006 the Nobel Prize in Physiology and Medicine. It was initially recognized that the dsRNA molecules were being processed to 21-28nt small interfering RNAs (siRNAs), which were then guiding the sequence specific degradation of mRNA (163,164). The elucidation of the structure of siRNAs was pivotal for the subsequent discovery that the RNAi mechanism was active in mammalian cells (165), as any attempts to introduce long dsRNA into mammalian cells would lead to the induction of the antiviral

interferon response and usually cell death (166). The current status of knowledge for the RNAi pathway can be summarized as following: Initially dsRNA is cleaved by the Dicer enzyme generating 21- to 28-nucleotide siRNA duplexes that contain 2 nucleotide 3′-overhangs with 5′-phosphate and 3′-hydroxyl termini. This product is then loaded into a protein complex named the RISC loading complex of which the protein Argonaute 2 (Ago2) is a key component. The RISC complex then separates the two strands and an active RISC complex containing the guide (antisense) strand is produced. The active RISC recognizes the target mRNA through hybridization with the guide strand and Ago2 catalyses the cleavage of the target mRNA. The cleavage itself occurs 10 nucleotides from the 5'-end of the guide siRNA strand (167).

The use of synthetic siRNAs exogenously administrated to cells is an easy way to achieve down-regulation of target genes and the rules for the design of these efficient synthetic oligos have been investigated. Thus, a synthetic siRNA should have a low G/C content, have a bias towards low internal stability at the sense strand 3′-terminus, lack of inverted repeats, and sense strand base preferences with an A nucleobase at positions 3, "U" at position 10, a base other than "G" at position 13 and "A" at position 19 (168).

Recently, siRNAs designed against promoter regions have also been found to induce gene silencing in mammalian cells through a transcriptional mechanism as opposed to post-transcriptional, designated by transcriptional gene silencing (TGS) (68,169). This silencing comes about due to heterochromatin formation, which occurs by the action of an epigenetic remodeling complex guided to the specific target loci by the small RNA. Argonaute 1 (Ago1) seems to be involved directly in this remodeling complex since after 24h exposure to the small RNA it increases its presence at the promoter with subsequent increase in H3K27 trimethylation and H3K9 dimethylation (170). When targeting is maintained for at least 3 days, DNA methylation increases at the target locus which seems to correlate to long term silencing (171). Interestingly it now seems that for RNA-targeted TGS to take place there needs to be transcription going through the promoters with production of promoter-associated RNAs (pRNAs) which can then be recognized by the antisense strand of the siRNA (172,173). This mechanism brings new insights into the use of single-stranded antisense oligonucleotides for targeting pRNA in order to achieve long-term gene silencing without even having to resort to siRNAs. A study where only the 21-bp antisense (guide) strand was necessary to direct TGS hints at this possibility (174).

1.3.3 Splice switching

Manipulation of the process of RNA splicing has a tremendous potential which can be immediately recognized by the fact that around 95% of human genes are alternatively spliced and at the same time 50% of disease causing mutations affect splicing (175,176).

Alternative splicing is a way of regulating gene expression by generation of multiple proteins from a single pre-mRNA transcript. Splicing and alternative splicing rely on essential sequence elements in pre-mRNA such as splice-sites, branch points, polypyrimidine tracts, and auxiliary splicing enhancer or silencer sequences existing both in exons and introns (designated exonic/intronic splicing enhancers, ESE/ISE or exonic/intronic splicing silencers, ESS/ISS). These sequences are recognized by the splicing machinery and auxiliary proteins such as SR proteins and hnRNP (177).

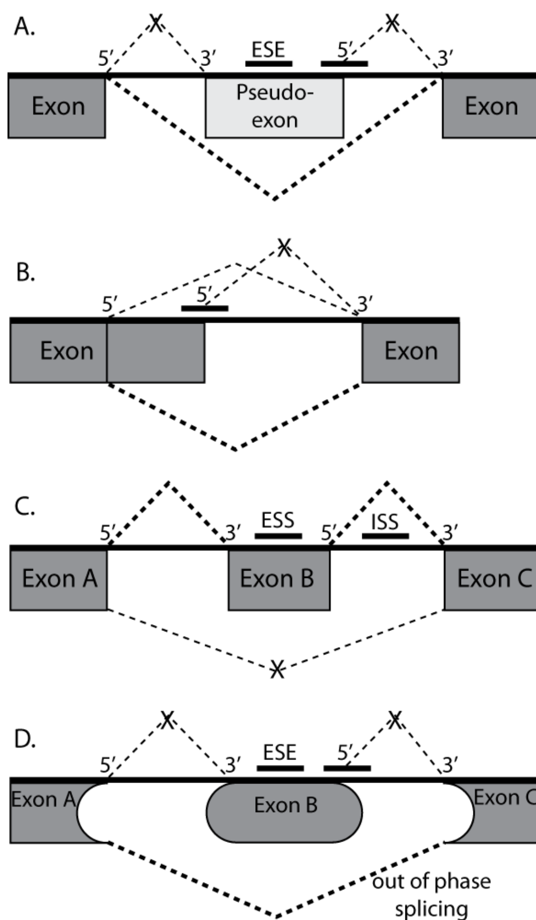

Figure 8. Overview on splicing modulation by SCOs. A) exclusion of an activated pseudo-exon; B) Forcing the use of an alternative 5'-splice site; C) exon inclusion; D) exclusion of a constitutive exon leading to an out-of-phase splicing and protein down-regulation.

To affect mRNA splicing, AONs need to hybridize to the target pre-mRNA and act through a steric-block mechanism without eliciting any RNA degradation pathway. Thus, there is a potential to use AON technology for diseases caused by aberrant splicing or when altering normal splicing can mitigate a disease-causing mutation. These specific AONs are frequently named splice-switching oligos (SSOs) and can operate in different ways such as: a) blocking of a mutation-induced cryptic 5'- or 3'-splice site for restoration of normal splicing; b) blocking of exon-intron junctions and/or ESEs to induce exon skipping (this strategy can be used to exclude

pseudoexons, exclude mutated exons, exclude one or more exons for restoration of an open reading frame caused by a frameshift deletion, or even for downregulation purposes by excluding an exon leading to an out-of-phase splicing); c) blocking of ESS or ISS to enhance exon inclusion.

Blocking of cryptic splicing was one of the first demonstrations for the use of AONs to alter splicing. This was shown in the case of β-thalassemia where mutations in intron 1 and 2 of β-globin lead to activation of cryptic splice sites that disrupt normal splicing thus leading to a disrupted protein production. Targeting of an AON to the cryptic splice sites reverted the splicing back to normal in erythroid cells restoring β-globin expression (178).

Regarding exon exclusion it is best exemplified when considering the Duchenne Muscular Dystrophy disease (DMD) (179). DMD is caused by deletions and non-sense mutations causing frame-shifts and subsequent premature termination codons leading to disruption of normal protein production. However deletions that maintain the reading frame give rise to a shorter but functional form of the dystrophin protein causing the milder Becker muscular dystrophy (BMD) (180). This fact was exploited in a strategy for DMD where by using AONs targeting consensus splice-sites or ESS, one or more exons, including the mutation, could be excluded thereby restoring the reading frame. This would then partially correct the dytrophin transcript reverting the disease to the milder Becker form. This strategy has been evaluated in cells and *in-vivo* in a DMD mouse model where a non-sense mutation in exon 23 leads to premature termination during translation. *In-vivo* systemic administration of AONs of different chemistries such as PS-2'*O*-Me-RNA and PMOs has led to the observation of revertant muscle fibers (regain of dystrophin production) in several muscle groups, body wide (181-183). This strategy has also been used in patient-derived muscle cells *in-vitro* where targeting and induction of exon skipping was achieved for multiple exons (184,185). This work has subsequently led to the presentation of highly promising results from studies (phase 1-2a trials) in DMD patients treated locally (186,187) and, more recently, systemically with SSOs (188).

