From Department of Laboratory Medicine
Karolinska Institutet, Stockholm, Sweden

DEVELOPMENT OF NONVIRAL GENE DELIVERY AND OF ANTI-GENE REAGENTS

Rongbin Ge

Karolinska Institutet

Stockholm 2007

Published and printed by Karolinska University Press
Box 200, SE-171 77, Stockholm, Sweden

ISBN 978-91-7357-326-9

Published and printed by

www.reproprint.se
Gårdsvägen 4, 169 70 Solna

To my parents and friends

ABSTRACT

Although significant progress has been made in the basic science and applications of various non-viral gene delivery systems, the majority of non-viral approaches are still much less efficient than viral vectors, especially for in vivo gene delivery, Therefore, non-viral gene delivery must be developed in order to increase the efficiency of *in vivo* delivery. Moreover, a novel anti-gene reagent was developed for sequence-specific gene silencing.

Peptide Nucleic Acid (PNA), a mimic of oligonucleotide with a neutral pseudopeptide backbone consisting of repeating *N*- (2-amino-ethyl)-glycine units, can invade double-stranded DNA to generate a stable single-strand D-loop. Locked Nucleic Acid (LNA) are synthetic analogs of nucleic acids, which contain a bridging methylene carbon between the 2' and 4' positions of the ribose ring. Over the years, a variety of chemically modified small molecules have been developed. Among them, PNA and LNA have shown great promise for a number of applications.

In order to further develop a technology for linking functional entities to non-viral vectors, the Bioplex technology, we investigated how hybridization of PNA to supercoiled plasmids would be affected by the binding of multiple PNA-peptides to DNA strands. Cooperative effects were found at a distance of up to three bases. With a peptide present at the end of one of the PNA, steric hindrance occurred, reducing the increase in binding rate when the distance between the two sites was less than two bases. In addition, we found enhanced kinetics when PNA binding to overlapping sites on opposite DNA strands were added, without the use of further chemically modified bases in the PNAs (I).

We also generated a novel, sequence-specific anti-gene molecule "Zorro LNA", in which a 14-mer LNA binds to the coding strand, while a 16-mer connected LNA binds to the template strand. Our data suggest that the "Zorro LNA" induced effective and specific strand invasion into DNA duplexes and potent inhibition of gene transcription, also in mammalian cells. This offers a novel type of anti-gene drug, which is easy to synthesize (II). We also found that the Zorro LNA construct efficiently inhibited pol III-dependent transcription in a cellular context, including *in vivo* in a mouse model. Thus, this new form of gene silencer could potentially serve as a versatile regulator of pol III-dependent transcription, including various forms of shRNAs (III).

Finally, In order to further evaluate the *in vivo* activity of Zorro LNA and trace its long-term effect in inhibiting gene expression, we inserted two adjacent Zorro LNA-binding sites flanked by loxP sites within the transcribed region of a reporter gene. As previously observed, after Zorro LNA was hybridized to the plasmid, it significantly induced gene silencing in the mammalian cells. Both in transfected cells and in mouse model the silencing effect was lost when Cre-recombinase expressing plasmids were subsequently transfected. This suggests that binding of Zorro LNA is stable over a period of days.

LIST OF PUBLICATIONS

I. **Ge R**, Heinonen JE, Svahn MG, Mohamed AJ, Lundin, KE, Smith CIE. From The cover: Zorro Locked Nucleic Acid (LNA) induces sequence-specific gene silencing. *FASEB J*. 2007 Jun;21(8):1902-14

II. Lundin KE, **Ge R**, Svahn MG, Tornquist E, Leijon M, Brandén LJ, Smith CIE. Cooperative strand invasion of supercoiled plasmid DNA by mixed linear PNA and PNA-peptide chimeras. *Biomol Eng*. 2004 Apr; 21(2):51-9.

III. **Ge R**, Svahn MG, Simonson OE, Mohamed AJ, Lundin, KE, Smith CIE. Sequence-specific inhibition of RNA polymerase III-dependent transcription using Zorro Locked Nucleic Acid (LNA) *Submitted*

IV. **Ge R**, Simonson OE, Mohamed AJ, Lundin, KE, Smith CIE. Cre-loxP-mediated recombination leads to re-expression of silenced gene induced by Zorro LNA in a mouse model. *Submitted*

TABLE OF CONTENTS

1 INTRODUCTION 1
1.1 DNA DELIVERY SYSTEM 1
1.1.1 Viral delivery 1
1.1.2 Non-viral delivery 1
1.1.3 Bioplex 2
1.1.4 DNA delivery is a multiple process 3
1.1.5 Perspective 3
1.2 TRIPLEX-FORMING OLIGONUCLEOTIDES (TFOs) 4
1.3 PEPTIDE NUCLEIC ACIDS 4
1.3.1 Introduction 4
1.3.2 Thermodynamics 6
1.3.3 Uptake of PNA in eukaryotic cells 7
1.3.4 PNA-mediated transcription arrest 7
1.4 LOCKED NUCLEIC ACIDS 8
1.4.1 Introduction 8
1.4.2 Antisense effect of LNA 9
1.4.3 Antigene effect of LNA 9
1.4.4 LNA in therapeutic development and diagnostics 9
1.4.5 Prospective 10
1.5 Eukaryotic transcription 10
1.5.1 Transcription 10
1.5.2 RNA polymerases 11
1.5.3 Regulated transcription initiation 12
1.5.4 The structure of transcription factors 13
1.5.5 Chromatin 13
1.5.6 Post transcriptional modification 14
1.6 GENE SILENCING 16
1.6.1 Transcriptional Gene Silencing 16
1.6.2 Post-Transcriptional Gene Silencing (PTGS) 19
1.6.3 Nucleic acid-based gene silencing 19
1.6.4 RNA Interference 20
1.7 Cre-loxP recombination 24
1.7.1 Cre recombinase 24
1.7.2 Lox P site 24
1.7.3 The Cre-lox system in action 24
2 AIMS OF THE PRESENT STUDY 26
3 METHODS 27
3.1 Quantification of bound PNA using the Thiazole orange derivative 27
3.2 PNA binding assay 27
3.3 LNA binding assay 27
3.4 Use of destabilized green fluorescent protein (d2EGFP) 28
3.5 Microinjection and immunofluorescence 28
3.6 Hydrodynamic transfection 28
3.7 Intramuscular injection 29
4 RESULTS 30

4.1 PAPER I 30
4.2 PAPER II 30
4.3 PAPER III 31
4.4 PAPER IV 31
5 CONCLUSION 33
6 Acknowledgements 34
7 REFERENCES 35

LIST OF ABBREVIATIONS

BS	Binding site
CLL	Chronic Lymphocytic Leukaemia
CRC	Chromatin remodelling complex
Cre	Cre Recombinase
EGS	External guide sequence
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
hnRNP	Heterogeneous ribonucleoprotein particle
LNA	Locked Nucleic Acid
MF-AS	Morpholino antisense
miRNA	Micro RNA
PAP	Poly-A Polymerase
PNAs	Peptide Nucleic Acids
poly A	polyadenosine
PS-ODN	Phosphorothioate oligodeoxynucleotide
PTGS	Post-Transcriptional Gene Silencing
RITS	RNA-induced transcriptional silencing
snRNA	Small nuclear RNA
shRNA	Small hairpin RNA
siRNA	Small interfering RNA
sisiRNA	Small internally segmented interfering RNA
TFO	Triplex-forming oligonucleotide
Tm	Melting Temperature
TO	Thiazole orange

1 INTRODUCTION

1.1 DNA DELIVERY SYSTEM

Introduction of DNA into mammalian cells is a powerful tool for studying the mechanism of gene transcription, and for gene therapy. The process of introducing DNA into cells for the purpose of gene expression is called transfection or gene delivery. For this purpose, it is common to use plasmid DNA and variety of transfection methods(1). These transfection reagents are used to stimulate cellular attachment, intracellular release and subsequent nuclear translocation of the plasmid DNA(2, 3). An ideal gene delivery method needs to meet 3 major criteria: (i) it should protect the transgene against degradation by nucleases in intercellular matrices, (ii) it should bring the transgene across the plasma membrane and into the nucleus of target cells, and (iii) it should have no detrimental effects(4).

1.1.1 Viral delivery

Nonpathogenic, attenuated viruses can be used as delivery systems for DNA molecules, especially plasmids(5). Viral vectors are able to mediate gene transfer with both high efficiency and the possibility of long-term gene expression. Commonly used viral systems for gene transfer include Retroviruses, Lentiviruses, Adenoviruses and Adeno-associated viruses. As every virus has unique characteristics, it is possible to select a virus that is optimal for each specific type of gene delivery(6). Viral vectors are tailored to their specific applications but generally share a few key properties: (i) safety, (ii) low toxicity, (iii) stability, (iii) Cell type specificity. Gene expression using viral vectors has been achieved with high transfection efficiencies in tissues such as liver(7). Viruses are currently used in more than 70% of human clinical gene therapy trials worldwide(8). However, unfortunately, the acute immune response, immunogenicity, and insertion mutagenesis uncovered in gene therapy clinical trials have raised serious safety concerns about some commonly used viral vectors. Such toxicities have also been observed in numerous animal models(9-11). The limitation in the size of the transgene that recombinant viruses can carry and issues related to the production of viral vectors present additional practical challenges present additional practical challenges.

1.1.2 Non-viral delivery

Methods of non-viral gene delivery have also been explored using mechanical and chemical approaches (Fig.1.1). Mechanical approaches including:

- Direct injection of naked DNA(12) – Conceptually simple, DNA goes directly into the nucleus, laborious (one cell at a time)
- Electroporation(13, 14) – based on high-voltage electrical pulses to transiently permeabilize cell membranes
- Gene gun(15, 16) – particle bombardment-DNA coated microparticles are accelerated to high velocity to penetrate cell membranes:
- Hydrodynamic delivery(17, 18) – delivering naked DNA to hepatocytes (rapid injection via tail vein)

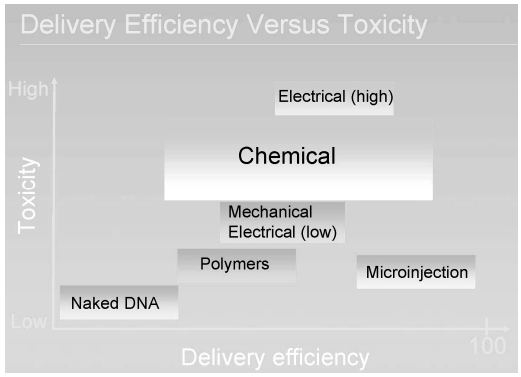

Figure 1.1: DNA delivery efficiency versus toxicity

The chemical approaches(19-21) use synthetic or naturally occurring compounds as carriers to deliver the transgene into cells (Table 1.1). Although significant progress has been made in the basic science and applications of various non-viral gene delivery systems, the majority of non-viral approaches are still much less efficient than viral vectors, especially for in vivo gene delivery.

Transfection reagent	Function	Name	Sequence specifity	Selected examples
Cationic lipid	DNA condensing activity	Lipoplex	No	Lipofectamine
Cationic polymer	DNA condensing activity	Polyplex	No	Polyethleneimine
Nucleic acid fused to a functional moiety	Including or excluding DNA condensing activity	Bioplex	Yes	Nucleic acid-Nuclear localization signal (NLS) fusion

Table 1.1: Nomenclature and characteristics of transfection complexes. Reproduced from *Branden LJ et al.* (22)

1.1.3 Bioplex

Bioplex is a new technology intended to substantially improve the cellular uptake of naked-DNA during transfection. The concept is based upon using synthetic oligonucleotides which serve as anchors for different functions attached directly to the plasmid. Examples are nuclear localization signals (NLS), RGD for receptor mediated endocytosis and HA2 for endosomal disruption(21).

The technology is based on the use of two particular aspects of PNA (or analogues thereof), namely the high sequence specificity, making these molecules most suitable as genetic anchors, and the possibility of making a continuous peptide synthesis, allowing PNA and a peptide moiety to become adjacent without involving cumbersome linking chemistry and purification of the conjugated peptides. It is then possible to attach and/or specificity (**Fig. 1.2**).

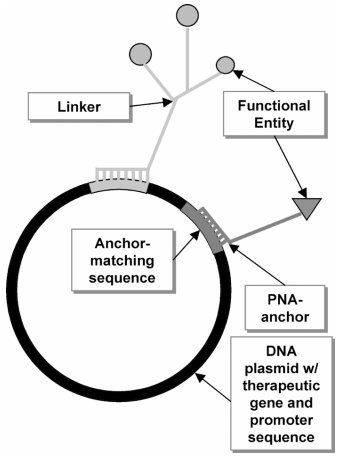

Figure 1.2: The Bioplex concept. Site-specific PNAs are hybridized to the plasmid by strand invasion. Branched or linear linkers couple functional entities to the PNA anchors and thereby to the plasmid. Reproduced from *Svahn MG et al.* (23).

1.1.4 DNA delivery is a multiple process

Transfer of exogenous DNA into the cell is greatly impeded by the size and charge of the DNA. Furthermore, DNA delivery is a multi-step process (Table 1.3), DNA must evade biochemical degradation and several cellular barriers before it can have the desired effect in the cell. Only DNA that survives all these barriers has the potential to be functional. As a result, the number of DNA molecules decreases at each step of the journey to the nucleus. Therefore, identifying and overcoming each barrier along the pathways can significantly increase DNA delivery efficiency. Equally important as efficient delivery is targeted delivery, in which specific molecular agents are used to enhance the delivery of DNA to a specific target.

1	DNA condensation and complexation: Negatively charged DNA molecules are usually condensed and/or complexed with cationic transfection reagents
2	Uptake of the complexes by the cell usually through endocytosis: Involves binding, internalization, formation of endosome, development of lysosome, lysis/degradation
3	Dissociation from the complexes and entering into the nucleus: Entry is thought to occur through nuclear pore complexes. DNA may also be recruited into the nucleus during the physical process of cell division

Table 1.2: DNA delivery is a multi-step process

1.1.5 Perspective

Ideal DNA delivery properties:

- Ease of assembly
- Capability of bypassing or escaping from endocytotic pathways
- Efficient decomplexation or “unpacking”
- Efficient nuclear targeting

- High, persistent and adjustable expression

1.2 TRIPLEX-FORMING OLIGONUCLEOTIDES (TFOS)

In earlier studies, it has been shown that triplex-forming oligonucleotides (TFOs) and their derivatives are able to invade the major groove of oligopyrimidine•oligopurine regions in double-stranded DNA and interfere with gene transcription initiation and elongation(24-26). However, this Hoogsteen-binding dependent invasion is considered slow and there is room for improvement of the intracellular efficiency of TFOs(27). Moreover, it was recently reported that a nucleotide excision repair machinery is targeted to helical distortions and altered hydrogen-bonding pattern caused by Hoogsteen binding(28). Certain synthetic polyamides binding to the minor groove constitute an alternative to TFOs(29). However, further exploration of other Watson-Crick binding reagents could be rewarding. Over the years, a variety of chemically modified small molecules have been developed. Among them, PNA and LNA have shown great promise for a number of applications.