As for exon inclusion, a nice example is represented by a strategy against spinal muscle atrophy (SMA). SMA is caused by mutations in the SMN1 gene. However, in humans two copies of the SMN gene are expressed (SMN1 and 2) which differ only in 5 nucleotides, two of them present in exons. In SMN2 a translationally silent cytosine to thymidine exchange at position 6 of exon 7 is responsible for the skipping of exon 7 during splicing. This results in abrogation of an ESE site (189), weakening of the

upstream 3'-splice site (190) and consequent exclusion of exon 7, yielding an unstable truncated protein, SMNΔ7. Since SMN2 is similar to SMN1, if exon 7 could be included this would result in a functional SMN2 protein capable of compensating for the mutated SMN1. Hence, strategies using SSOs have been devised to achieve exon 7 inclusion. One strategy uses the concept of competition between 5'-splice site of exon 6 and the 3'-splice sites of exons 7 and 8, thus SSOs targeting the 3'-splice site of exon 8 could induced an alteration in splicing pattern in favor of inclusion of exon 7 (190). In another strategy an exon-7-targeted SSO was linked to a peptide domain designed to mimic the splicing activation domains of serine-arginine–rich proteins (SR proteins). Similarly, another SSO contained an extra domain with a non-complementary tail with RNA sequences that corresponded to ESE sequences that are recognised by splicing proteins. These last two elegant strategies were designated by exon specific splicing enhancement by small chimeric effectors (ESSENCE) (191) and targeted oligonucleotide enhancer of splicing (TOES) (192) and both have shown successful inclusion of exon 7. A recent study has shown that injections of an SSO (2'-methoxy-ethyl RNA based ON) against the intronic splicing silencer of the 5' region of SMN2 intron 7 into the cerebral lateral ventricles of mice resulted in an increase of SMN2 protein and number of motor neurons in the spinal cord. In the same study intrathecal infusion of the SSO into cynomolgus monkeys was also performed to evaluate safety and tolerance of the method (193). Collectively these results give an important step in the direction of future clinical trials with SSOs for SMA.

Interestingly exon exclusion has also been used as a way to achieve down-regulation of a specific protein. This approach was used for the knockdown of myostatin, a negative regulator of muscle mass, in order to improve muscle-wasting conditions (such as the ones encountered in DMD). Thus SSOs were designed against indentified ESE regions of exon 2 which, if skipped, would lead to an out-of-phase splicing between exon 1 and exon 3 with subsequent truncation of the open reading frame and transcript destabilization. The strategy was proven efficient *in-vitro* in C2C12 mouse myoblasts and *in-vivo* after systemic injection of the SSOs into the mouse (194).

It is also worth mentioning an interesting example of splice modification based on biasing a natural alternative splicing in favor of a specific transcript to produce a desired outcome. The *bcl-x* gene generates two isoforms by alternative splicing, the pro-apoptotic *bcl-xS* and the anti-apoptotic *bcl-xL*, which is overexpressed in various cancers (195). Alternative splicing takes place due to the usage of alternative 5'-splice sites in the first coding exon. Thus, an SSO targeting the 5'-splice site of *bcl-xL* exon II

was shown to redirect the splicing towards the pro-apoptotic *bcl-xS* (196,197). This can regulate cancer cell survival and sensitize them to chemotherapeutic treatments (198).

1.3.4 Anti-gene

The anti-gene strategy is based on the sequence specific targeting of the chromosomal dsDNA, in this way acting upstream of the antisense strategy. For this purpose, different types of oligonucleotides have been developed with capacity to bind in a sequence specific way to the major groove of the duplex, designated triplex-forming-oligonucleotides (TFO), or to invade the duplex DNA, displacing one of the DNA strands.

1.3.4.1 Triplex Forming Oligos (TFOs)

The first reports on the binding of short oligonucleotides to the major groove of dsDNA, forming a triple-helix structure, dates back to 1987 in work done simultaneously by Dervan and colleagues (199) and Heléne and colleagues (200). However, recognition of a nucleic-acid triple structure actually came about two decades earlier when Poly(U) and poly(A) were found to form a stable 2:1 structure in presence of $MgCl_2$ (201).

The triple helix is formed by specific hydrogen bonding, referred to as Hoogsteen bonds (202), of a TFO with the polypurine sequence in a polypurine:polypyrimidine dsDNA motif. Binding of the TFO can be realized in two ways: parallel and anti-parallel to the purine-binding strand. In the parallel mode (i.e. both TFO and polypurine strand are in the 5'→3' direction) the TFO can bind through a TC or GT motif. With the parallel TC-motif the TFO forms T:A*T and C:G*C+ triplets in the Hoogsteen configuration. A condition for this binding is that the cytosine is protonated (C+), which due to the pKa of the imino group being lower than 7 makes this binding dependent of slight acidic conditions. With the parallel GT-motif the TFO forms C:G*G and T:A*T triplets also in the Hoogsteen configuration. Concerning the anti-parallel mode (i.e. TFO in the 3'→5' orientation and polypurine strand in the 5'→3' direction) the TFO can also bind through the GT-motif or a GA-motif. With the GT-motif the TFO forms C:G*G and T:A*T triplets in the reverse-Hoogsteen orientation. With the GA-motif the TFO forms C:G*G and T:A*A triplets also in the reverse-Hoogsteen orientation.

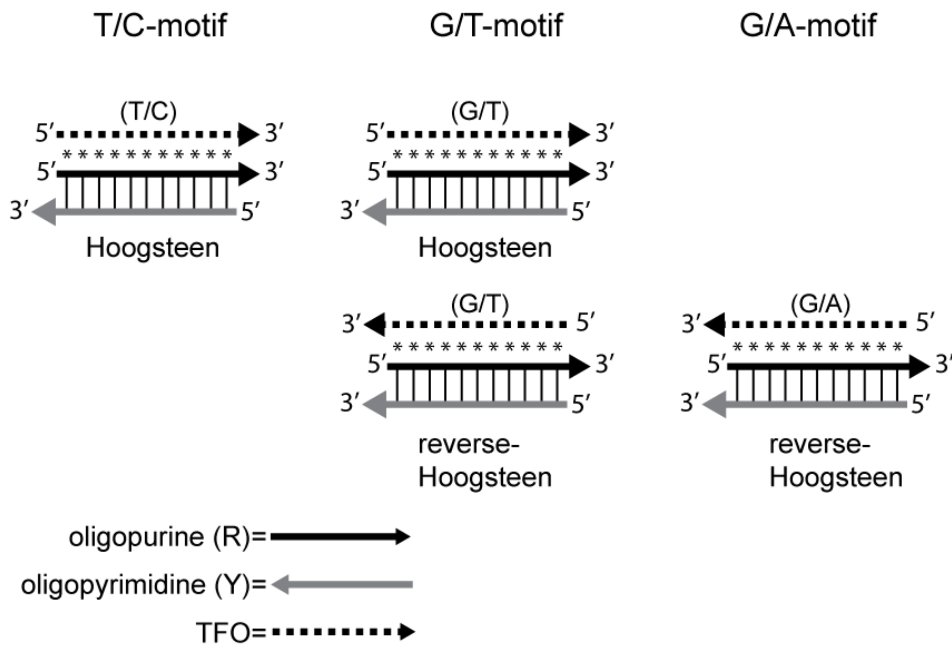

Figure 9. The different orientations for the three possible triplex motifs.