1.3 PEPTIDE NUCLEIC ACIDS

1.3.1 Introduction

During the past decade several hundred different analogs, containing natural nucleobases on a modified backbone, have been synthesized. A pioneering example of this is peptide nucleic acid (PNA), an analog with a noncyclic peptide-like backbone. The original aminoethylglycine-based PNA was first described by Buchardt(30). Using molecular modeling the Danish group removed the sugar phosphate backbone and replaced it with a polyamide of N-(2-aminoethyl) glycine. So PNA (Fig. 1.3) is an oligonucleotide analog with a neutral pseudopeptide backbone consisting of repeating *N*- (2-amino-ethyl)-glycine units(30). It is chemically stable and resistant to hydrolytic (enzymatic) cleavage and thus not expected to be degraded inside a living cell. PNA is capable of sequence-specific recognition of DNA and RNA obeying the Watson-Crick base pairing rules(31), and the hybrid complexes exhibit extraordinary thermal stability and unique ionic strength effects.

It may also recognize duplex homopurine sequences of DNA to which it binds by strand invasion, forming an extremely stable PNA-DNA-PNA triplex with a looped-out DNA strand(32-34) (Fig. 1.4). Strand invasion is most efficient when pyrimidine PNAs are connected by a flexible linker to form a bisPNA in which one PNA strand hybridizes to DNA by Watson-Crick base pairing, while the other binds to the same strand by Hoogsteen mode(33, 35). When targeting DNA containing several copies of PNA binding sites (BSs), it is possible to achieve a cooperative effect for the PNA binding. A report showed that cooperative strand displacement by bis-PNAs bound to DNA sequences, positioned in the immediate vicinity of each other, but on *opposite* DNA strands, occurs at a distance of up to two bases(36). PNA oligomers also show greater specificity in binding to complementary DNAs, with a PNA/DNA base mismatch being more destabilizing than a similar mismatch in a DNA/DNA duplex. This binding strength and specificity also applies to PNA/RNA duplexes(31). PNA appears to be resistant to both cellular nucleases and proteases(37).

Moreover, it has recently been reported that further optimized PNA(38), which is extended outside the bis-region, widens the application for strand invasion and inhibits transcription *in vitro*(38, 39). These properties of PNA should be advantageous for use as anti-gene drugs to inhibit gene activity at the transcriptional level. Based on *in vitro* cell-free experiments, it is generally considered that triplex invasion complexes are effective inhibitors of transcription initiation as well as transcription elongation, especially when positioned on the template strand(38-41). Several interesting studies by Boffa *et al*. have reported cellular anti-gene effects on *c-myc* transcription of mixed purine-pyrimidine sequence PNAs(42-44). Given that linear PNAs show considerably reduced binding affinities as compared to bisPNAs these results are remarkable. The exact mechanism accounting for the observed effect has, however, not been delineated and the binding stochiometry of PNA to the DNA target was not demonstrated(45). Other potential applications for duplex recognition by PNA include activation of gene transcription(46, 47)and introduction of mutations(48). However, to date, we are not aware of any convincing data showing that PNA induces gene transcription by simply increasing the accessibility of RNA polymerases, through the displacement of the two DNA strands as an artificial promoter. One paper reported that PNA induced human gammaglobin gene expression *in vivo*(47), but we noticed that the anchor site for PNA binding in that paper overlapped with a binding site for a transcriptional repressor, Oct-1, thus offering an alternative explanation. To this end, a recent report describes the elegant use of anti-gene PNAs, which target transcriptional start sites, demonstrating that linear PNA work very efficiently at high PNA to target site ratios(49).

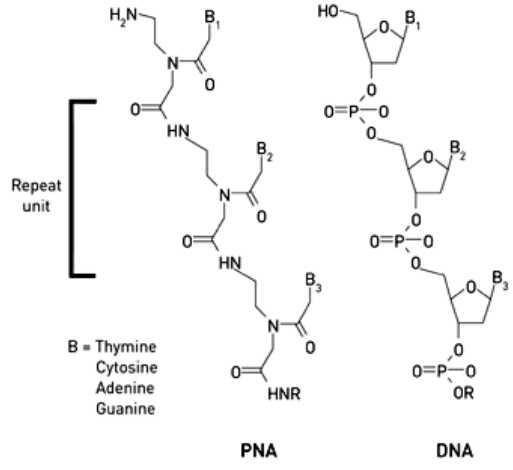

Figure 1.3: Molecular structures of PNA and DNA

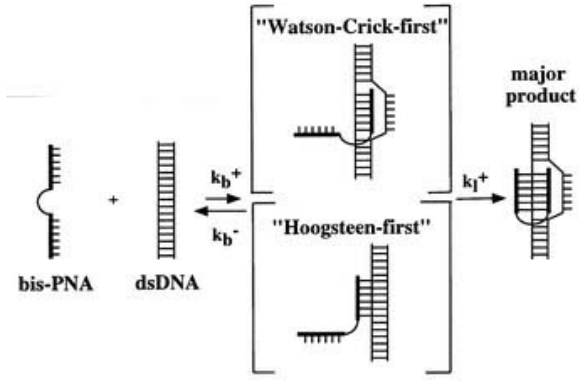

Figure 1.4: D-loop formation by bisPNA inside dsDNA (Hoogsteen and Watson-Crick binding) Reproduced from *Demidov VV et al.* (37).

1.3.2 Thermodynamics

The thermal stability of PNA/DNA and PNA/RNA duplexes is higher compared to DNA/DNA and DNA/RNA duplexes (Table 1.3). The stronger binding is attributed to the lack of charge repulsion between the neutral PNA strand and the DNA or RNA strand. The achiral backbone of PNA allows for a greater flexibility and may also influence binding.

Duplex	Tm
15-mer PNA/DNA	69 C
15-mer DNA/DNA	54 C
15-mer PNA/RNA	72 C
15-mer DNA/RNA	50 C

Table 1.3: Reproduced from *Nielsen PE et al.*(31)

Another important consequence of the neutral backbone is that PNA/DNA duplexes are insensitive to variations in salt concentration (Table 1.4). This is in contrast to DNA/DNA duplexes, which are highly dependent on ion concentration. At low salt concentration, PNA can bind to a target sequence at temperatures where DNA hybridization is suboptimal.

Salt (Na Cl) Concentration	PNA/DNA Tm	DNA/DNA Tm
0 mM	72 C	38 C
140 mM	69 C	56 C
1000 mM	65 C	65 C

Table 1.4: Reproduced from *Nielsen PE et al.*(31)

1.3.3 Uptake of PNA in eukaryotic cells

Cellular uptake is a major barrier to successful use of oligonucleotides inside cells. Fortunately, several methods have been developed to promote entry of PNAs into cells.

One strategy for achieving cellular uptake involves annealing PNAs to negatively charged DNA oligonucleotides and then complexing the heteroduplex with cationic lipid(50). In this variation of standard protocols for delivering RNA or DNA into cells, the lipid binds to the DNA, allowing it to pass through the cell membrane. PNAs delivered into cells by this method have been shown to inhibit telomere synthesis by human telomerase(51) and to bind mRNA and inhibit expression of luciferase(52), human caveolin(53) and human progesterone receptor. The inhibition of mRNA by PNAs delivery by this strategy is as efficient and as prolonged as inhibition by analogous siRNAs(53). Advantages of this method are that the PNA does not require chemical modification and standard transfection protocols can be used. A disadvantage is that use of lipid adds an extra experimental step. It is likely that this simple method has not become more widely used because its successful use requires that a laboratory be familiar with the chemical properties of PNAs and possesses expertise performing transfections of mammalian cell culture.

Spontaneous uptake of PNAs by cells can be achieved by attachment of peptide sequences that promote translocation across cell membranes(54, 55). One advantage of this approach is that lipid is not necessary, making the procedure more straightforward. Another advantage of this approach is that the peptide might also be used to promote nuclear uptake. Disadvantages are that additional synthetic steps are needed to add the peptide to the PNA and relatively high concentrations of PNA are required. In animals, uptake of PNAs tagged with four lysine residues has been shown to occur in a variety of tissues in mice and alter splicing(56). Development of conjugates between PNAs and peptides or peptoids may ultimately be the method needed to achieve breakthrough results in vivo. PNAs with minimal modification can enter cultured cells and alter splicing, but only when high concentrations are present in the growth medium(57)

1.3.4 PNA-mediated transcription arrest

PNAs can arrest transcriptional processes by virtue of their ability to form a stable triplex structure or a strand-invaded or strand-displacement complex with DNA(58). Such complexes can create a structural hindrance to block the stable function of RNA polymerase and thus are capable of working as antigene agents. It is important that the PNA target (DNA homopurine) must be present in the gene of interest(58). One of the major obstacles for applying PNA as an antigene agent is that the strand invasion or the formation of strand displacement complex is rather slow at physiological salt concentrations. But it was found out that under physiological salt conditions, binding of PNA to supercoiled plasmid DNA is faster compared to linear DNA(59). This result is relevant to the fact that the transcriptionally active chromosomal DNA usually is negatively supercoiled, which can act as a better target for PNA binding in vivo. It was also found that the binding of PNA to dsDNA is enhanced when the DNA is being transcribed. This transcription-mediated PNA binding occurs about three-fold as efficiently when the PNA target is situated on the nontemplate strand instead of the template strand(59). As transcription mediates template strand-associated (PNA)

2/DNA complexes that can arrest further elongation, the action of RNA polymerase results in repression of its own activity, i.e., suicide transcription(60).

1.4 LOCKED NUCLEIC ACIDS

1.4.1 Introduction

Modifications to native nucleic acids can be introduced in the nucleobase, the sugar ring or the phosphodiester backbone(61). In an effort to increase binding affinity towards RNA by conformational restriction, many sugar-modified nucleic acids have been prepared(62). Model building revealed an O2′- to C4′-linked analogue to be of interest, owing to its conformationally locked C3′-*endo* sugar conformation, which is supposedly ideal for recognition of RNA; a 2′-*O*,4′-*C*-methylene-linked ribonucleotide derivative was subsequently synthesized(63, 64) and named LNA(63, 64).

LNA bases contain a bridging methylene carbon between the 2' and 4' positions of the ribose ring(63-67) (Fig. 1.5). This constraint preorganizes the oligonucleotide backbone thereby increasing the T_m values by as much as 10 C per LNA base replacement. Multiple LNA substitutions allow T_m values to be precisely tailored for specific applications. The introduction of LNA bases also increases resistance to digestion by nucleases(68). LNA contains natural phosphodiester linkages and, therefore, resembles natural nucleic acids with respect to, for example, adequate aqueous solubility, Watson-Crick mode of binding, and ease of automated synthesis using conventional phosphoramidite chemistry. Since LNA bases are introduced by standard DNA/RNA synthesis protocols, the corresponding oligonucleotide can be generated as pure LNA or as mixed LNA/DNA/RNA chimeras and the introduction of a relatively small number of LNA bases can dramatically enhance the affinity of an oligonucleotide for its complement. The extremely high binding affinity of LNA, LNA/DNA mixmers, LNA/RNA mixmers and LNA/PS-DNA mixmers toward complementary DNA and RNA is the most important characteristic property of LNA(69). Moreover, LNAs have been demonstrated to be very efficient in binding to complementary nucleic acids and to serve as active, nontoxic antisense agents *in vitro* and in cultured mammalian cells(70-72) as well as *in vivo*(73). LNAs have also been used as decoys(74), aptamers(75), LNAzymes(76), agents to attach functional moieties to the plasmid DNA(77) and DNA correcting agents(78) as well as antigene reagents(27, 79). LNAs have furthermore been successfully applied to FISH (fluorescence *in situ* hybridization)(80).

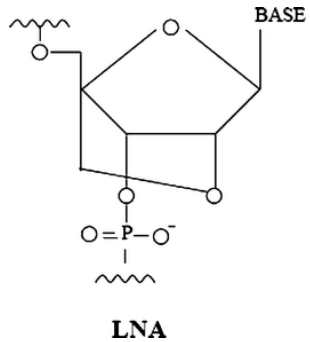

Figure 1.5: Molecular structures of LNA

1.4.2 Antisense effect of LNA

LNA in antisense experiments was evaluated against siRNA, leading to the conclusion that LNA antisense oligonucleotides combined with other modifications such as phosphorothioate linkages might rival the current very popular siRNA approach for gene silencing *in vitro* and *in vivo*(81). There is one study directly comparing the inhibitory effect of siRNA, LNA/DNA/LNA gap-mers, phosphorothioate DNA, and 2'-*O*-methyl-RNA on the expression of vanilloid receptor subtype 1 in cells(71). A new finding indicates that weak base pairing at the 5'-end is an important selection criterion for determining which siRNA strand will be used(82). Therefore, the exact positioning and the overall number of LNA monomers will be very crucial for optimizing LNA-containing siRNA. Recently, Dr. Jørgen Kjems has improved the biological efficiency of the siRNA itself, by introducing various types of 2'-modefied locked nucleic acids in both strands of the siRNA and 1-2 nicks in the backbone of the sense strand. This type of siRNA, termed "small internally segmented interfering RNA (sisiRNA), has a potent, long-lasting gene-silencing effect whilst reducing the number of off-target effects.

1.4.3 Antigene effect of LNA

Antigene effect normally relates to interference with processes that involve chromosomal dsDNA. These include inhibition of transcription, inhibition of replication, and inhibition of other interactions between protein and dsDNA. The task of hybridizing to chromosomal dsDNA that is imbedded in positively charged proteins is obviously not straightforward. Two types of hybridization can be envisaged between dsDNA and single-stranded LNA oligonucleotides, namely, binding to the dsDNA by the formation of a triple-helical complex or binding to the dsDNA by single-strand targeting, thus displacing the other DNA strand by a strand invasion process.