Realization of the anti-gene strategy with the use of TFO needs, as we have seen above, specific targeting to polypurine:polypyrimidine rich regions. This can be seen as a limitation, however, such sequences have been recognized to occur quite frequently in almost every gene, being over-represented in promoter regions but also available in intronic regions (203,204).

To achieve gene down-regulation, TFOs have been targeted to promoter regions, thus interfering with transcription factor binding (205,206) or formation of the initiation complex (207), or they have been targeted to the transcribed regions where they need to arrest transcription elongation (208). TFO inhibition of transcription has been described for some endogenous genes in *in-vitro* and cell culture studies. Some examples are c-myc (209), tie1 (205), ICAM-1 (210), ets2 (206), however the efficiencies have not been great and there is no solid-proof *in-vivo* data yet for the TFO anti-gene strategy.

This fact is probably related to some hurdles that TFOs encounter in the cellular environment. As such, intracellularly, the TFO needs to have the capacity to resist nucleases, ability to form triplexes at physiological pH, overcome charge repulsion between TFO and dsDNA, cope with the chromatin environment and be stable enough to compete with endogenous DNA binding proteins. Some of these barriers are being dealt with the use of modified nucleic acids in the TFOs in the same way as already referred to in the classical antisense strategies.

1.3.4.2 Duplex Invasion

Duplex invasion associated to transcription inhibition, in the context of the chromosomal DNA, was first reported for a linear PNA based oligomer targeted against the CAG triplet repeats of the androgen receptor and TATA-binding protein genes (211). It was after also found to target specific c-myc regions (212,213).

Corey and co-workers have also used linear PNA and LNA to target the progesterone and the androgen receptor genes (214-216).

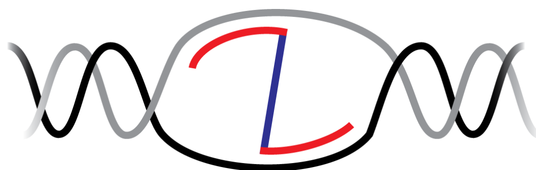

Figure 10: Schematic representation of the Zorro-LNA concept. Two LNA rich oligonucleotides (in red) are hybridized simultaneously to the sense and antisense strand. The oligos can be interconnected by a linker region (in blue) composed by nucleotides or other chemistries (eg. PEG).

In our lab we have not been able to achieve transcription inhibition with linear PNAs or LNAs. However a new concept was developed using LNAs targeted to both the sense and antisense strand and where the 5'-end of one LNA oligo is hybridized to the 3'-end of the other. This formed the designated Zorro-LNA structure with capacity to invade the DNA duplex and having additional binding stability provided by the ability to hybridize to both strands (217). This construct has however only worked so far when two adjacent binding sites are present in the DNA and was only demonstrated for one sequence.

1.4 NUCLEIC ACIDS ANALOGS

Evolution of antisense and anti-gene gene therapy rests not only on the shoulders of gene delivery development but also, and quite importantly, on the shoulders of nucleic acid chemistry advances. While initial antisense experiments where done with unmodified DNA oligonucleotides it was soon realized that they needed to be made more efficient to achieve clinical significance. Hence, novel molecules have been obtained through modifications of the nucleic acids at the nucleobase, the sugar ring or phosphodiester backbone. They have been designed with the objective to improve certain properties such as the process of synthesis, nuclease resistance (resistance to degradation), affinity and selectivity towards other nucleic acids (mostly regarding binding kinetics to RNA or DNA), and in some cases even the ability to cross biological membranes.

For the purposes of this thesis only the modifications most generalized in the antisense field will be covered with focus on PNAs and LNAs.

Among the first modifications made to nucleic acids was an alteration to the phosphodiester backbone where one of the non-bridging phosphate oxygen atoms is replaced with a sulfur atom. These phophorothioate containing oligonucleotides (**first generation** PS-ONs) were mainly thought to increase the stability against nucleolytic degradation (218). However, they also improve the pharmacokinetic properties of the oligonucleotides, when used systemically, by increasing the persistence time in circulation as a result of the binding to serum proteins, which prevents rapid renal excretion (219). However, this latter property is connected to a recognized tendency of PS-ONs to unspecifically bind to proteins which can provoke toxicity effects (219). They also have a reduced affinity towards complementary RNA in comparison to unmodified DNA (220).

Nonetheless their advantages over the completely unmodified DNA have granted PS-oligos with the longest history in clinical tests with most of the advanced studies being performed with this modified ON.

Accordingly, and as already mentioned, the only AON approved by the FDA to date is the 21-mer phosphorothioate Vitravene (fomivirsen) (161). Two additional PS-AONs, Genasense (221), and Affinitak (222), have reached advanced stages of clinical trials against cancer, targeting Bcl-2 and PKC-α, respectively. The results of the clinical trials were however disappointing (223,224).

To overcome some of the PS limitations a new generation of nucleic acid modifications emerged. The **second generation ONs** were focused on increasing the binding affinity and reducing toxicity effects. These nucleic acids had modifications on their 2'-*O* positions and the most preeminent were the 2'-*O*-methyl (225) and 2'-*O*-methoxy-ethyl (MOE) (226) RNAs. These modifications confer a much less affinity towards proteins, however, a drawback of fully modified 2'-*O*-Me or MOE AONs is their inability to activate the RNase H mechanism. This problem has been tackled by the introduction of the gapmer concept already discussed previously.

These modifications conferred nuclease resistance, however, when AONs are additionally modified with PS this results in further improvements. MOE-AONs have been more widely used than 2'-*O*-methyl-AONs and are currently under evaluation in clinical trials, some examples being the targeting of apolipoprotein B-100 for the potential treatment of hypercholesterolemia (227) and targeting of alpha 4 integrin (VLA-4) for treatment of multiple sclerosis (228,229).

Over the years a variety of other nucleic acid modifications have been derived, again driven by the premises of increased affinity and improved stability. Thus, the concept of conformational restriction has been applied as well as completely different chemical moieties substituting the furanose ring have been developed. In analogy to the previous first/second-generation terms these have been designated by **third-generation modifications** or 3rd Gen-AONs. Included are Locked Nucleic Acids (LNA), Peptide Nucleic Acids (PNA), and morpholinos (PMO).

Morpholinos were developed in the late 80's and they were seen as a solution to the production costs of existing DNA analogues (230). They used less expensive ribonucleosides and the synthesis steps did not involve expensive catalysts and postcoupling oxidation steps. A morpholino is a non-charged DNA analog, in which the ribose is replaced by a morpholino moiety and phosphoroamidate intersubunit linkages are used instead of phosphodiester bonds. Both the constrained ring unit and the non-ionic character make morpholino-RNA duplexes more stable than DNA-RNA duplexes. A fully modified PMO oligomer does not elicit RNase H, they have been nevertheless quite successfully used in steric blocking mechanisms in the field of splice correction, the most preeminent example being their use in the DMD scenario (66,231), and for knocking out viral gene expression and replication by translational arrest (232,233). PNA and LNA are discussed in more detail in the sections below.

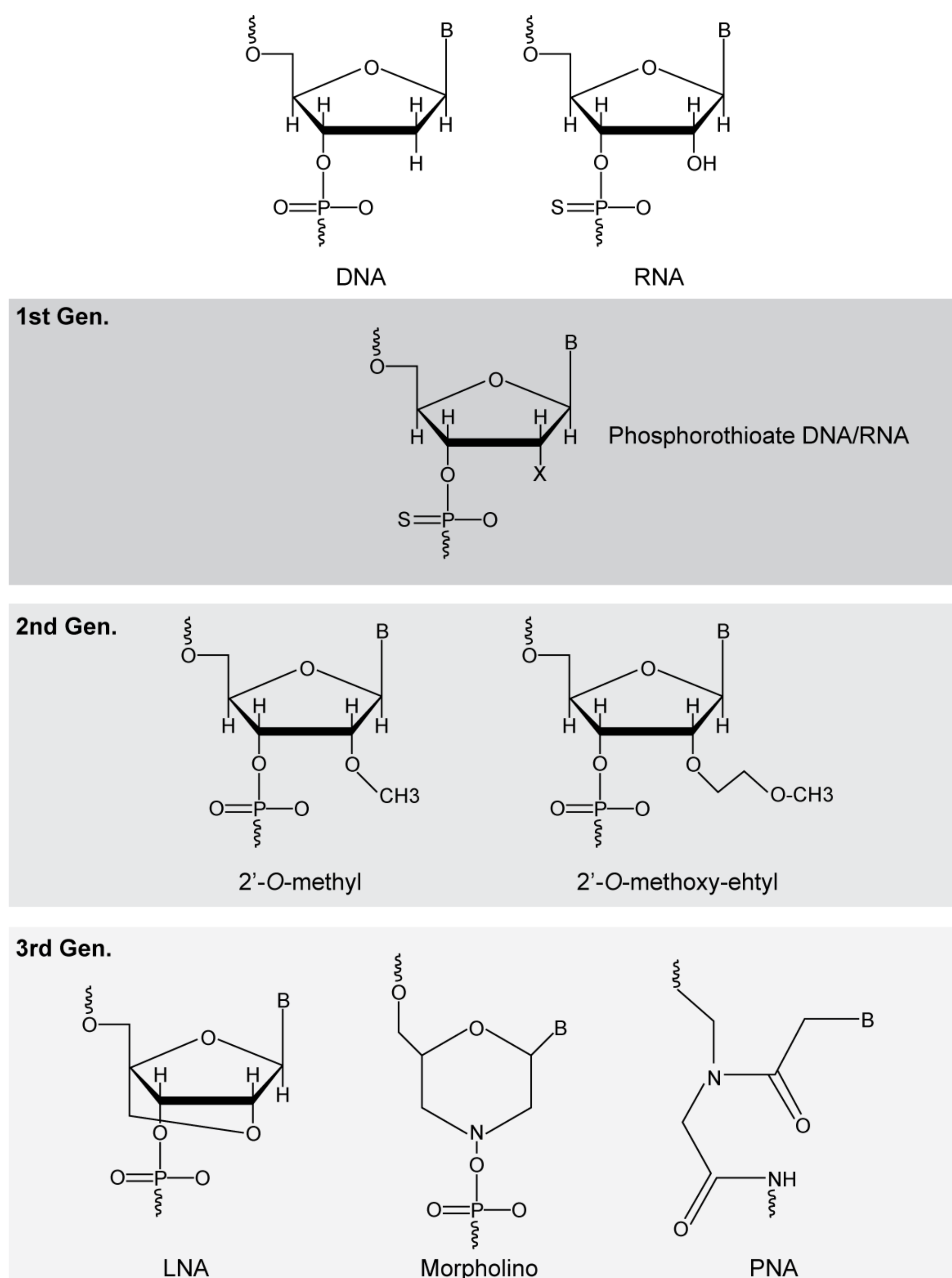

Figure 11. The basic nucleic acids and examples of modifications used in antisense oligonucleotides.

First generation involved the modification of the phosphate backbone by substitution of a non-bridging oxygen by sulfur atom; Second generation involved modifications at the 2'-*O* position; Third generation is more broad involving modifications restricting the sugar ring conformations and complete substitutions of the furanose ring.

1.4.1 PNA

PNA was developed by Ole Buchardt and co-workers in the 80's (234). It is a nucleic acid analog composed of repeated N-(2-aminoethyl)glycine units with the nucleobases attached to the glycine nitrogen *via* a methylenecarbonyl linker (235). The nature of the backbone-resembling amino-acid linkages confers to the molecule a neutral charge in comparison to the intrinsic negative charge of nucleic acids provided by their phosphate linkage backbone.

PNA molecules have the ability to bind to complementary DNA or RNA oligonucleotides through Watson-Crick base paring (236). Due to the lack of electrostatic repulsions between PNA-DNA hybrids, the thermal stability (measured as melting temperatures, Tm) of such duplexes is virtually independent of ionic strength and in general higher than the corresponding DNA-DNA hybrid.

PNA has also the capacity to strand-invade a dsDNA, forming very stable $(PNA)_2$/DNA triplex structures with homopurine sequences and where the non-complementary DNA strand remains as a loose loop structure (237). In this mode of binding one strand of PNA binds via Watson-Crick base pairing while the other binds the same strand via Hoogsteen interactions. This strand-invasion capacity is further enhanced when two pyrimidine PNAs are connected through a flexible linker forming a bisPNA (PNA clamp) molecule.

PNA oligomers have also been found to be very stable in human serum and cellular extracts, being resistant to nuclease and protease degradation (238).

These PNA properties have made them useful as antisense and anti-gene molecules. *In-vitro* cell-free experiments have shown that PNA leads to inhibition of transcription initiation as well as elongation, especially when positioned on the template strand, and also translational block has been observed (239-241).

A drawback associated with the use of PNA is their lack of cellular uptake, which has hampered its development into a therapeutic option. However, PNAs can be conjugated with peptides increasing their membrane permeability (242). Such PNA-peptide conjugates have been used as anti-gene agents targeting transcriptional start sites in the progesterone receptor gene in cells. Inhibition of transcription was achieved when PNA was used at high ratios to the target site (215). PNA-peptide conjugates have also been used as splice switching oligos (SSOs) (243-245).

1.4.2 LNA

LNA is a nucleic acid analog containing a bridging methylene carbon between the 2' and 4' positions of the ribose ring. This bridge leads to a conformational restriction (C3'-*endo*) which pre-organizes the LNA monomers in an N-type conformation (246-248).

If a DNA or RNA oligonucleotide has some of its monomers substituted by LNA this will push the nucleotides flanking the LNA bases into a N-type conformation (especially the nucleotides following the LNA in the 3' direction). As such, a partially LNA modified oligo will have its phosphate backbone pre-organized due to the C3'-*endo* locked conformation. This in turn leads to a decrease of entropy upon duplex formation while contributing to a more efficient stacking of the nucleobases (increased loss of enthalpy upon duplex formation) (249). In conclusion an LNA-DNA or LNA-RNA duplex will have much higher Tm then their homodimer counterparts due to favorable enthalpy and entrophy effects. Worth noting is that an LNA-RNA duplex can have a slightly higher binding affinity than a corresponding LNA-DNA duplex. This is also explained in part by the RNA-like conformation imparted by the locked sugar moiety both in the single stranded and duplex state. While a DNA-DNA duplex has a B-form helix structure, RNA-RNA duplexes have an A-form structure and DNA-RNA duplexes are in an intermediate state between A- and B-forms. LNA modifications impose an A-type geometry (due to their locked N-type conformation), thus they slightly favor duplex formation with RNA strands (250).