Incorporation of one LNA nucleotide centrally in TFOs significantly increases the binding affinity of the TFOs(83). Increasing the number of LNAs in the TFO induced increases in melting temperatures of 4-5 °C per modification at pH 6.6. Also, at pH 7.2, transitions were detectable, demonstrating that triplex formation occurred even at neutral pH. The best results were obtained with TFOs with sequences of alternating DNA and LNA nucleotides, whereas fully modified LNA TFOs showed no propensity at all to hybridize to dsDNA(84). Directing an LNA TFO, with alternating DNA and LNA nucleotides, against part of a dsDNA recognized by the NF-κB transcription factor induced inhibition of binding of NF-κB to the target dsDNA at pH 7.0 by formation of a dsDNA·LNA triplex(84). Crinelli *et al*.(85) have applied various LNA oligomers as decoys for transcription factor NF-κB binding to dsDNA. Inclusion of one or two terminal LNA nucleotides, outside the NF-κB recognition sequence, appreciably improved the protection against nuclease digestion without interfering with transcription factor binding. When LNA nucleotides were included in the NF-κB sequence, further stabilization against degradation was observed.

1.4.4 LNA in therapeutic development and diagnostics

The strengths of LNA are being exploited for preclinical investigations and therapeutic development. LNAs can inhibit gene expression at the level of

translation(86), alter splicing in mouse models(87), inhibition of telomerase activity(88). The mouse experiment demonstrating inhibition of translation also suggested that LNAs containing phosphorothioate substitutions can exhibit significant hepatotoxicity(86). It is possible, however, that revised LNA designs might avoid this problem. An LNA targeting Bcl-2 is being tested in Phase I/II trials for the treatment of chronic lymphocytic leukemia(89, 90).

In clinical trials, AONs are routinely delivered naked, and several class-related toxicities have been described for PS oligonucleotides, such as hypotension, fever, asthenia, complement activation, and thrombocytopenia(91). However, effective doses are generally below the maximum tolerated dose and slow i.v. infusion appears to be well tolerated in humans(92). The toxicity of LNA oligonucleotides has been examined in rodents(73) showed that fully modified LNA, LNA/DNA mixmer, or LNA/DNA/LNA gapmer AONs do not elicit any histologically detectable toxicity or changes in core body temperature when injected into the striatum of rat brains. Most interesting, LNA has been entered into clinical trials and SPC2996 is the first of a new class of LNA-based anti-cancer drugs and observered down-regulation of Bcl-2 in the treatment of Chronic Lymphocytic Leukaemia (CLL) (www.santaris.com).

As mentioned above, LNAs display unique hybridization properties and provide decreased susceptibility to nucleases. Both these properties are highly advantageous for a molecular tool for diagnostic applications. Easy-to-use SNP assays based on the LNA technology have been designed and implemented. LNA is particularly well-suited for this purpose because it leads to better single-nucleotide mismatch discrimination than that obtained with DNA. For a review on SNP analyses using LNA, see Mouritzen *et al.*(93). Several LNA genotyping assays have been reported, including screening for an apolipoprotein B (apoB) R3500Q mutation and two mutations in apolipoprotein E(94, 95) and for the factor V Leiden mutation(96).

1.4.5 Prospective

LNA substitutions couple the high affinity binding that characterizes recognition by PNAs with retention of the phosphodiester backbone of DNA or RNA. Therefore, it is supposed that LNAs would be ideal candidates for examining whether efficient inhibition of gene expression could be extended beyond PNAs to negatively charged, synthetic oligonucleotides.

1.5 EUKARYOTIC TRANSCRIPTION

1.5.1 Transcription

The central dogma of molecular biology stipulates that DNA gives rise to RNA which in turn gives rise to protein. It follows that a primary process in the molecular biology of the cell is production of RNA from DNA, i.e. the process of transcription. The failure of this process to occur, or to decode faithfully the information encoded in the DNA, would render the cell non-viable. In eukaryotic cells, most genes are regulated (during development and differentiation, in response to specific cellular signals), at least in part, at the level of transcription initiation. Transcription and its regulation are fundamentally essential processes in the cell.

Eukaryotic transcription is more complicated than prokaryotic transcription, because eukaryotes have evolved much more complicated transcriptional regulatory mechanisms. For instance, in eukaryotes the genetic material (DNA), and therefore transcription, is primarily localized to the cell nucleus, where it is separated from the cytoplasm (in which translation occurs) by the nuclear membrane. DNA is also present in mitochondria and mitochondria utilize a specialized RNA polymerase for transcription. This allows for the temporal regulation of gene expression through the sequestration of the RNA in the nucleus, and allows for selective transport of RNAs to the cytoplasm, where the ribosomes reside.

1.5.2 RNA polymerases

The process of transcription involves polymerisation of nucleoside triphosphates into RNA, in a DNA template-dependent manner, and in eukaryotes it is catalysed by three RNA polymerase enzymes, RNA polymerase I, RNA polymerase II and RNA polymerase III. RNA polymerase I is the most prominent polymerase activity in the cell, followed by polymerase II while polymerase III contributes the least polymerase activity.

RNA polymerases are huge, multi-component complexes of over 10 protein subunits and are around 500 kDa in size. There is considerable relatedness between the three eukaryotic RNA polymerases and to the prokaryotic *E. coli* RNA polymerase, especially between the largest and second largest subunits. RNA polymerase II differs from the others in that the largest subunit has a carboxy-terminal extension called the carboxy-terminal domain (CTD). The CTD contains a highly repeated heptapeptide,

Tyr-Ser-Pro-Thr-Ser-Pro-Ser

which can be heavily phosphorylated. This phosphorylated domain is essential for transcription by RNA polymerase II in most eukaryotes, and it also links the processes of transcription and RNA processing(97).

The basal eukaryotic transcription complex includes the RNA polymerase and additional proteins that are necessary for correct initiation and elongation.

The three nuclear RNA polymerase of eukaryotes have distinct roles and properties (Fig. 1.6)

- RNA polymerase I synthesizes a pre-rRNA 45S, which matures into 28S, 18S and 5.8S rRNAs, which will form the major RNA sections of the ribosome.(98)
- RNA polymerase II synthesizes precursors of mRNAs and most snRNA and microRNAs. This is the most studied type, and due to the high level of control required over transcription, a range of transcription factors are required for its binding to promoters. (99)
- RNA polymerase III synthesizes tRNAs, rRNA 5S and other small RNAs found in the nucleus and cytosol. (100)

There are other RNA polymerase types in mitochondria and chloroplasts.

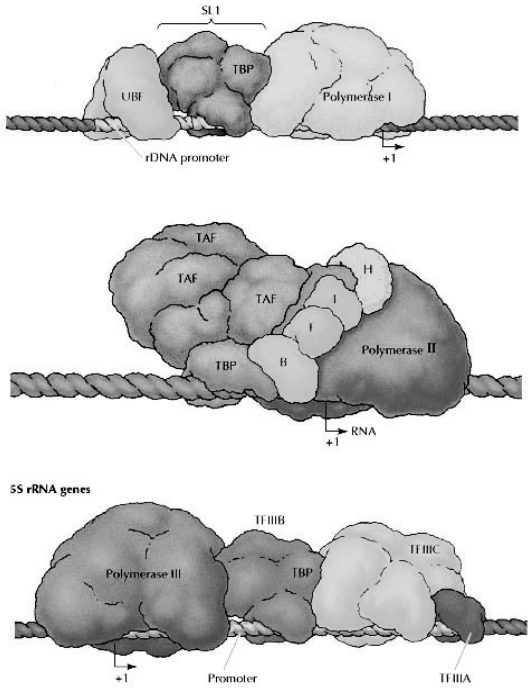

Figure 1.6: Three nuclear RNA polymerases of eukaryotes. Reproduced from *Cooper GM et al.*(101)

1.5.3 Regulated transcription initiation

Transcription initiation can be subject to activation or repression. Gene promoters contain transcription factor-binding sites necessary for constitutive expression but these are often interspersed with factors regulating inducible expression. There are many possible strategies for activation and repression and some are listed below:

Activation

a. An activator binds a site in the promoter and stimulates formation of the stable transcription initiation complex.

b. An activator binds and stimulates the activity of the transcription initiation complex.

c. An activator binds the appropriate site in the promoter but only has a significant effect once it binds another protein, or co-factor, or becomes modified.

d. An activator binds the promoter but can only act once the chromatin structure of the promoter has been remodeled.

Repression

a. A repressor molecule binds a site used by an activator thus preventing it from interacting with the DNA.
b. A repressor sequesters the activator molecule before it can bind the promoter.
c. A repressor neutralizes the activity of the activator.
d. A repressor molecule can directly repress transcription by binding to and preventing assembly of the transcription initiation complex.
e. A repressor molecule induces a tight chromatin structure around the promoter.

1.5.4 The structure of transcription factors

A number of protein domains have been identified which interact with double stranded DNA. These include zinc finger domains, helix-turn-helix domains, leucine zippers and the helix-loop-helix.

Motif	**Examples**
Zinc finger	TFIIA, Sp1, Steroid hormone receptor
Helix-turn-helix	Homeodomains
Leucine zipper	GCN4, JUN, C/EBP
Helix-loop-helix	MyoD

1.5.5 Chromatin

RNA polymerases do not transcribe naked DNA since cellular DNA is packed into nucleosomes (Fig. 1.7) as well as interacting with a myriad of different DNA-binding protein(102). However, studies have shown that the rate of elongation of RNA polymerase II *in vitro* on naked DNA is very similar to the rate measured *in vivo* inferring that DNA must be cleared of protein during transcription(103). To allow transcription nucleosomes must either fall off the DNA template or dissociate temporarily. A useful model is that nucleosomes and DNA are in dynamic association such that changes in local chromatin structure, including post-translational modification of histones, would cause an opening up of the promoter DNA to allow transcription complexes access to the template. Recent studies have come up with a model where nucleosomes may skip around the RNA polymerase complex without actually leaving the DNA(104). It is clear, however, that DNA which is being actively transcribed is usually in a more "open" chromatin conformation than transcriptionally silent DNA.

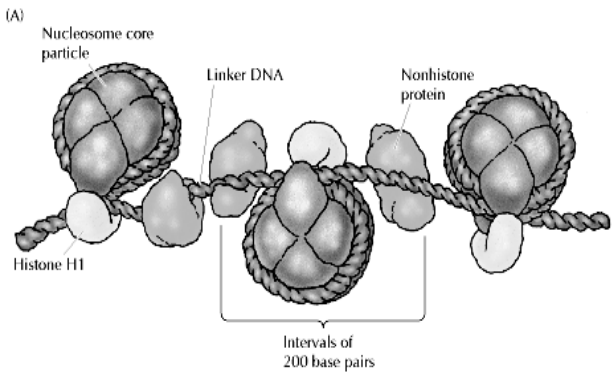

Figure 1.7: Structure of nucleosome and chromatin. Reproduced from *Cooper GM et al.(101)*

Open chromatin is also associated with hyperacetylation of histones by HATs (histone acetyltransferases). Hyperacetylated histones display a decreased tendency to pack together thus loosening chromatin structure. Conversely, histone deacetylases (HDACs) remove acetyl groups from lysine residues in histones H3 and H4 resulting in surface charge changes which then restrict histone mobility on the DNA. This probably renders promoter sequences inaccessible to the transcriptional machinery(105).

A third chromatin change that may alter transcriptional activity is CpG methylation, however, this modification is not present in all eukaryotes. Undermethylated DNA characterizes transcriptionally active chromatin while methylation induces silencing(106). It has been found that methylated CpG residues are recognised by proteins which serve to target HDACs thus causing repression of gene expression(106).

Finally, experiments in yeast (genetic and biochemical studies) flies and human cells (biochemical studies) have revealed a set of huge multiprotein complexes with ATP-dependent chromatin restructuring capacities called chromatin remodelling complexes (CRCs)(107). Three complexes from *Drosophila* have been identified, NURF (nucleosome remodelling factor), CHRAC (chromatin accessibility complex), and ACF (ATP-dependent chromatin assembly and remodelling factor). These are all members of the ISWI family of CRCs(108). NURF can mediate ATP-dependent binding of transcription factors to chromatin templates, creating DNase I hypersensitive sites. CHRAC has a nucleosome spacing activity. ACF helps to assemble properly spaced nucleosome arrays and facilitate activator binding to chromatin. Another ubiquitous family of CRCs, the SWI-SNF family was first identified in yeast. SWI-SNF complexes are important regulators of RNA polymerase II and, like NURF and ACF, perturb nucleosome spacing and open up promoter chromatin(107).

It has become clear that transcription factors, co-factors, HATs/HDACs and CRCs often interact in quite complex ways to effect up- or down-regulation of gene expression by making local changes in the accessibility of promoter chromatin(104). In addition to specific elongation factors there is strong evidence that transcription elongation also requires HATs and CRCs, such as SWI/SNF and FACT which removes the outer nucleosome histones H2A and H2B thus loosening chromatin structure. These proteins seem to "hitch a ride" on the elongating RNA polymerase and when these elongation-specifc HATs open the chromatin structure this openness is maintained by the elongating polymerase complex. Future studies on chromatin structure and transcription should provide a wealth of information on the subtleties of eukaryotic transcriptional regulation.

1.5.6 Post transcriptional modification

Post transcriptional modification is a genetic process in cell biology by which, in eukaryotic cells precursor messenger RNA is converted into mature messenger RNA (mRNA) during the larger process of protein synthesis so ultimately there will be resulting functional proteins. This process is vital for the efficient and correct

translation of the genome by ribosomes, the next step of protein synthesis, and for the export of the mRNA from the nucleus for translation.

The three main modifications are 5' capping, 3' polyadenylation, and RNA splicing (Fig. 1.8). While in the nucleus, pre-mRNA is associated with a variety of proteins in complexes known as heterogeneous ribonucleoprotein particles (hnRNPs).

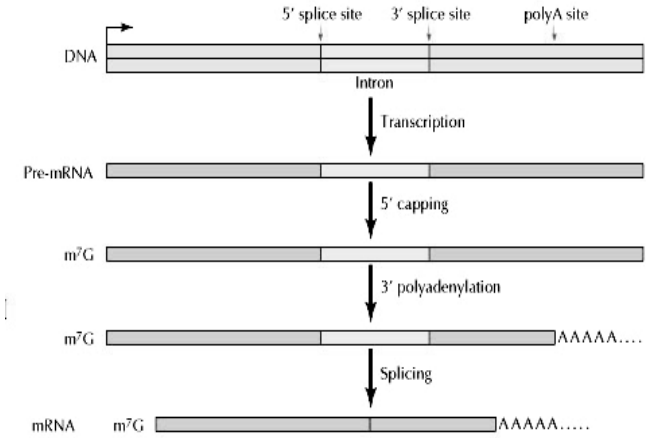

Figure 1.8: Processing of eukaryotic messenger RNAs. Reproduced from *Cooper GM et al.(101)*

Addition of the 5' cap is the first step in pre-mRNA processing. This step occurs co-transcriptionally, that is, while the RNA molecule is still being transcribed, after the growing RNA strand has reached 30 nucleotides. The process is catalyzed by a capping enzyme that associates with the carboxyl-terminal domain of RNA polymerase II, the main enzyme involved in mRNA transcription.