These LNA properties conferring high affinity and sequence specificity makes them an excellent nano-technological tool for creating self-assembling nano-structures. Moreover, they have other useful properties, such as an enhanced serum stability (251) a slower plasma and renal clearance (252) as well as signs of low toxicity (252,253), that gives them a high therapeutic potential.

In the antisense field LNAs have been used quite successfully, especially when integrated into gap-mer designs. They have proven to be efficient in *in-vivo* settings when targeting apolipoprotein-B in the liver of mice and non-human primates (254) and as anticancer agents targeted to survivin (255), which is currently being evaluated in phase I clinical trials (256).

Interestingly LNAs have also been used to improve the biological efficiency of siRNAs by the introduction of LNA monomers in both strands of siRNA. LNA substitution should be kept to a minimum and out of the central region of the RNA

(257). They were also found to alter strand-bias through selectively increasing the affinity of the closing base-pair at the 5'-end of the siRNA sense strand (258).

In the anti-gene field LNA has been used to modify TFOs. LNA substituted TFOs have an increased triplex thermostability although a fully LNA substituted TFO hampers triplex stability (259). Moreover, increased affinities are also seen at neutral pH, which is important for the TFO parallel motif (homopyrimidine-type TFOs) and for the efficient binding in cellular conditions (260-262).

Linear LNAs have been used by Corey's group to arrest transcription of the androgen and progesterone receptor genes (216).

2 AIMS

The aims of this Ph.D studies were to further develop the potential of nucleic acids for gene delivery and gene regulation with focus on the following areas:

I- Improving the capacity for nuclear import of exogenous nucleic acids;

II- Develop new and efficient cell delivery methods for oligonucletide-based strategies;

III- Develop nucleic-acids/oligonucleotides as a new splice-correction therapeutic option for XLA;

IV- Further develop an anti-gene strategy based on the use of LNA-modified oligonucleotides and study the concept of bisLNAs.

3 METHODOLOGY

This section covers some relevant methods used in this thesis papers.

3.1 PREPARATION OF STREPTAVIDIN-OLIGONUCLEOTIDE COMPLEXES

Streptavidin-Alexa488 (Molecular Probes) + biotinylated oligonucleotide constructs were formed by incubating 3µg STV (Streptavidin) reconstituted in 1x phosphate buffer saline (PBS, pH 7.4) with 2-4 times molar amount of biotinylated oligo constructs. The volume was adjusted with nuclease free water (Qiagen) to have a final concentration of 329ng STV/µl and the incubation proceeded for 2h at r.t. and if not used the same day the constructs were stored at 4°C.

3.2 *XENOPUS* OOCYTE MICROINJECTIONS, NUCLEAR DISSECTION AND FLUORESCENCE MICROSCOPY

The *Xenopus* oocyte offers excellent conditions for the study of nucleo-cytoplasmic transport of different materials (synthetic or biological) and has been previously used for early studies on nuclear transport of karyophilic proteins and U-snRNAs (263,264). They are easy to microinject and retain the nuclear membrane integrity throughout the experiment, moreover the nucleus is relatively easy to isolate and analyze due to its size.

Prior to microinjections the prepared STV-oligo constructs were spun down at 16,000 xg for 5 minutes. A volume of 41.4nl of STV-oligo constructs were injected in the cytoplasm of the oocyte using the Nanoinjector 2000™ pump (World Precision Instruments, Inc) followed by incubation at 19°C in OR2 buffer for 4h. After incubation, oocyte nuclei were manually dissected with metal tweezers in nuclear dissection buffer (140mM KCl; 0.5mM $MgSO_4$; 20mM Tris-HCl pH 7.2) and further processed for either western blot or fluorescence microscopy. For microscopy the oocytes were placed in a petri dish filled with nuclear dissection buffer, manually dissected and immediately imaged. Visible light and fluorescent photos were taken with the use of a Stereo microscope Leica MZ6 equipped with a Leica stereofluorescent module and a Leica Video/photo tube to which was attached a Canon powershot S40 Camera. Photos were captured using the program Canon remote capture version 2.5.1.11.

3.3 TRANSFECTIONS OF STREPTAVIDIN-OLIGO COMPLEXES

In order to easily introduce Streptavidin-oligonucleotide conjugates into mammalian cells a method for the intracellular delivery of proteins was used based on creation of protein delivery complexes with a cationic amphiphile molecule (PULSin reagent). This allowed us to use an easy transfection-like based method instead of performing cell-by-cell microinjections.

U2OS cells were seeded on top of coverslips in a 24-well plate with DMEM+10% FCS the day before transfection so that they were confluent or close to confluency the next day. Streptavidin-Alexa488 (Molecular Probes) + oligonucleotide constructs (Figure 2) were formed by incubating 2µg STV (Streptavidin) reconstituted in 1xPBS (pH 7.4) buffer with 2-4 times molar amount of biotinylated oligo constructs for 2h at r.t. 20mM Hepes buffer (pH 7.4) was then used to bring the total volume up to 100µl and then 3.5µl of PULSin transfection reagent (PolyPlus-Transfection, New York, USA) was added. The mixture was vortexed and left to incubate for 15min at r.t. During this incubation time the cells were washed twice with PBS and 900µl of pre-warmed OPTI-mem I (Invitrogen) was added to the wells. The PULSin complexes were then added to the cells and left to incubate for 4h. Subsequently the medium was discarded and the cells washed twice with pre-warmed OPTI-MEM I. Finally, 400µl of OPTI-MEM I was added to the wells after which the cells were further incubated for 2-3h. Cells were then analysed by fluorescence microscopy and confocal microscopy.

3.4 HELA/LUC705 SPLICE CORRECTION ASSAY

This splice correction assay uses a HeLa cell line clone stably expressing a luciferase transcript interrupted by a mutated b-globin intron (HeLa/Luc705 system)(88). The mutation creates an aberrant 5' splice site while activating an upstream 3' cryptic splice site. This leads to production of a non-functional luciferase mRNA containing a fragment of the original intron due to the defective splicing. By introduction of a validated antisense oligonucleotide against the mutation site on the pre-mRNA, the splice mechanism is redirected to its normal pattern by the simple steric block due to the hybridization of the oligo with the pre-mRNA. This allows the production of correct Luciferase which can be easily measured by testing for the enzyme activity or using RT-PCR to detect corrected Luc mRNA transcripts. The method is widely used to test for the efficiency of new oligonucleotide transfection reagents.

3.5 SEMI-QUANTITATIVE RT(REVERSE-TRANSCRIPTION)-PCR FOR DETECTION OF SPLICE CORRECTION

This is a semi-quantitative technique to verify the amount of splice-corrected mRNAs in relation to the non-corrected mRNAs. The different splicing patterns obtained are differentiated as bands of distinct sizes in agarose gels. For this a set of PCR primers need to be designed flanking the RNA sequence that is going to be affected by the splice pattern modifications, in our case, induced by the oligonucleotide treatments. It was used for the relative quantification of Luciferase (paper I, II and III), dystrophin (paper II) and Btk (paper III) spliced transcripts.

3.6 PHYSICAL CHARACTERIZATION: DYNAMIC LIGHT SCATTERING AND ZETA POTENTIAL STUDIES

Dynamic Light scattering (DLS) is a technique for measuring the size distribution profile of particles in solution. Particle size is determined from fluctuations in scattered light intensity due to the Brownian movement of the particles (265). Zeta potential is related to the surface charge of a particle in simple systems and can be affected by changes in the environment (pH, salt, adsorption of proteins). It provides information about possible charge interactions of the particle with, in the case of gene/drug delivery systems, cellular surfaces or biological fluids such as when the particle is injected in the blood system.

3.7 FLUOROMETRY

In paper II a method based on the fluorescence detection of Cy5-labeled oligonucleotides was used to quantify the total amount of oligonucleotide efficiently taken up by cells *in-vitro*. This method uses a standard curve based on the fluorescence emission of known amounts of Cy5-oligonucleotides to quantify the amount of oligonucleotide internalized by the cells after the transfection with the oligo-complexes. The cells are washed and lysed to release the oligonucleotide after which the fluorescence emission from the lysate is measured using a fluorescence reader.

3.8 FLOW CYTOMETRY

Flow cytometry is a technique for the simultaneous multiparametric analysis of physical and chemical cell characteristics. It is based on light scattering but also allows

fluorescence detection from single cells. Flow cytometry allows the high-throughput analysis and quantification of different parameters.

Flow cytometry was used in paper II for analysis of single-cell transfection efficiencies based on the detection of cellular internalization of a fluorescent oligonucleotide, and used in paper II for analysis of expression of Btk protein after intracellular staining with a fluorescent antibody marker.

3.9 STABILITY STUDIES

In paper II stability studies of PF14-oligo complexes are done using incubations for relatively long periods of time (8 weeks) at temperatures of 40ºC and 60ºC. After this time the complexes are used in a transfection experiment to access their efficiency. Storage at high temperatures increases the rate of degradation mimicking reactions that could take years to occur (266). For this reason this method is routinely used to assess the long-term stability of formulations (267).

3.10 ISOLATION OF PRIMARY MOUSE B-LYMPHOCYTES

Spleens from BTKmut/BAC mice and wild-type (C57BL/6 strain) were collected and B-lymphocytes purified using an EasySep® mouse B-cell enrichment kit through negative selection (StemCell Technologies). The purity of isolated B-lymphocytes was checked by staining of the cell population with a PE-anti-mouse CD19 (BD Pharmingen) and analysed by FACS. Purity levels were always above 90%. During the experimental procedures B-lymphocytes were maintained in culture using the medium IMDM with 15% FCS and supplemented with 50µM β-mercaptoethanol.

3.11 CELL ELECTROPORATIONS

The electroporation technique uses a short externally applied electrical field to cells in suspension in order to increase the electrical conductivity and permeability of the cell plasma membrane. This enables the access of foreign material (proteins, DNA) to the intracellular environment and even to the nucleus. This method is very useful when dealing with *in-vitro* transfections of primary cells, which are known to be quite difficult to transfect using common delivery reagents (eg. lipid and polymer based reagents). Electroporation was used in paper III for transfection of primary human monocytes and primary mouse B-lymphocytes with oligonucleotides.

3.12 AGAROSE GEL ELECTROPHORESIS FLUORESCENT BINDING ASSAY (GEFA)

This assay was set-up in order to detect and quantify fluorescently labeled oligonucleotides hybridized/bound to plasmid DNA. By running an hybridized plasmid in an agarose gel the smaller free oligonucleotides can be separated from the bigger plasmid DNA. The fluorescent signal coming from the plasmid DNA band (due to the bound oligos) can then be quantified by using a standard curve of known amounts of fluorescently-labeled oligonucleotides run in the same agarose gel in the same conditions. This quantification cannot however be regarded as 100% accurate since the environment in close proximity can influence fluorescent labels. Thus, a fluorescent label in an oligonucleotide hybridized to plasmid DNA is surrounded by negative charges and in close proximity to the double-stranded DNA phosphate backbone. This could be different from the standard curve conditions used where the oligos are at most hybridized to a complementary single-stranded oligonucleotide. We cannot exclude that this different environment might affect the fluorescent label properties. Nevertheless this method of quantification can be seen as a good approximation.

3.13 S1 NUCLEASE DIGESTION ASSAY

The S1 digestion assay provides a way to verify and quantify the double-strand invasion capacity of an oligonucleotide. S1 nuclease preferentially cuts single-stranded DNA. Hybridization of an oligonucleotide with dsDNA by means of duplex invasion leads to the displacement of one of the DNA strands. This now single stranded region of DNA will be accessible for S1 digestion which will create a nick in the plasmid DNA and loss of supercoilness. By quantifying the amount of supercoiled and nicked plasmid it is possible to determine the efficiency of the duplex invasion/hybridization of the oligonucleotide in question.

4 RESULTS, DISCUSSION AND PERSPECTIVES

4.1 PAPER I

A synthetic snRNA m_3G-CAP enhances nuclear delivery of exogenous proteins and nucleic acids

The objective of this study was to develop an alternative to the NLS peptide based nuclear delivery strategies for nucleic-acids and non-viral vectors. We reasoned that the high density of positive charges usually found in NLS peptides can be of problem when these peptides are used in conjunction with DNA for the purposes of improved nuclear uptake. Thus, a strong association of the positively charged NLS with the negatively charged DNA phosphate backbone could result in at least a partial blocking to the recognition of the NLS by the transport receptor proteins (importins). We therefore decided to explore the use of a different type of NLS signal entirely based on a nucleic-acid structure found in the 5'-end of endogenous U snRNAs. The hypermethylated 5'-cap structure, 2,2,7-trimethylguanosine-cap (m_3G-CAP), acts as a signal for the nuclear import of fully assembled U-snRNPs where they will function in the splicing mechanisms.

We developed a completely synthetic m_3G-CAP which we could couple to oligoribonucleotides, and established systems for assessing its effect on the nuclear import of non-permeable cargoes across the intact nuclear membrane. The systems were based on the coupling of biotinylated capped-oligos to a fluorescent streptavidin. This created a construct with MW over 60KDa which is above the nuclear permeability cut-off. We then analysed the nuclear import capacity in *Xenopus* oocytes using microinjections. The usefulness of this system rests on the fact that the oocyte being a non-dividing cell maintains the nuclear membrane integrity throughout the course of the experiments. In addition, it is relatively easy to microinject and analysis of the uptake of fluorescently labeled streptavidin in the oocyte nucleus is fairly straightforward. Using this system we observed a marked fluorescence coming from the nucleus of oocytes injected with CAP-containing streptavidin in opposition to no fluorescence in non-capped streptavidin. We also used a mammalian cell system based on the delivery of streptavidin into cells and localization of the constructs using epi-fluorescent and confocal microscopy, again revealing the efficiency of the m_3G-CAP in supporting the nuclear delivery of non-permeating molecules.

Additionally, we used a splice correction assay based on the HeLa/Luc705 system (88) to test our CAP signal in a functional assay. Since the splicing mechanism only occurs in the nucleus we wanted to test if splice-correcting oligonucleotides equipped with the nuclear transport entity could have an advantage over oligonucleotides not equipped with the transport system. Indeed, although the correction levels were low we observed a significant increase of around 3-4 fold in splice correction efficiency. Although we employed short oligos in this last assay, and diffusion is in principle still possible through the nuclear pores, we believe that in suboptimal conditions, such as low transfection efficiencies or less efficient oligos, higher intranuclear concentrations should be beneficial. It is also important to note that, *in-vitro*, very high intracellular concentrations can be easily achieved using transfection reagents, whereas *in-vivo*, in a clinical context, intracellular oligonucleotides levels may be a limiting factor.