The second step is the cleavage of the 3' end of the primary transcript following by addition of a polyadenosine (poly-A) tail. This end of the pre-mRNA contains a sequence of around 50 nucleotides that acts as a signaling region recognised by a protein complex. The protein complex promotes association of other proteins including those involved in the cleavage and Poly-A Polymerase (PAP), the enzyme responsible for the addition of the tail. PAP binding is required before the cleavage can occur, enforcing the tight coupling of the two events.

RNA splicing is the process by which introns, are removed from the pre-mRNA and the remaining exons connected to re-form a single continuous molecule (mature mRNA). Although most RNA splicing occurs after the complete synthesis and end-capping of the pre-mRNA, transcripts with many exons can be spliced co-transcriptionally. The splicing reaction is catalyzed by a large protein complex called the spliceosome assembled from proteins and small nuclear RNA (snRNA) molecules that recognize splice sites in the pre-mRNA sequence. Many pre-mRNAs, including those encoding antibodies, can be spliced in multiple ways to produce different mature

mRNAs that encode different protein sequences. This process is known as alternative splicing, and allows production of a large variety of proteins from a limited amount of DNA(101).

1.6 GENE SILENCING

Gene silencing is a general term describing epigenetic processes of gene regulation. The term gene silencing is generally used to describe the "switching off" of a gene by a mechanism other than genetic mutation. That is, a gene which would be expressed (turned on) under normal circumstances is switched off by some form of machinery in the cell.

Genes are regulated at either the transcription or post-transcriptional level. Transcriptional gene silencing is the result of histone modifications, creating an environment of heterochromatin around a gene that makes it inaccessible to transcriptional machinery (RNA polymerase, transcription factors, etc.).

Post-transcriptional gene silencing is the result of mRNA of a particular gene being destroyed. The destruction of the mRNA prevents translation to form an active gene product. A common mechanism of post-transcriptional gene silencing is RNAi(109).

Both transcriptional and post-transcriptional gene silencing are used to regulate endogenous genes. Mechanisms of gene silencing also protect the organism's genome from transposons and viruses. Gene silencing thus may be part of an ancient immune system protecting from such infectious DNA elements.

1.6.1 Transcriptional Gene Silencing

1.6.1.1 Genomic imprinting

Genomic imprinting is a genetic phenomenon by which certain genes are expressed in a parent of origin-specific manner. Forms of genomic imprinting have been demonstrated in insects, mammals and flowering plants.

In diploid organisms somatic cells possess two copies of the genome. Each autosomal gene is therefore represented by two copies, or alleles, with one copy inherited from each parent at fertilization. For the vast majority of autosomal genes, expression occurs from both alleles simultaneously. However, a small proportion (<1%) of genes are imprinted, meaning that expression occurs from only one allele. The expressed allele is dependent upon its parental origin. For example, the gene encoding insulin-like growth factor II (IGF2/Igf2) is only expressed from the allele inherited from the father(110).

1.6.1.2 RNA-induced transcriptional silencing

RNA-induced transcriptional silencing (RITS) is a form of RNA interference by which short RNA molecules - microRNA (miRNA) or small interfering RNA (siRNA) - trigger the downregulation of transcription of a particular gene or genomic region. This is usually accomplished by modification of histones, often by histone methylation, or by the induction of heterochromatin formation. The protein complex that binds the

miRNA and interacts with histones and DNA is known as the "RITS complex".(111, 112)(Fig. 1.9)

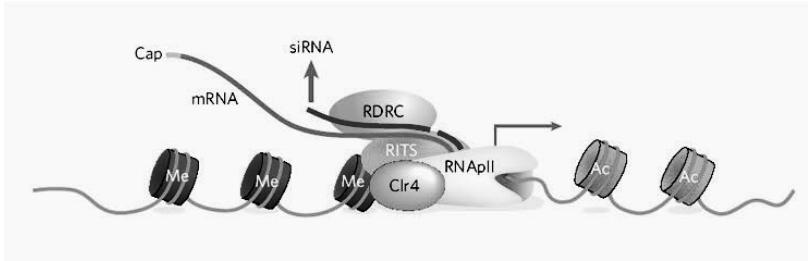

Figure 1.9: Transcription-coupled RNA interference. Reproduced from *Cooper GM et al.(101)*

The maintenance of heterochromatin regions by RITS complexes has been described as a self-reinforcing feedback loop, in which RITS complexes stably bind the methylated histones of a heterochromatin region and induce co-transcriptional degradation of any nascent mRNA transcripts, which are then used as RNA-dependent RNA polymerase substrates to replenish the complement of siRNA molecules(113). Heterochromatin formation, but possibly not maintenance, is dependent on the ribonuclease protein dicer, which is used to generate the initial complement of siRNAs(114)

1.6.1.3 Paramutation

Paramutation, in epigenetics, is an interaction between two alleles of a single locus, resulting in a heritable change of one allele that is induced by the other allele(115, 116). Paramutation violates Mendel's first law, which states that in the process of the formation of the gametes (egg or sperm) the allelic pairs separate, one going to each gamete, and that each gene remains completely uninfluenced by the other(117). In paramutation an allele in one generation heritably affects the other allele in future generations, even if the allele causing the change is itself not transmitted. What may be transmitted in such a case are RNA's such as siRNA, miRNA or other regulatory RNAs. These are packaged in egg or sperm and cause paramutation upon transmission to the next generation. This means that RNA is a molecule of inheritance, just like DNA.

The molecular basis of paramutation is being unraveled. Paramutation may share common mechanisms with other epigenetic phenomena, such as gene silencing, and imprinting. It has been proposed that paramutation is RNA-directed(118). Stability of the chromatin states associated with paramutation and transposon silencing requires the mop1 gene, which encodes an RNA-dependent RNA polymerase. This polymerase is required to maintain a threshold level of the repeat RNA, which causes the paramutation. Exactly how the RNA does this is not understood, but like other epigenetic changes, it involves a covalent modification of the DNA and/or the DNA-bound histone proteins without changing the DNA sequence of the gene itself.

1.6.1.4 Antigene reagent

Selective artificial control of gene expression is a longstanding dream in biotechnology and human therapy. Oligonucleotides seem perfectly suited for this purpose because of their unique base–base recognition properties. In the antisense approach, short oligonucleotides are designed to bind to specific sequences of an mRNA of interest via Watson–Crick duplex formation, to block gene expression on the level of translation. In the antigene approach (Fig. 1.10), oligonucleotides bind sequence selectively to genomic, double-stranded DNA and interfere with transcription and the DNA processing machinery via triple helix formation.

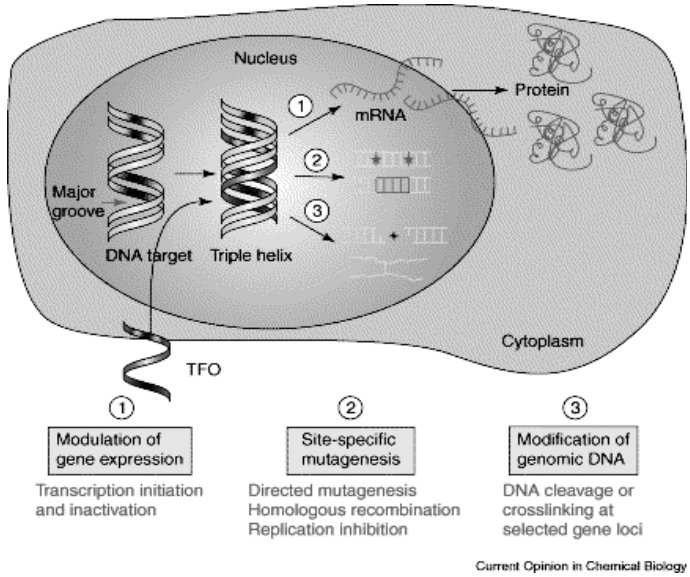

Figure 1.10. Basic antigene concepts and corresponding applications. Gene expression can be upregulated or downregulated on the level of transcription via selective triple-helix formation, preferably at promoter sites. Selective modification of the genome includes site-specific mutagenesis via triplex delivered mutagens (e.g. psoralen), or homologous recombination through triplex delivered donor DNA via DNA repair. Further applications arise from triplex-targeted chemical modification of the gene (e.g. cleavage, site-specific cross-linking or alkylation). Reproduced from *Buchini S. et al*(119)

Antigene agents that could recognize specific sequences in chromosomal DNA would have many applications, including specific inhibition or activation of gene expression, introduction or correction of mutations, and analysis of chromosome structure and function(50). There are two intrinsic advantages of the antigene over the antisense:

- There are only two target copies of DNA per diploid cell as compared with the hundreds to thousands of mRNA copies that have to be targeted in the antisense approach. This should dramatically reduce the amount of oligonucleotide needed for activity.
- Not only transcriptional activation and deactivation but also gene knockout as well as targeted mutagenesis, targeted recombination and sequence-selective manipulation of genomic DNA can be achieved.

Despite the exciting applications of chromosomal recognition, there have been few reports of potent effects inside cells. Antigene agents face special challenges relative to current approaches that target mRNA. They must enter the nucleus and locate their target sequence, actions that require them to overcome the barriers posed by chromatin structure and the double helix itself(50).

1.6.2 Post-Transcriptional Gene Silencing (PTGS)

Post-transcriptional gene silencing(120, 121) in plants, quelling in fungi, and RNA interference in animals: are responses to various types of foreign nucleic acids including viruses, transposons, transgenes and dsRNA(122). These processes represent natural defence mechanisms against viruses and transposons(123).

Small RNAs accumulate during both virus and transgene-induced gene silencing, indicating convergence of these two branches of silencing before the formation of the sequence-specific ribonuclease(124). In plants, dsRNA that triggers PTGS can be produced in the nucleus by transcription through inverted repeat (IRs) or through the action of RdRP, which is postulated to use pre-existing dsRNA or 'aberrant' sense RNAs(125, 126) as templates for the synthesis of antisense RNAs. Several reports which suggested that PTGS can be induced by dsRNA, that small RNAs are produced and that cognate DNAs become methylated suggested that TGS and PTGS are mechanistically linked(127).

The cellular machinery does not seem to discriminate between dsRNAs consisting of coding and promoter sequences and the dsRNAs is digested to 21–25 nt small-interfering RNAs (siRNAs) which play an integral role in homology-dependent RNA turnover and methylation of homologous DNA coding sequences in plants(128). When homologous mRNAs are present, the small antisense RNAs may anneal and guide degradation of these mRNAs, but prior to degradation, an RNA-dependent RNA polymerase (RdRP) may elongate some of the guide RNAs on the mRNA, after which the dsRNA part is degraded(129, 130)

1.6.3 Nucleic acid-based gene silencing

Ever since the first demonstration that antisense oligodeoxynucleotide (AS-ODN) could silence gene expression more than 25 years ago, hope was borne for developing specific targeting strategies for research and therapeutic use(131). Several tools now exist to knock-down the expression of specific genes. Virtually all these tools are comprised of nucleic acids, or nucleic acid analogs, capable of base-pairing in a Watson-Crick fashion with a target sequence on either DNA or RNA. Modification of nucleic acids confers increased resistance to nucleases, provides different affinities for either DNA or RNA and/or increases specificity for a given target. Most tools developed today target and bind to the mRNA of a specific gene resulting in post-transcriptional gene silencing (PTGS) (Table 1.5).

Tools	Mechanism
Antisense oligodeoxynucleotide (**AS-ODN**)	Induction of RNase H
Antisense RNA (**AS-RNA**)	Protein-dependent cleavage
2'-O-methyl phosphorothioate antisense oligodeoxynucleotide (**AO**)	Inhibition of splicing (exon skipping)
DNAzymes	Protein-independent catalytic cleavage
miRNA	RNAi (inhibition of translation)
PNA	Inhibition of translation
Phosphorothioate oligodeoxynucleotide (**PS-ODN**)	Induction of RNase H
Ribozyme	Protein-independent catalytic cleavage
siRNA	RNA interference (RNAi) (cleavage)
shRNA	RNAi (cleavage)

Table 1.5: Post-transcription silencing tools

There are three main pathways whereby these molecules can induce PTGS by targeting the RNA(132):

- They can catalytically cleave the target RNA in absence of accessory proteins (e.g. ribozymes)
- They induce a protein-dependent degradation of a specific RNA once bound to their target (e.g. AS-RNAs, AS-ODNs, siRNAs)
- They block the translation of the mRNA by preventing protein binding (e.g. miRNAs, AS-RNAs, AS-ODNs)

1.6.4 RNA Interference

Despite the hype surrounding RNA interference over the past 6 years, post-transcriptional gene silencing (PTGS) from dsRNA has been documented more than 15 years ago in plants and is not a new phenomena(133, 134). It was then observed by several teams that expression of AS-RNAs or RNA from homologous genes completely abolished expression of genes with complementary sequences in plants, this

was then termed co-suppression(135). It was only in 1998 that this phenomena re-surfaced with a brand new name, RNA interference, in a publication by Fire and colleagues(109). In this work, they showed that a few molecules of dsRNA could abolish the expression of a complementary gene in *C. elegans* .

1.6.4.1 The siRNA pathway

Follow-up work on dsRNA-induced gene silencing in *Drosophila* embryo extracts revealed that long dsRNAs were processed into short 21-25 nt RNA duplexes with 2-nt 3'end overhangs and 5'-phosphate and 3' hydroxyl groups, termed short interfering RNAs (siRNAs) (Fig. 1.11A)(136, 137). While long dsRNAs are processed efficiently into siRNAs by various organisms, they, however, induce a non-specific interferon response in mammalian cells(138). The structure and characteristics of processed siRNAs are reminiscent of RNAse-III activity. It was later found that an evolutionary conserved RNAse-III-like enzyme named Dicer was responsible for long dsRNA processing that is required for siRNA activity (Fig. 1.11B)(139, 140)

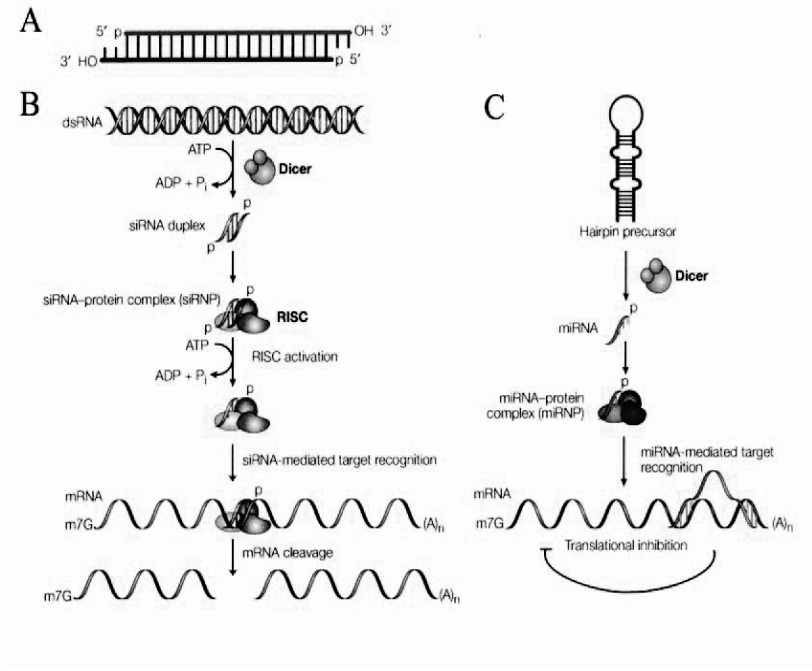

Figure 1.11 : RNAi post-transcriptional gene silencing mechanisms. Reproduced from *Dykxhoorn et al., 2003(141)*

The way RNAi works is that long dsRNAs or small hairpin RNAs (shRNAs) of 19-25 nt in length are recognised by Dicer in the cytoplasm. Although some evidence points to that there may be a nuclear component to RNAi that regulates chromatin remodelling(142), reports have generally shown that both Dicer and RNA-induced silencing compex (RISC) activity are restricted to the cytoplasm(143, 144). Dicer will then cleave in an ATP-dependent manner the long dsRNAs in precisely sized fragments(141). If shRNAs are processed by Dicer, their stem loop will be cleaved to

reveal the characteristic 2-nt-3' overhang(145). The resulting siRNAs bound to Dicer will then pass through an siRNA-protein complex (siRNP) comprised of a helicase that will unwind and separate each homologous strand and direct them to RISC, the catalytic complex responsible for target RNA cleavage(82). Although Dicer associates with RISC, it does not participate in catalytic activity for target mRNA cleavage. Each RISC contains only one of the two strands of the siRNA, but both strands of the duplex may successfully induce RNAi if an appropriate target is available(146). There is, however, recent evidence indicating that preferentially the strand, which is less tightly paired to its complement at the 5' end, is incorporated into RISC, while the other strand is degraded(82). Interestingly, while artificial approaches for espressing or introducing siRNA in cells work well to induce RNAi, no naturally occurring siRNAs have yet been found in mammals(141).

1.6.4.2 The miRNA pathway

Like siRNAs, micro RNAs (miRNA), or small temporal RNAs (stRNAs) named after RNAs that are temporally regulated, are capable of PTGS through translational repression(147-149). MiRNAs are produced from Dicer-processing of shRNAs having imperfect sequence homology to the opposite strand of the duplex (Fig. 1.11C). The miRNA pathway shares many components of the siRNA pathway but differs in two main aspects(150). For one, only the Dicer-processed antisense strand of the miRNA accumulates in the cell and is incorporated into the miRNA-protein complex (miRNP)(151, 152). Secondly, PTGS is achieved by miRNAs through binding with partial sequence complementarity to their target RNA. It is this detail that can skew the response between target cleavage or translational repression(150). Unlike siRNAs, miRNAs have been found in worms, flies and humans(149) Most extensively studied are lin-4 (lineage-abnormal-4) and let-7 (lethal-7) RNAs that control timing and sequence of postembryotic development in *C. elegans*(153). They act as protein expression regulators by modulating transcription of their homologous mRNAs at precise moments in development(154). Their discovery has opened our eyes to a world of tiny non-coding RNAs that act as molecular switches to fine-tune gene expression(155).

1.6.4.3 RNAi design and expression systems (shRNA)

SiRNAs are expensive to synthesize, are rapidly degraded once transfected into cells, their silencing effects are transient in mammalian cells and not all siRNAs have the same cleavage efficiency(156). This is why expression systems have been developed to stably express siRNAs or shRNAs and several factors need to be taken into account for proper siRNA or shRNA design(157-159). The 2 main promoter systems utilised today are: U6 and H1 promoters, both of which rely on Pol III for transcription and have given equally satisfactory results for producing siRNAs and shRNAs(141, 160). After deciding on a promoter system, one must next choose between producing a shRNA from a single promoter (Fig. 1.12A) or producing siRNAs that associate in *trans* from transcription products of tandem promoters (Fig.

1.12B). While shRNAs expressed from Pol III promoters have the disadvantage of requiring processing into siRNAs by Dicer, certain individuals have reported that fewer molecules of shRNAs than siRNAs are required to achieve RNAi(145, 148, 160). This observation suggests that Dicer binding may facilitate entry of the processed shRNA into RISC(141, 148).

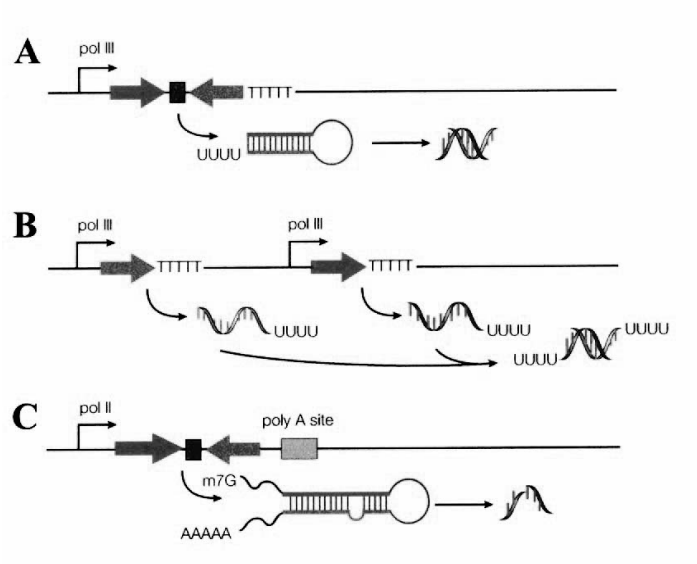

Figure 1.12 : RNAi expression systems. Reproduced from *Dykxhoorn et al., 2003*(141)

For other studies, it may be interesting to silence gene expression at the protein level. To this effect, one may consider using miRNAs that can be produced from inducible Pol II promoters expressing shRNAs with imperfect hairpin structures(161). An important consideration is that Pol II promoters could not form functional siRNAs, but work well as miRNAs (Fig. 1.12C). The main drawback to using such a system is that these lengthier shRNAs (> 30 bp) may trigger an interferon response in mammalian cells and result in the non-specific degradation of RNA(162, 163). Another important part of designing a proper system for silencing specific genes through RNAi is the identification of accessible target sites. As for AS-RNAs and ribozymes, there is a correlation between RNase-H accessible target sites and RNAi efficiency(164). Therefore initial screening of the target RNA for good cleavage sites using AS-ODNs as described in the previous sections may help save a considerable amount of time.

1.7 CRE-LOXP RECOMBINATION

The Cre/loxP system is used as a genetic tool to control site specific recombination events in genomic DNA. This system has allowed researchers to manipulate a variety of genetically modified organisms to control gene expression, delete undesired DNA sequences and modify chromosome architecture.

The system is based on the Cre protein, a site-specific DNA recombinase. Cre can catalyse the recombination of DNA between specific sites in a DNA molecule. These sites, known as loxP sequences, contain specific binding sites for Cre that surround a directional core sequence where recombination can occur.

When cells that have loxP sites in their genome express Cre, a reciprocal recombination event will occur between the loxP sites(165). The double stranded DNA is cut at both loxP sites by the Cre protein and then ligated back together. It is a quick and efficient process. The efficiency of recombination depends on the orientation of the loxP sites. For two lox sites on the same chromosome arm, inverted loxP sites will cause an inversion, while a direct repeat of loxP sites will cause a deletion event. If loxP sites are on different chromosomes it is possible for translocation events to be catalysed by Cre induced recombination.

1.7.1 Cre recombinase

The Cre (Cyclization Recombination) protein consists of 4 subunits and two domains : The larger carboxyl C-terminal domain, and smaller amino N-terminal domain. The total protein has 341 amino acids. The C domain is similar in structure to the domain in the Integrase family of enzymes isolated from Bacteriophage λ. This is also the catalytic site of the enzyme.

1.7.2 Lox P site

Lox P is a site on the Bacteriophage P1 consisting of 34 bp. There exists an asymmetric 8 bp sequence in between with two sets of palindromic, 13 bp sequences flanking it(166, 167). The detailed structure is given below.

13bp **8bp** **13bp**

ATAACTTCGTATA - GCATACAT -TATACGAAGTTAT

1.7.3 The Cre-lox system in action

In initial studies in the Bacteriophage P1 system, it was noticed that the gene to be excised was flanked by two loxP DNA sequences or sites. The presence of the Cre enzyme resulted in the Phage P1 chromosome assuming a structure that allowed the two loxP sites to come in such close contact that the site-specific recombination mechanism was capable of taking place. This site-specific recombination resulted in the excision of the flanked gene or DNA sequence from the P1 chromosome into a circular structure. The Cre-mediated recombination can be achieved in various kinds of eukaryotic cells, such as yeast(168), plant(169) and mammalian cells(170). Furthermore, Cre recombinase can also be stably expressed in transgenic mice(171, 172) suggesting that it is not toxic like many other recombinases. (Fig. 1.13)

.

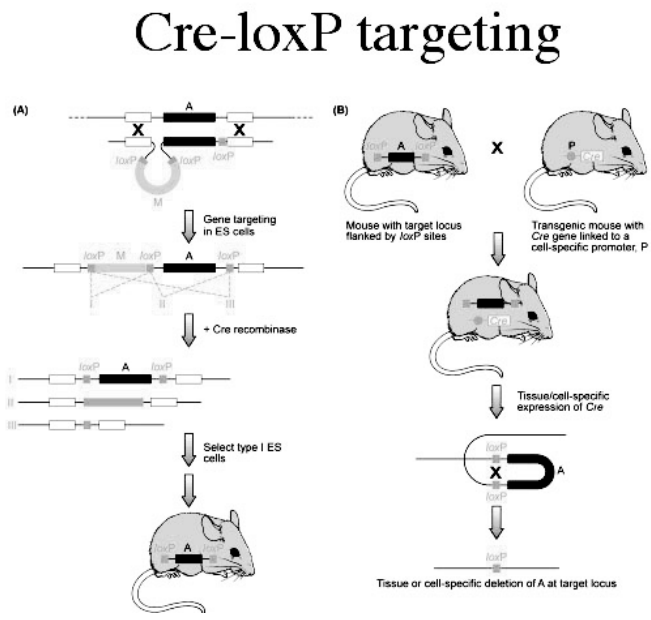

Figure 1.13 : The Cre-lox system in action (www.geocities.com)

2 AIMS OF THE PRESENT STUDY

The aims of the study were to develop methods for non-viral gene delivery and especially for the Bioplex technology. Although significant progress has been made in the basic science and applications of various non-viral gene delivery systems, the majority of non-viral approaches are still much less efficient than viral vectors, especially for in vivo gene delivery. Therefore, non-viral gene delivery must be further developed in order to increase the efficiency of *in vivo* delivery. Moreover, a second aim was to develop novel, anti-gene reagents for sequence-specific gene expression.

The specific findings were:

- We found that cooperative effects of PNA were at a distance of up to three bases and binding kinetics increased when two PNAs binding to overlapping sites on opposite DNA strands were used.
- We generated a novel sequence-specific anti-gene molecule "Zorro LNA", which bound specifically to DNA duplexes and induced potent inhibition of gene transcription, also in a cellular context. This offers a novel type of anti-gene drug, which is easy to synthesize.
- We found that the Zorro LNA construct efficiently inhibited pol III-dependent transcription in a cellular context, including *in vivo,* in a mouse model. Thus, this new form of gene silencer could potentially serve as a versatile regulator of pol III-dependent transcription, including various forms of shRNAs.
- Cre-loxP-mediated recombination leads to re-expression of silenced genes, an inhibition induced by Zorro LNA in a mouse model

3 METHODS

3.1 QUANTIFICATION OF BOUND PNA USING THE THIAZOLE ORANGE DERIVATIVE

The fluorescence signal from DNA-bound TO-labeled probes differs depending on both the ionic strength and the conformation of the DNA (single-stranded (ss) or double-stranded (ds)). Consequently, the standard curve used must contain PNA bound to ds DNA. To create this, the TO-labeled PNA was hybridized to a 45 bp long ds DNA fragment containing one BS. The two DNA strands were mismatched over the PNA BS. Using a five-fold molar excess of DNA over PNA and preventing competition by the complementary DNA strand, it was assumed that 100% binding of added PNA had been achieved. The gels were then analyzed in the Fluoro-S gel documentation system using the fluorescein filter and the Quantity One software (Bio-Rad). Quantification was done using the band analysis method with a rolling disc lane background subtraction.

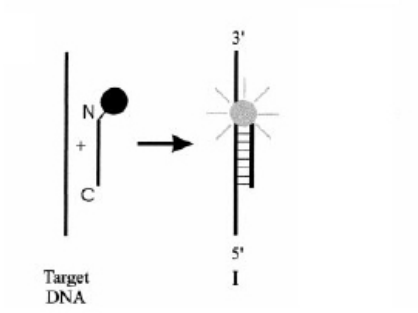

Figure 3.1: The "light-up" labeled-PNA probe

3.2 PNA BINDING ASSAY

PNA binding to the target duplex was measured by using a gel mobility shift assay. Oligonucleotides containing the PNA binding sites were synthesized. The duplex DNA was prepared by mixing both complementary oligonucleotides at a ratio of 1:1 in TE buffer (10 mM Tris/1 mM EDTA, pH 8.0) and incubating the solution at 95°C for 5 minutes and then cooling it down to room temperature slowly. A fixed concentration of duplex DNA containing the PNA binding site (1 μM) was incubated with increasing concentration of the PNA in 20 μl a solution containing 20 mM sodium phosphate at 37°C overnight, respectively. The reactions were analyzed by electrophoresis in a 15% native polyacrylamide gel.