It should be noted that these results were achieved using a less efficient oligo based on a non-phosphorothioated 2'-*O*-methyl RNA oligo in part due to the inability to conjugate and purify in a satisfactory way PS-based CAP-oligos. Nonetheless, owing to some toxicity that is attributed to PS-based oligonucleotides, due to for example nonspecific protein binding (268,269), it is feasible to think that new nucleic acid chemistries can abolish the need in the future for the PS modification. This however does not preclude the development of new synthesis options that will allow the capping of PS-oligos, which are under development at the moment.

Another important issue is the fact that for *in-vivo* applications the CAP structure will most likely need to be modified to better withstand degradation conditions in serum and inside the cell. Triphosphate linkages are most likely easily degraded if completely exposed to extra-cellular environments, additionally, inside the cell there are de-capping enzymes, such as Dcp2 (270), that could influence the half-life of the CAP. Therefore, developing CAP analogues with improved degradation resistance is of uttermost importance for future *in-vivo* applications of the CAP concept.

Besides its use in oligonucleotide delivery the CAP structure can be used as integral part of a plasmid DNA delivery vector. The most obvious choice for us would be to integrate it in a system developed in our lab where one can easily attach different functional entities to plasmid DNA, via hybridization of PNA or LNA anchors (271). Being negatively charged as the DNA, any unspecific electrostatic interactions that happen when using the classical NLS peptides are not relevant for the CAP, which can then be totally available for interaction with its specific import receptor.

4.2 PAPER II

PepFect 14, a novel cell-penetrating peptide for oligonucleotide delivery in solution and as solid formulation

The most commonly used oligonucleotides, such as DNA/RNA, PNA, LNA, PMO based, have in general low membrane penetration and cellular uptake. This fact made the development of methods to enhance their cellular internalization of uttermost importance so that not only the therapeutic but also the biological-tool potential of nucleic-acids could be realized.

In this paper we have developed a new cell penetrating peptide as a delivery vector for PS-2'-*O*-methyl-RNA splice correcting oligos, employing a non-covalent complexation strategy. CPPs have been recognized for their capacity to deliver several cargoes to cells both *in-vitro* and *in-vivo* with quite low toxicities associated (272). CPP-cargo complexes are thought to internalize preferentially by endocytosis mechanisms, however one limitation has been their rather extensive entrapment inside endosomal vesicles (273).

The employed CPP builds on previous peptide modifications, such as stearylation, which was shown to increase complexation and endosomal release. Previous transfection efficiencies with stearyl-TP10 (274) and stearyl-$(RxR)_4$ (275) were however somewhat lower than with Lipofectamine 2000 besides being affected by serum proteins. Thus, the developed CPP was based on the previous stearylated TP10 but modifying lysines and isoleucines for ornithines and leucines, respectively. The reasoning behind the exchange for ornithines was based on earlier reports showing that poly-L-ornithine demonstrated superior transfection efficiency (up to 10-fold) compared to equivalent poly-L-lysine-based systems. This increase in efficiency was related to the higher affinity for DNA and the ability to make more stable complexes at lower charge ratios (276). Furthermore, it was hypothesized that ornithine, being a nonstandard amino acid, would be less prone to serum proteases, and thus could retain activity in serum conditions.

The new CPP, PF14, was initially used for delivery of a SCO in a splice correction reporter system, HeLa/Luc705 (already described in paper I). The efficiency of delivery, correlated to luciferase correction and gain in enzyme activity, was higher than for Lipfectamine 2000 when transfections were made both without and with serum. PF14-SCO complexes were also shown to be efficient for exon skipping in

differentiated mdx mouse myotubes which carry a point mutation in exon 23 of the dystrophin gene.

Uptake of PF14-SCO complexes was attributed to processes of endocytosis. Transfections done in presence of chloroquine showed an increase in the splice-correction activity, while SCO uptake remained nearly unchanged. This discrepancy between uptake and biological activity in the presence of chloroquine, a well-known endosomolytic agent, suggests the involvement of endocytic vesicles in the uptake of the nanocomplexes. Importantly, live cell confocal imaging confirmed this results since the nanocomplexes extensively colocalized with labeled dextran, a marker for fluid-phase endocytosis. The nanocomplexes were characterized in terms of size giving an average of 300-400nm and surface Zeta potential which had a negative value. The negative surface potential raises some interesting questions in terms of the exact mechanism of interaction with the cell membrane and the mechanism of uptake. In fact the cell membrane negative potential could in principle pose an obstacle to the interaction of another negatively charged particle, due to electrostatic hindrance. There are however reports on other types of nanocomplexes, based on gold nanoparticles, which have an increased efficiency in uptake depending on their negative potential and the amount of oligonucleotide load they carry. This uptake was hypothesized to depend on the adsorption of specific proteins to the surface of the nanoparticle (277). Other reports have also shown that cationic liposome and plasmid DNA complexes are negatively charged under optimal transfection conditions (278,279). A more detailed study on the uptake mechanism of the PF14-SCO complexes is perhaps warranted in light of these facts.

Another interesting property of the PF14-SCO complexes is the possibility to form solid formulations using a solid dispersion technique based on solvent evaporation (in this case water) by speed drying under elevated temperature (55–60ºC) and reduced pressure. The solid dispersion complexes almost completely retained the same activity as freshly prepared complexes in a splice-correction-assay. The success of the solid dispersion was dependent on the type and amount of excipient used, with 3.33% of lactose being the best condition.

The possibility of forming efficient solid formulations is of course a step towards the direction of having an oral drug delivery system.

4.3 PAPER III

Correction of *BTK* splicing mutations in X-linked agammaglobulinema (XLA) by an exon-skipping strategy

In this manuscript our objective was to design splice correction oligonucleotides (SCOs) to correct a *BTK* splicing defect deriving from a mutation in the intron 4 of the gene. It was previously identified in our group a specific A→T mutation in intron 4 of the *BTK* gene which creates a novel splice donor site. This, together with a pre-existing cryptic splice acceptor site in the same intron 4, results in the addition of a pseudo-exon sequence of 109bp between exons 4 and 5 (280). This specific aberrant splicing event completely abolishes BTK protein production.

The inclusion of this pseudo-exon due to the aberrant splicing suggested that exon skipping with the use of splice-correcting ONs could be considered as a treatment option for this XLA scenario.

We decided to establish an *in-vivo* model for this *BTK* aberrant splice and for that it was created a BAC (bacterial artificial chromosome) transgenic mouse harbouring the full human *BTK* locus with the referred point mutation. The transgenic mouse was confirmed to express the aberrant *BTK* pre-mRNA in B-lymphocytes.

The strategy was to exon-skip the created pseudo-exon in intron 4 by redirecting the splicing to the proper 5'- and 3'-splice sites. Initially, we used a model cell line expressing a Luciferase gene interrupted by the mutated *BTK* intron 4. We tested a number of different PS-2'-*O*-methyl RNA AONs to distinct sites including the aberrant 5'- and 3'-splice sites and also several exonic splice enhancer sites (ESE) in the formed pseudo-exon. The ESEs were identified using different published bioinformatic algorithms (281-283). The initial screening allowed the identification of the most promising oligos which were then designed as LNA modified 2'-*O*-methyl RNAs in order to increase their potency, and in preparation for future *in-vivo* studies where LNAs have been quite successful in other applications (252,254,255,284). The LNAs proved to be quite efficient in the model cell line.