3.3 LNA BINDING ASSAY

Cy3/Cy5 labeled LNAs binding to the target sites of plasmid were detected using Molecular Imager FX equipment (Bio-Rad, Hercules, CA). 1 μg of plasmid DNA was mixed with 10-fold molar excess of LNA oligomers in 4 μl phosphate buffer (20 mM, pH 6.8) with the final LNA concentration of 1 μM and incubated at 37°C overnight, respectively. Physiological condition of hybridization: 150 mM phosphate buffer (pH 7.4). This reaction was analyzed by electrophoresis in Novex® 4-20% polyacrylamide TBE gels (Invitrogen).

3.4 USE OF DESTABILIZED GREEN FLUORESCENT PROTEIN (D2EGFP)

The green fluorescent protein (GFP) is a widely used reporter in gene expression and protein localization studies. GFP is a stable protein; this property allows its accumulation and easy detection in cells. However, this stability also limits its application in studies that require rapid reporter turnover. Destabilized GFP was created by fusing amino acids 422-461 of the degradation domain of mouse ornithine decarboxylase (MODC) to the C-terminal end of an enhanced variant of GFP (EGFP). The fusion protein, unlike EGFP, was unstable in the presence of cycloheximide and had a fluorescence half-life of 2 h. Western blot analysis indicated that the fluorescence decay of EGFP-MODC-(422-461) correlated with degradation of the fusion protein. Therefore, destabilized EGFP can be used to directly correlate gene induction with biochemical change(173).

3.5 MICROINJECTION AND IMMUNOFLUORESCENCE

The plasmid (2 μg) was incubated with, or without, 10-fold molar excess of LNA (2.5 μM) in 20 mM phosphate buffer (pH 6.8) at 37 °C overnight. The LNA-bound plasmid and mock-treated plasmid were adjusted to a concentration of 0.1 μg/μl. NIH-3T3 cells were grown to 50% confluence on 22 mm cover-slips and were co-injected with 0.25 μg/μl pDsRed2-N1 (Invitrogen) and 0.1 μg/μl LNA-bound plasmid or mock-treated plasmid, respectively, into the nucleus using an Eppendorf 5246 Microinjector. Sixteen hours after microinjection, cells were visualized by a Carl Zeiss fluorescence microscope. For microinjection of LNA into NIH-3T3 cells stably carrying the plasmid with binding sites, cells were plated on the coverslips for 16 hours. One μg/μl of LNA oligomers was mixed with DsRed2-N1, respectively and microinjected into the nucleus. Sixteen hours after microinjection, cells were analyzed for fluorescence.

3.6 HYDRODYNAMIC TRANSFECTION

Hydrodynamic transfections of plasmids in PBS were carried out as described (17, 18). Briefly, 1.8 mL of DPBS (with no $MgCl_2$ or $CaCl_2$) were mixed with 1 μg of pEGFPluc plasmid, 1 μg of pEGFPluc plasmid and 4 μg of shRNA plasmids alone or prehybrdized with LNA, respectively. This mixture was introduced by tail vein injection over a period of 5 sec to inbred 25- to 30-g female NMRI mice. Live, anesthetized mice were imaged for 10 sec to 5 min using an intensified CCD camera (IVIS Imaging System, Xenogen) at day 1, day 3 and day 6 after injection, respectively. This image is comprised of a pseudocolor image representing intensity of emitted light (red most intense and blue least intense) superimposed on a grayscale reference image (for orientation). All animal experiments were approved by the local ethical committee at Karolinska Institutet.

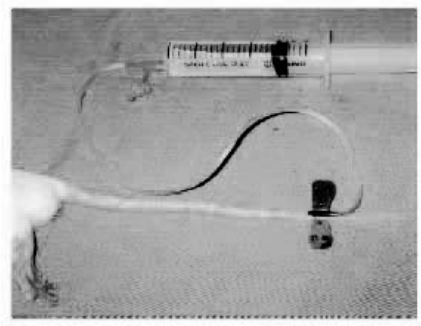

Figure 3.2: "Hydrodynamics-Based Transfection" uses hydrostatic pressure induced by a rapid intravenous injection of a large volume of DNA solution into an animal to accomplish gene transfer into cells. Reproduced from *Maruyama H et al*(174)

3.7 INTRAMUSCULAR INJECTION

50 µl of physiological saline solution were mixed with 5 µg of pEGFPluc plasmid, 5 µg of pEGFPluc plasmid and 20 µg of shRNA plasmid alone or prehybridized with LNA, respectively.This mixture was injected into the tibialis anterior muscle (*sin et dex*). Injection pressure and time were constant between the injections and dorsal flexation of the talocrural joint was observed as sign for correct injection. Live, anesthetized mice were imaged for 1 min using an intensified CCD camera (IVIS Imaging System, Xenogen, MA) at 24h post injection. All animal experiments were approved by the local ethical committee at Karolinska Institutet.

4 RESULTS

4.1 PAPER I

Cooperative strand invasion of supercoiled plasmid DNA by mixed linear PNA and PNA-peptide chimeras

PNA is a DNA analog with broad biotechnological applications, and possibly also treatment applications. Its suggested uses include that of a specific anchor sequence for tethering biologically active peptides to plasmids in a sequence-specific manner. Such complexes, referred to as Bioplex, have already been used to enhance non-viral gene transfer in vitro. To investigate how hybridization of PNAs to supercoiled plasmids would be affected by the binding of multiple PNA-peptides to the same strand of DNA, we have developed a method of quantifying the specific binding of PNA using a PNA labeled with a derivative of the fluorophore thiazole orange (TO). Cooperative effects were found at a distance of up to three bases. With a peptide present at the end of one of the PNAs, steric hindrance occurred, reducing the increase in binding rate when the distance between the two sites was less than two bases. In addition, we found increased binding kinetics when two PNAs binding to overlapping sites on opposite DNA strands were used, without the use of chemically modified bases in the PNAs.

4.2 PAPER II

Zorro Locked Nucleic Acid (LNA) induces sequence-specific gene silencing

LNA is a synthetic analog of nucleic acids, which contains a bridging methylene carbon between the 2' and 4' positions of the ribose ring. In this study, we generated a novel sequence-specific anti-gene molecule "Zorro LNA", (Fig. 4.1) in which a 14-mer LNA binds to the coding strand, while a 16-mer connected LNA binds to the template strand. We have verified that the "Zorro LNA" induced effective and specific binding into DNA duplexes and potent inhibition of gene transcription, also in a cellular context. By comparing the Zorro LNA with linear LNA, as well as an optimized bisPNA oligonucleotide directed against the same target sites, respectively, we found that the Zorro LNA construct was unique in its ability to arrest gene transcription in mammalian cells. To our knowledge, this is the first time that in mammalian cells, gene transcription was blocked by a nucleic acid analog in a sequence-specific way using low, but saturated binding of a blocking agent. This offers a novel type of anti-gene drug, which is easy to synthesize.

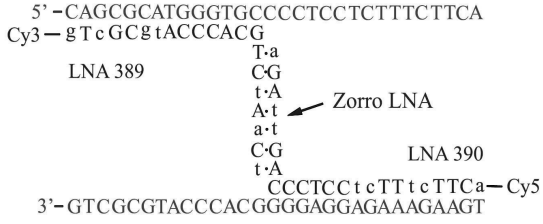

Figure 4.1: Schematic presentation of Zorro LNA.

4.3 PAPER III

Sequence-specific inhibition of RNA polymerase III-dependent transcription using Zorro Locked Nucleic Acid (LNA)

RNA polymerase III (pol III) -dependent transcripts are involved in many fundamental activities in a cell, such as splicing and protein synthesis. They also regulate cell growth and influence tumor formation. During recent years vector-based systems for expression of short hairpin (sh) RNA under the control of a pol III promoter have been developed for molecular therapy. Therefore, there is an increasing interest in means to regulate pol III-dependent transcription. Recently, we have developed a novel anti-gene molecule "Zorro LNA", which strand invades into super-coiled DNA, simultaneously hybridizes to both strands and potently inhibits RNA polymerase II derived transcription. We have now applied Zorro LNA in an attempt to also control U6 promoter-driven expression of shRNA. We found that the Zorro LNA construct efficiently inhibited pol III-dependent transcription in a cellular context, including *in vivo,* in a mouse model (Fig. 4.2). Thus, this new form of gene silencer could potentially serve as a versatile regulator of pol III-dependent transcription, including various forms of shRNAs.

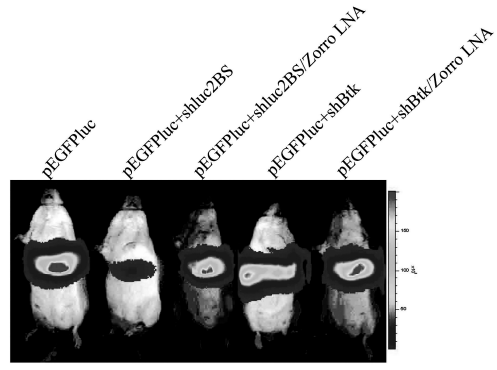

Figure 4.2: Effect of Zorro LNA on pol III-dependent transcription in mice using hydrodynamic transfection.

4.4 PAPER IV

Cre-loxP-mediated recombination leads to re-expression of silenced gene induced by Zorro LNA in a mouse model

Zorro LNA is a new, small molecule that targets both DNA strands simultaneously and induces efficient gene silencing. It has the potential of becoming a new drug for the treatment of human genetic disease. In order to further evaluate the *in vivo* effect of Zorro LNA and trace the long-term effect of Zorro LNA in inhibiting gene expression, we inserted two Zorro LNA binding sites flanked by loxP sites within the transcribed region of a reporter gene. As previously observed, after Zorro LNA was hybridized to the plasmid, it significantly induced gene silencing in the mammalian cells. Both in transfected cells and in mouse model (Fig. 4.3) the silencing effect was lost when Cre-

expressing plasmids were transfected. This suggests that binding of Zorro LNA is stable over a period of days.

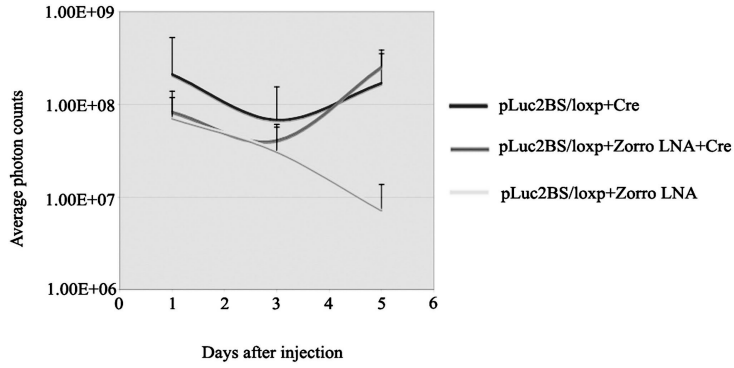

Figure 4.3: Hydrodynamic infusion of Zorro LNA-bound pLuc2BS/loxp followed by a sequencial injection of Cre plasmid

5 CONCLUSION

In this work, we tried to develop non-viral gene delivery and a novel anti-gene reagent. The following conclusions are based on our findings.

In order to develop a technology for linking functional entities to non-viral vectors, the Bioplex technology, we investigated how hybridization of PNAs to supercoiled plasmids would be affected by the binding of multiple PNA-peptides to DNA strands. Cooperative effects were found at a distance of up to three bases. With a peptide present at the end of one of the PNAs, steric hindrance occurred, reducing the increase in binding rate when the distance between the two sites was less than two bases. In addition, we found increased binding kinetics when two PNAs binding to overlapping sites on opposite DNA strands were used, without the use of further chemically modified bases in the PNAs.

We generated a novel sequence-specific anti-gene molecule "Zorro LNA", in which a 14-mer LNA binds to the coding strand, while a 16-mer connected LNA binds to the template strand. Our data suggested that the "Zorro LNA" induced effective and specific binding into DNA duplexes and potent inhibition of pol II-derived gene transcription, also in mammalian cells. We also found that the Zorro LNA construct efficiently inhibited pol III-dependent transcription in a cellular context, including *in vivo* in a mouse model. Finally, a Cre-loxP-mediated recombination model showed re-expression of the silenced gene, induced by Zorro LNA, in a mouse model and suggested that binding of Zorro LNA is stable over a period of days.

6 ACKNOWLEDGEMENTS

I would like to express my gratitude to everyone who has made the thesis possible:

A special thanks to Professor C.I.Edvard Smith, for giving me a chance to work in his lab, for your generosity, support, encouragement discussion and for providing me with excellent research facility.

Associate professor, Abdalla J. Mohamed, and Dr. Karin E. Lundin, my supervisors, for your great supervision, kindness and support and for a nice collaboration.

Associate professor, Beston Nore and a close associate colleague and mentor, for your sincere passionate support and sharing your outstanding scientific knowledge.

All former and present members in Edvard Smith's lab: Mathias Svahn, Oscar Simonson, Pedro Moreno, Maroof Hasan, Iulian Oprea, Anna Berglof, Emelie Bloomberg, Jose Arteaga, Leonardo Vargas, Liang Yu, Joana Viola, Juhana Heinonen, Nawaz Hossain Lars Branden, and Jessica Lindvall for scientific support and a nice working atmosphere.

All my friends outside the lab: Zongwei Wang, Hairong Song, Feng Wang, Zaimei Peng. We have shared a lot of fun during the years. I also appreciate your sincere help and encouragement.

My beloved family, Mama, Baba for your love and support during the years and for always being there no matter what.

Finally, Dr. Aria Olumi, my present supervisor for accepting me as a research fellow in Harvard Medical School and proving me with time and material to finish my Ph.D thesis.

7 REFERENCES

1. **Erbacher, P., Remy, J. S., and Behr, J. P. (1999) Gene transfer with synthetic virus-like particles via the integrin-mediated endocytosis pathway. *Gene Ther* 6, 138-145**
2. **Xu, Y., and Szoka, F. C., Jr. (1996) Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry* 35, 5616-5623**
3. **Zelphati, O., and Szoka, F. C., Jr. (1996) Mechanism of oligonucleotide release from cationic liposomes. *Proc Natl Acad Sci U S A* 93, 11493-11498**
4. **Gao, X., Kim, K. S., and Liu, D. (2007) Nonviral gene delivery: what we know and what is next. *Aaps J* 9, E92-104**
5. **Kamiya, H., Tsuchiya, H., Yamazaki, J., and Harashima, H. (2001) Intracellular trafficking and transgene expression of viral and non-viral gene vectors. *Adv Drug Deliv Rev* 52, 153-164**
6. **Kaplitt, M. G., and Pfaff, D. W. (1996) Viral Vectors for Gene Delivery and Expression in the CNS. *Methods* 10, 343-350**
7. **Ghosh, S. S., Gopinath, P., and Ramesh, A. (2006) Adenoviral vectors: a promising tool for gene therapy. *Appl Biochem Biotechnol* 133, 9-29**
8. **Walther, W., and Stein, U. (2000) Viral vectors for gene transfer: a review of their use in the treatment of human diseases. *Drugs* 60, 249-271**
9. **Kay, M. A., Glorioso, J. C., and Naldini, L. (2001) Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. *Nat Med* 7, 33-40**
10. **Favre, D., Provost, N., Blouin, V., Blancho, G., Cherel, Y., Salvetti, A., and Moullier, P. (2001) Immediate and long-term safety of recombinant adeno-associated virus injection into the nonhuman primate muscle. *Mol Ther* 4, 559-566**
11. **Timme, T. L., Hall, S. J., Barrios, R., Woo, S. L., Aguilar-Cordova, E., and Thompson, T. C. (1998) Local inflammatory response and vector spread after direct intraprostatic injection of a recombinant adenovirus containing the herpes simplex virus thymidine kinase gene and ganciclovir therapy in mice. *Cancer Gene Ther* 5, 74-82**
12. **Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A., and Felgner, P. L. (1990) Direct gene transfer into mouse muscle in vivo. *Science* 247, 1465-1468**
13. **Heller, L. C., Ugen, K., and Heller, R. (2005) Electroporation for targeted gene transfer. *Expert Opin Drug Deliv* 2, 255-268**
14. **Neumann, E., Schaefer-Ridder, M., Wang, Y., and Hofschneider, P. H. (1982) Gene transfer into mouse lyoma cells by electroporation in high electric fields. *EMBO J* 1, 841-845**
15. **Yang, N. S., Burkholder, J., Roberts, B., Martinell, B., and McCabe, D. (1990) In vivo and in vitro gene transfer to mammalian somatic cells by particle bombardment. *Proc Natl Acad Sci U S A* 87, 9568-9572**
16. **Yang, N. S., and Sun, W. H. (1995) Gene gun and other non-viral approaches for cancer gene therapy. *Nat Med* 1, 481-483**

17. **Liu, F., Song, Y., and Liu, D. (1999) Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther* 6, 1258-1266**
18. **Zhang, G., Budker, V., and Wolff, J. A. (1999) High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum Gene Ther* 10, 1735-1737**
19. **Neu, M., Fischer, D., and Kissel, T. (2005) Recent advances in rational gene transfer vector design based on poly(ethylene imine) and its derivatives. *J Gene Med* 7, 992-1009**
20. **Liu, D., Ren, T., and Gao, X. (2003) Cationic transfection lipids. *Curr Med Chem* 10, 1307-1315**
21. **Branden, L. J., Mohamed, A. J., and Smith, C. I. (1999) A peptide nucleic acid-nuclear localization signal fusion that mediates nuclear transport of DNA. *Nat Biotechnol* 17, 784-787**
22. **Branden, L. J., and Smith, C. I. (2002) Bioplex technology: novel synthetic gene delivery system based on peptides anchored to nucleic acids. *Methods Enzymol* 346, 106-124**
23. **Svahn, M. G., Lundin, K. E., Ge, R., Tornquist, E., Simonson, E. O., Oscarsson, S., Leijon, M., Branden, L. J., and Smith, C. I. (2004) Adding functional entities to plasmids. *J Gene Med* 6 Suppl 1, S36-44**
24. **Besch, R., Giovannangeli, C., and Degitz, K. (2004) Triplex-forming oligonucleotides - sequence-specific DNA ligands as tools for gene inhibition and for modulation of DNA-associated functions. *Curr Drug Targets* 5, 691-703**
25. **Faria, M., Wood, C. D., Perrouault, L., Nelson, J. S., Winter, A., White, M. R., Helene, C., and Giovannangeli, C. (2000) Targeted inhibition of transcription elongation in cells mediated by triplex-forming oligonucleotides. *Proc Natl Acad Sci U S A* 97, 3862-3867**
26. **Faria, M., Wood, C. D., White, M. R., Helene, C., and Giovannangeli, C. (2001) Transcription inhibition induced by modified triple helix-forming oligonucleotides: a quantitative assay for evaluation in cells. *J Mol Biol* 306, 15-24**
27. **Brunet, E., Alberti, P., Perrouault, L., Babu, R., Wengel, J., and Giovannangeli, C. (2005) Exploring cellular activity of locked nucleic acid-modified triplex-forming oligonucleotides and defining its molecular basis. *J Biol Chem* 280, 20076-20085**
28. **Vasquez, K. M., Christensen, J., Li, L., Finch, R. A., and Glazer, P. M. (2002) Human XPA and RPA DNA repair proteins participate in specific recognition of triplex-induced helical distortions. *Proc Natl Acad Sci U S A* 99, 5848-5853**
29. **Dervan, P. B., and Edelson, B. S. (2003) Recognition of the DNA minor groove by pyrrole-imidizole polyamides. *Curr. Opin. Struct. Biol.* 13, 284**
30. **Nielsen, P. E., Egholm, M., Berg, R. H., and Buchardt, O. (1991) Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science* 254, 1497-1500**
31. **Egholm, M., Buchardt, O., Christensen, L., Behrens, C., Freier, S. M., Driver, D. A., Berg, R. H., Kim, S. K., Norden, B., and Nielsen, P. E. (1993)**

PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. ***Nature*** **365, 566-568**

32. **Hanvey, J. C., Peffer, N. J., Bisi, J. E., Thomson, S. A., Cadilla, R., Josey, J. A., Ricca, D. J., Hassman, C. F., Bonham, M. A., Au, K. G., and et al. (1992) Antisense and antigene properties of peptide nucleic acids.** ***Science*** **258, 1481-1485**
33. **Peffer, N. J., Hanvey, J. C., Bisi, J. E., Thomson, S. A., Hassman, C. F., Noble, S. A., and Babiss, L. E. (1993) Strand-invasion of duplex DNA by peptide nucleic acid oligomers.** ***Proc Natl Acad Sci U S A*** **90, 10648-10652**
34. **Demidov, V. V., Yavnilovich, M. V., Belotserkovskii, B. P., Frank-Kamenetskii, M. D., and Nielsen, P. E. (1995) Kinetics and mechanism of polyamide ("peptide") nucleic acid binding to duplex DNA.** ***Proc Natl Acad Sci U S A*** **92, 2637-2641**
35. **Cherny, D. Y., Belotserkovskii, B. P., Frank-Kamenetskii, M. D., Egholm, M., Buchardt, O., Berg, R. H., and Nielsen, P. E. (1993) DNA unwinding upon strand-displacement binding of a thymine-substituted polyamide to double-stranded DNA.** ***Proc Natl Acad Sci U S A*** **90, 1667-1670**
36. **Kurakin, A., Larsen, H. J., and Nielsen, P. E. (1998) Cooperative strand displacement by peptide nucleic acid (PNA).** ***Chem Biol*** **5, 81-89**
37. **Demidov, V. V., Potaman, V. N., Frank-Kamenetskii, M. D., Egholm, M., Buchard, O., Sonnichsen, S. H., and Nielsen, P. E. (1994) Stability of peptide nucleic acids in human serum and cellular extracts.** ***Biochem Pharmacol*** **48, 1310-1313**
38. **Bentin, T., Larsen, H. J., and Nielsen, P. E. (2003) Combined triplex/duplex invasion of double-stranded DNA by "tail-clamp" peptide nucleic acid.** ***Biochemistry*** **42, 13987-13995**
39. **Kaihatsu, K., Shah, R. H., Zhao, X., and Corey, D. R. (2003) Extending recognition by peptide nucleic acids (PNAs): binding to duplex DNA and inhibition of transcription by tail-clamp PNA-peptide conjugates.** ***Biochemistry*** **42, 13996-14003**
40. **Nielsen, P. E., Egholm, M., and Buchardt, O. (1994) Sequence-specific transcription arrest by peptide nucleic acid bound to the DNA template strand.** ***Gene*** **149, 139-145**
41. **Mologni, L., Nielsen, P. E., and Gambacorti-Passerini, C. (1999) In vitro transcriptional and translational block of the bcl-2 gene operated by peptide nucleic acid.** ***Biochem Biophys Res Commun*** **264, 537-543**
42. **Boffa, L. C., Scarfi, S., Mariani, M. R., Damonte, G., Allfrey, V. G., Benatti, U., and Morris, P. L. (2000) Dihydrotestosterone as a selective cellular/nuclear localization vector for anti-gene peptide nucleic acid in prostatic carcinoma cells.** ***Cancer Res*** **60, 2258-2262**
43. **Cutrona, G., Carpaneto, E. M., Ulivi, M., Roncella, S., Landt, O., Ferrarini, M., and Boffa, L. C. (2000) Effects in live cells of a c-myc anti-gene PNA linked to a nuclear localization signal.** ***Nat Biotechnol*** **18, 300-303**
44. **Boffa, L. C., Morris, P. L., Carpaneto, E. M., Louissaint, M., and Allfrey, V. G. (1996) Invasion of the CAG triplet repeats by a complementary peptide nucleic acid inhibits transcription of the androgen receptor and TATA-binding protein genes and correlates with refolding of an active**

nucleosome containing a unique AR gene sequence. *J Biol Chem* 271, 13228-13233

45. **Nielsen, P. E. (2004) PNA Technology. *Mol Biotechnol* 26, 233-248**
46. **Mollegaard, N. E., Buchardt, O., Egholm, M., and Nielsen, P. E. (1994) Peptide nucleic acid.DNA strand displacement loops as artificial transcription promoters. *Proc Natl Acad Sci U S A* 91, 3892-3895**
47. **Wang, G., Xu, X., Pace, B., Dean, D. A., Glazer, P. M., Chan, P., Goodman, S. R., and Shokolenko, I. (1999) Peptide nucleic acid (PNA) binding-mediated induction of human gamma-globin gene expression. *Nucleic acids research* 27, 2806-2813**
48. **Faruqi, A. F., Egholm, M., and Glazer, P. M. (1998) Peptide nucleic acid-targeted mutagenesis of a chromosomal gene in mouse cells. *Proc Natl Acad Sci U S A* 95, 1398-1403**
49. **Janowski, B. A., Huffman, K. E., Schwartz, J. C., Ram, R., Hardy, D., Shames, D. S., Minna, J. D., and Corey, D. R. (2005) Inhibiting gene expression at transcription start sites in chromosomal DNA with antigene RNAs. *Nature chemical biology* 1, 216-222**
50. **Kaihatsu, K., Janowski, B. A., and Corey, D. R. (2004) Recognition of Chromosomal DNA by PNAs. *Chemistry & Biology* 11, 749-758**
51. **Herbert, B., Pitts, A. E., Baker, S. I., Hamilton, S. E., Wright, W. E., Shay, J. W., and Corey, D. R. (1999) Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death. *Proc Natl Acad Sci U S A* 96, 14276-14281**
52. **Doyle, D. F., Braasch, D. A., Simmons, C. G., Janowski, B. A., and Corey, D. R. (2001) Inhibition of gene expression inside cells by peptide nucleic acids: effect of mRNA target sequence, mismatched bases, and PNA length. *Biochemistry* 40, 53-64**
53. **Liu, Y., Braasch, D. A., Nulf, C. J., and Corey, D. R. (2004) Isoform-specific inhibition of cellular gene expression by peptide nucleic acid. *Biochemistry* 43, 1921**
54. **Simmons, C. G., Pitts, A. E., Mayfield, L. D., Shay, J. W., and Corey, D. R. (1997) Synthesis and membrane permeability of PNA-peptide conjugates. *Bioorganic & Medicinal Chemistry Letters* 7, 3001-3006**
55. **Pooga, M., Soomets, U., Hallbrink, M., Valkna, A., Saar, K., Rezaei, K., Kahl, U., Hao, J. X., Xu, X. J., Wiesenfeld-Hallin, Z., Hokfelt, T., Bartfai, T., and Langel, U. (1998) Cell penetrating PNA constructs regulate galanin receptor levels and modify pain transmission in vivo. *Nat Biotechnol* 16, 857-861**
56. **Sazani, P., Gemignani, F., Kang, S. H., Maier, M. A., Manoharan, M., Persmark, M., Bortner, D., and Kole, R. (2002) Systemically delivered antisense oligomers upregulate gene expression in mouse tissues. *Nat Biotechnol* 20, 1228-1233**
57. **Sazani, P., Kang, S. H., Maier, M. A., Wei, C., Dillman, J., Summerton, J., Manoharan, M., and Kole, R. (2001) Nuclear antisense effects of neutral, anionic and cationic oligonucleotide analogs. *Nucleic acids research* 29, 3965-3974**
58. **Nielsen, P. E. (2005) Gene targeting using peptide nucleic acid. *Methods Mol Biol* 288, 343-358**

59. **Larsen, H. J., and Nielsen, P. E. (1996) Transcription induced binding of peptide nucleic acid (PNA) to double-stranded DNA: sequence-specific suicide transcription.** ***Nucleic Acids Res.*** **24, 458**

60. **Larsen, H. J., and Nielsen, P. E. (1996) Transcription-mediated binding of peptide nucleic acid (PNA) to double-stranded DNA: sequence-specific suicide transcription.** ***Nucleic acids research*** **24, 458-463**

61. **Freier, S. M., and Altmann, K. H. (1997) The ups and downs of nucleic acid duplex stability: structure-stability studies on chemically-modified DNA:RNA duplexes.** ***Nucleic acids research*** **25, 4429-4443**

62. **Herdewijn, P. (1999) Conformationally restricted carbohydrate-modified nucleic acids and antisense technology.** ***Biochim Biophys Acta*** **1489, 167-179**

63. **Singh, S. K., Kumar, R., and Wengel, J. (1998) Synthesis of Novel Bicyclo[2.2.1] Ribonucleosides: 2'-Amino- and 2'-Thio-LNA Monomeric Nucleosides.** ***J Org Chem*** **63, 6078-6079**

64. **Koshkin, A. A., and Wengel, J. (1998) Synthesis of Novel 2',3'-Linked Bicyclic Thymine Ribonucleosides.** ***J Org Chem*** **63, 2778-2781**

65. **Obika, S., Nanbu, D., Hari, Y., Andoh, J.-i., Morio, K.-i., Doi, T., and Imanishi, T. (1998) Stability and structural features of the duplexes containing nucleoside analogues with a fixed N-type conformation, 2'-O,4'-C-methyleneribonucleosides.** ***Tetrahedron Letters*** **39, 5401**

66. **Braasch, D. A., and Corey, D. R. (2001) Locked nucleic acid (LNA): fine-tuning the recognition of DNA and RNA.** ***Chem Biol*** **8, 1-7**