We confirmed the efficiency of our LNA-SCO by initially using monocyte cells purified from an XLA patient bearing the *BTK* intron 4 mutation. Monocytes express BTK but do not need it for their development and survival. Thus using *ex-vivo* transfections we observed correction of *BTK* transcript and restoration of protein production to some extent. These results confirmed that the splice correction strategy

was working not only in our luciferase model system but also in the context of the full human *BTK* gene.

Importantly, *ex-vivo* transfections of purified BAC transgenic mouse B-cells with the LNA-SCOs showed that splice correction was also efficient in the context of B-lymphocytes. We obtained correction at the level of the *BTK* mRNA and also protein production. To our knowledge this is the first BAC mouse model of a splicing mutation in a full human gene.

In terms of a future therapeutic application two strategies could be possible. One alternative could be an *ex-vivo* approach in which case the pro-pre B-lymphocyte population could be mobilized, collected and transfected with the SCOs. After this step the cells could be re-infused and by regaining BTK production the developmental steps could be completed. A particularly attractive feature of XLA is the fact that the most mature cells of the B-cell lineage, the plasma cells, do not express BTK protein, and hence are not dependent on this signal transducer for their survival. This means that if immature primary B-cells expressing BTK can be generated following SCO treatment, upon immunization these cells could mature into long-lived plasma cells, which could confer protection far beyond the actual treatment period. Owing to that there are efficient vaccines available for bacterial agents such as *Hemophilus influenzae* and pneumococci, important pathogens for XLA patients, this could potentially have a great impact.

The other alternative is the *in-vivo* delivery of the SCOs. *BTK* mutations leading to loss of protein production lead to a developmental block of B-cells at the pro-pre B-cell stage leaving the patients with a severe systemic B-lymphocyte defect. Thus, SCOs would need to be accumulated in the bone marrow, where B-lymphocyte development takes place, and be taken up by pro-pre B-lymphocytes or earlier progenitor cells in the developmental ladder. Immunization would then be the course of action to follow as in the *ex-vivo* approach.

4.4 PAPER IV

Optimization of bis-LNA for supercoiled DNA binding and duplex invasion

This work is in line with earlier publications from our lab discussing the anti-gene properties of LNA based oligonucleotides designated Zorro-LNA (217,285). Our earlier reports indicated that for successful double-strand invasion two adjacent binding

sites were needed, whereas with one site binding was severely reduced. Moreover we have previously observed that linear LNAs were binding only to supercoiled plasmid DNA (which have a negative helical twist) and upon linearization of the plasmid DNA the binding would be lost, by a “kick-out” effect (286).

In consequence, we decided to investigate the design and use of a clamp-type of LNA (designated by bisLNA in analogy to bisPNA), to suppress the earlier identified limitations. The bisLNA was composed of a TFO unit (binding in the major groove by Hoogsteen interactions) and a DNA linker to the Watson-Crick binding unit. We first tested the influence of LNA modifications on each arm of the bis-construct. This was done using a quite short construct binding to 6 adjacent 10mer-sites introduced in a plasmid DNA. The following constructs were tested [TFO(arm)-WatsonCrick(arm)]: DNA-DNA; LNA-DNA; DNA-LNA; LNA-LNA. The degree of LNA modifications in each arm was always 50% since it was known from before that consecutive LNA bases impart an extreme rigidity to a TFO resulting in less binding efficiency (259,287). The results showed a major influence of having both arms modified with the LNA bases. In addition we also verified that while incubations with either TFO-arm or WC-arm alone did not produce any quantifiable binding, when incubating the separate arms together some level of binding was regained. This suggests that the increased binding/duplex invasion is possibly not only due to the local increase of concentration when TFO and Watson-Crick arms are linked together, but could also be attributed to local conformation changes on the target DNA-strand imparted by the LNA-modified TFO or duplex binding domains. It is conceivable that a possible pre-organization of the whole LNA•DNA:LNA structure into an A-type helix has a positive effect both for the triplex LNA strand and duplex LNA strand binding, since both preferably bind to, or induce, the A-type or A-type “like” helix.

Importantly, we verified that after successfully binding to plasmid DNA the bisLNA withstands some degree of linearization of the plasmid by restriction enzyme digestion. This result showed for the first time an LNA based construct capable of resisting the “kick-out” effect when the negative helical twist is lost from duplex DNA.

We went on to test a bisLNA design that could in fact bind to a single target site. The previous 10-mer bisLNA was unable to bind to a single site plasmid which was not so unexpected due to the low T_m values calculated for the Watson-Crick arm of the bisLNA (around 30°C according to calculations done using LNA Oligo Prediction Tools at http://www.exiqon.com/oligo-tools). Hence we designed a longer bisLNA with 15 bases in each arm, and a tail-clamp type of bisLNA with a 29 bases WC-arm

and a 15 bases TFO-arm. The results obtained for the duplex invasion capacity of these oligos showed a low binding for the 15-15 bisLNA, however when the tail-clamp (29-15) bisLNA was used we managed to achieve around 50% binding in close to physiological conditions (intranuclear salt concentrations and pH 7.3). When the incubation time was increased from 24h to 72h there was a further increase in binding. The tail-clamp LNA, despite the high percentage of LNA modification and the large size (29-mer for the duplex arm and additional 15-mer for the TFO arm) still showed a decent discrimination against a site with one point mutation, since binding efficiency was reduced quite significantly (close to 10 fold less binding efficiency).

We believe that in a cellular and genomic context the bisLNA might prove to have advantageous properties. These can come from the increased affinities towards negative supercoiled dsDNA under intranuclear salt and pH conditions, which would allow to better withstand the competition from DNA binding proteins, especially regarding the TFO domain. Moreover, and when comparing to the ssLNA strategies used thus far, the bisLNA has the advantage of being able to more efficiently scan the genomic sequence through the TFO domain. This domain provides the capacity of binding, much like dsDNA binding proteins, on the outside of the duplex through major groove interactions, thereby correctly positioning and locally increasing the concentration of the Watson-Crick bisLNA arm (duplex domain) for a more efficient double-strand invasion of the duplex genomic DNA.

Some points remain to be investigated and optimized since although we indeed for the first time achieve significant binding to a single site in close to physiological conditions the concentrations still remain high. It will be interesting to investigate the reason for the big difference seen between the bisLNA with and without the tail clamp. The melting temperatures (T_m) for the WC binding arms are expected to be already quite high for both constructs so other mechanisms or structural features could be playing a role in the increased binding seen for the tail-clamp bisLNA. Also, if the tail-clamp is an essential feature it would be interesting to see what would be the minimum size still giving significant binding. To increase the binding affinity, new LNA-based modifications (eg. functionalized 2'-amino-LNAs (288)) or mixed chemistries can be employed [such as intercalator units, eg. Twisted-Intercalating Nucleic Acids-TINA (289)]. In addition, it would be worthwhile to see if the bisLNA design can be integrated in the ZorroLNA construct in order to increase its binding affinity, while maintaining specificity, and confer the ability to bind to a single site with high efficiency. These experiments are underway in the lab.

The final goal is to reach sufficient affinity enabling us to target endogenous genes either in promoter regions or in the transcribed regions in order to arrest transcription and demonstrate the anti-gene strategy in cells and *in-vivo*.

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