67. **Vester, B., and Wengel, J. (2004) LNA (locked nucleic acid): high-affinity targeting of complementary RNA and DNA.** ***Biochemistry*** **43, 13233-13241**

68. **Frieden, M., Hansen, H. F., and Koch, T. (2003) Nuclease stability of LNA oligonucleotides and LNA-DNA chimeras.** ***Nucleosides, nucleotides & nucleic acids*** **22, 1041-1043**

69. **Jepsen, J. S., Sorensen, M. D., and Wengel, J. (2004) Locked nucleic acid: a potent nucleic acid analog in therapeutics and biotechnology.** ***Oligonucleotides*** **14, 130-146**

70. **Braasch, D. A., Liu, Y., and Corey, D. R. (2002) Antisense inhibition of gene expression in cells by oligonucleotides incorporating locked nucleic acids: effect of mRNA target sequence and chimera design.** ***Nucleic acids research*** **30, 5160-5167**

71. **Grunweller, A., Wyszko, E., Bieber, B., Jahnel, R., Erdmann, V. A., and Kurreck, J. (2003) Comparison of different antisense strategies in mammalian cells using locked nucleic acids, 2'-O-methyl RNA, phosphorothioates and small interfering RNA.** ***Nucleic acids research*** **31, 3185-3193**

72. **Frieden, M., Christensen, S. M., Mikkelsen, N. D., Rosenbohm, C., Thrue, C. A., Westergaard, M., Hansen, H. F., Orum, H., and Koch, T. (2003) Expanding the design horizon of antisense oligonucleotides with alpha-L-LNA.** ***Nucleic acids research*** **31, 6365-6372**

73. **Wahlestedt, C., Salmi, P., Good, L., Kela, J., Johnsson, T., Hokfelt, T., Broberger, C., Porreca, F., Lai, J., Ren, K., Ossipov, M., Koshkin, A., Jakobsen, N., Skouv, J., Oerum, H., Jacobsen, M. H., and Wengel, J. (2000) Potent and nontoxic antisense oligonucleotides containing locked nucleic acids.** ***Proc Natl Acad Sci U S A*** **97, 5633-5638**

74. **Crinelli, R., Bianchi, M., Gentilini, L., Palma, L., Sorensen, M. D., Bryld, T., Babu, R. B., Arar, K., Wengel, J., and Magnani, M. (2004) Transcription factor decoy oligonucleotides modified with locked nucleic acids: an in vitro study to reconcile biostability with binding affinity. *Nucleic acids research* 32, 1874-1885**
75. **Schmidt, K. S., Borkowski, S., Kurreck, J., Stephens, A. W., Bald, R., Hecht, M., Friebe, M., Dinkelborg, L., and Erdmann, V. A. (2004) Application of locked nucleic acids to improve aptamer in vivo stability and targeting function. *Nucleic acids research* 32, 5757-5765**
76. **Vester, B., Lundberg, L. B., Sorensen, M. D., Babu, B. R., Douthwaite, S., and Wengel, J. (2002) LNAzymes: incorporation of LNA-type monomers into DNAzymes markedly increases RNA cleavage. *J Am Chem Soc* 124, 13682-13683**
77. **Hertoghs, K. M., Ellis, J. H., and Catchpole, I. R. (2003) Use of locked nucleic acid oligonucleotides to add functionality to plasmid DNA. *Nucleic Acids Res* 31, 5817-5830**
78. **Parekh-Olmedo, H., Drury, M., and Kmiec, E. B. (2002) Targeted nucleotide exchange in Saccharomyces cerevisiae directed by short oligonucleotides containing locked nucleic acids. *Chem Biol* 9, 1073-1084**
79. **Brunet, E., Corgnali, M., Perrouault, L., Roig, V., Asseline, U., Sorensen, M. D., Babu, B. R., Wengel, J., and Giovannangeli, C. (2005) Intercalator conjugates of pyrimidine locked nucleic acid-modified triplex-forming oligonucleotides: improving DNA binding properties and reaching cellular activities. *Nucleic Acids Res* 33, 4223-4234**
80. **Silahtaroglu, A. N., Tommerup, N., and Vissing, H. (2003) FISHing with locked nucleic acids (LNA): evaluation of different LNA/DNA mixmers. *Molecular and cellular probes* 17, 165-169**
81. **Jepsen, J. S., and Wengel, J. (2004) LNA-antisense rivals siRNA for gene silencing. *Current opinion in drug discovery & development* 7, 188-194**
82. **Schwarz, D. S., Hutvagner, G., Du, T., Xu, Z., Aronin, N., and Zamore, P. D. (2003) Asymmetry in the assembly of the RNAi enzyme complex. *Cell* 115, 199-208**
83. **Obika, S., Hari, Y., Sekiguchi, M., and Imanishi, T. (2000) Synthesis and triplex forming ability of conformationally locked oligonucleotides containing unnatural nucleobases: efficient recognition of a C.G interruption. *Nucleic Acids Symp Ser*, 131-132**
84. **Obika, S., Uneda, T., Sugimoto, T., Nanbu, D., Minami, T., Doi, T., and Imanishi, T. (2001) 2'-O,4'-C-Methylene bridged nucleic acid (2',4'-BNA): synthesis and triplex-forming properties. *Bioorg Med Chem* 9, 1001-1011**
85. **Crinelli, R., Bianchi, M., Gentilini, L., and Magnani, M. (2002) Design and characterization of decoy oligonucleotides containing locked nucleic acids. *Nucleic acids research* 30, 2435-2443**
86. **Swayze, E. E., Siwkowski, A. M., Wancewicz, E. V., Migawa, M. T., Wyrzykiewicz, T. K., Hung, G., Monia, B. P., and Bennett, C. F. (2007) Antisense oligonucleotides containing locked nucleic acid improve potency but cause significant hepatotoxicity in animals. *Nucleic acids research* 35, 687-700**

87. **Roberts, J., Palma, E., Sazani, P., Orum, H., Cho, M., and Kole, R. (2006) Efficient and persistent splice switching by systemically delivered LNA oligonucleotides in mice.** ***Mol Ther*** **14, 471-475**
88. **Elayadi, A. N., Braasch, D. A., and Corey, D. R. (2002) Implications of high-affinity hybridization by locked nucleic acid oligomers for inhibition of human telomerase.** ***Biochemistry*** **41, 9973-9981**
89. **Frieden, M., and Orum, H. (2006) The application of locked nucleic acids in the treatment of cancer.** ***IDrugs*** **9, 706-711**
90. **Corey, D. R. (2007) RNA learns from antisense.** ***Nature chemical biology*** **3, 8-11**
91. **Levin, A. A. (1999) A review of the issues in the pharmacokinetics and toxicology of phosphorothioate antisense oligonucleotides.** ***Biochim Biophys Acta*** **1489, 69-84**
92. **Crooke, S. T. (2000) Potential roles of antisense technology in cancer chemotherapy.** ***Oncogene*** **19, 6651-6659**
93. **Mouritzen, P., Nielsen, A. T., Pfundheller, H. M., Choleva, Y., Kongsbak, L., and Moller, S. (2003) Single nucleotide polymorphism genotyping using locked nucleic acid (LNA).** ***Expert review of molecular diagnostics*** **3, 27-38**
94. **Jacobsen, N., Bentzen, J., Meldgaard, M., Jakobsen, M. H., Fenger, M., Kauppinen, S., and Skouv, J. (2002) LNA-enhanced detection of single nucleotide polymorphisms in the apolipoprotein E.** ***Nucleic acids research*** **30, e100**
95. **Jacobsen, N., Fenger, M., Bentzen, J., Rasmussen, S. L., Jakobsen, M. H., Fenstholt, J., and Skouv, J. (2002) Genotyping of the apolipoprotein B R3500Q mutation using immobilized locked nucleic acid capture probes.** ***Clinical chemistry*** **48, 657-660**
96. **Orum, H., Jakobsen, M. H., Koch, T., Vuust, J., and Borre, M. B. (1999) Detection of the factor V Leiden mutation by direct allele-specific hybridization of PCR amplicons to photoimmobilized locked nucleic acids.** ***Clinical chemistry*** **45, 1898-1905**
97. **Buratowski, S. (2003) The CTD code.** ***Nat Struct Biol*** **10, 679-680**
98. **Grummt, I. (1999) Regulation of mammalian ribosomal gene transcription by RNA polymerase I.** ***Prog Nucleic Acid Res Mol Biol*** **62, 109-154**
99. **Lee, Y., Kim, M., Han, J., Yeom, K. H., Lee, S., Baek, S. H., and Kim, V. N. (2004) MicroRNA genes are transcribed by RNA polymerase II.** ***EMBO J*** **23, 4051-4060**
100. **Willis, I. M. (1993) RNA polymerase III. Genes, factors and transcriptional specificity.** ***Eur J Biochem*** **212, 1-11**
101. **Cooper, G. M., and Hausman, R. E. (2007)** ***The Cell: A Molecular Approach*** **Sinauer Associates, Inc.;**
102. **Workman, J. L., and Kingston, R. E. (1998) Alteration of nucleosome structure as a mechanism of transcriptional regulation.** ***Annu Rev Biochem*** **67, 545-579**
103. **Grewal, S. I., and Elgin, S. C. (2002) Heterochromatin: new possibilities for the inheritance of structure.** ***Curr Opin Genet Dev*** **12, 178-187**
104. **Li, B., Carey, M., and Workman, J. L. (2007) The Role of Chromatin during Transcription.** ***Cell*** **128, 707-719**

105. Jenuwein, T., and Allis, C. D. (2001) Translating the histone code. *Science* 293, 1074-1080

106. Jones, P. L., Veenstra, G. J., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., Strouboulis, J., and Wolffe, A. P. (1998) Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat Genet* 19, 187-191

107. Bruno, M., Flaus, A., Stockdale, C., Rencurel, C., Ferreira, H., and Owen-Hughes, T. (2003) Histone H2A/H2B dimer exchange by ATP-dependent chromatin remodeling activities. *Mol Cell* 12, 1599-1606

108. Shen, X., Mizuguchi, G., Hamiche, A., and Wu, C. (2000) A chromatin remodelling complex involved in transcription and DNA processing. *Nature* 406, 541-544

109. Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* 391, 806-811

110. DeChiara, T. M., Robertson, E. J., and Efstratiadis, A. (1991) Parental imprinting of the mouse insulin-like growth factor II gene. *Cell* 64, 849-859

111. Schramke, V., Sheedy, D. M., Denli, A. M., Bonila, C., Ekwall, K., Hannon, G. J., and Allshire, R. C. (2005) RNA-interference-directed chromatin modification coupled to RNA polymerase II transcription. *Nature* 435, 1275-1279

112. Kato, H., Goto, D. B., Martienssen, R. A., Urano, T., Furukawa, K., and Murakami, Y. (2005) RNA polymerase II is required for RNAi-dependent heterochromatin assembly. *Science* 309, 467-469

113. Sugiyama, T., Cam, H., Verdel, A., Moazed, D., and Grewal, S. I. (2005) RNA-dependent RNA polymerase is an essential component of a self-enforcing loop coupling heterochromatin assembly to siRNA production. *Proc Natl Acad Sci U S A* 102, 152-157

114. Noma, K., Sugiyama, T., Cam, H., Verdel, A., Zofall, M., Jia, S., Moazed, D., and Grewal, S. I. (2004) RITS acts in cis to promote RNA interference-mediated transcriptional and post-transcriptional silencing. *Nat Genet* 36, 1174-1180

115. Hollick, J. B., Dorweiler, J. E., and Chandler, V. L. (1997) Paramutation and related allelic interactions. *Trends Genet* 13, 302-308

116. Stam, M., and Mittelsten Scheid, O. (2005) Paramutation: an encounter leaving a lasting impression. *Trends Plant Sci* 10, 283-290

117. Rassoulzadegan, M., Grandjean, V., Gounon, P., Vincent, S., Gillot, I., and Cuzin, F. (2006) RNA-mediated non-mendelian inheritance of an epigenetic change in the mouse. *Nature* 441, 469-474

118. Alleman, M., Sidorenko, L., McGinnis, K., Seshadri, V., Dorweiler, J. E., White, J., Sikkink, K., and Chandler, V. L. (2006) An RNA-dependent RNA polymerase is required for paramutation in maize. *Nature* 442, 295-298

119. Buchini, S., and Leumann, C. J. (2003) Recent improvements in antigene technology. *Current Opinion in Chemical Biology* 7, 717-726

120. Sijen, T., Vijn, I., Rebocho, A., van Blokland, R., Roelofs, D., Mol, J. N., and Kooter, J. M. (2001) Transcriptional and posttranscriptional gene silencing are mechanistically related. *Curr Biol* 11, 436-440

121. Cogoni, C., and Macino, G. (2000) Post-transcriptional gene silencing across kingdoms. *Curr Opin Genet Dev* 10, 638-643

122. Dalmay, T., Hamilton, A., Rudd, S., Angell, S., and Baulcombe, D. C. (2000) An RNA-dependent RNA polymerase gene in Arabidopsis is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. *Cell* 101, 543-553

123. Ketting, R. F., and Plasterk, R. H. (2000) A genetic link between co-suppression and RNA interference in C. elegans. *Nature* 404, 296-298

124. Fagard, M., Boutet, S., Morel, J. B., Bellini, C., and Vaucheret, H. (2000) AGO1, QDE-2, and RDE-1 are related proteins required for post-transcriptional gene silencing in plants, quelling in fungi, and RNA interference in animals. *Proc Natl Acad Sci U S A* 97, 11650-11654

125. Montgomery, M. K., and Fire, A. (1998) Double-stranded RNA as a mediator in sequence-specific genetic silencing and co-suppression. *Trends Genet* 14, 255-258

126. Li, Y. X., Farrell, M. J., Liu, R., Mohanty, N., and Kirby, M. L. (2000) Double-stranded RNA injection produces null phenotypes in zebrafish. *Dev Biol* 217, 394-405

127. Mallory, A. C., Reinhart, B. J., Bartel, D., Vance, V. B., and Bowman, L. H. (2002) A viral suppressor of RNA silencing differentially regulates the accumulation of short interfering RNAs and micro-RNAs in tobacco. *Proc Natl Acad Sci U S A* 99, 15228-15233

128. Hamilton, A. J., and Baulcombe, D. C. (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* 286, 950-952

129. Hunter, C. P. (1999) Genetics: a touch of elegance with RNAi. *Curr Biol* 9, R440-442

130. Kasschau, K. D., Xie, Z., Allen, E., Llave, C., Chapman, E. J., Krizan, K. A., and Carrington, J. C. (2003) P1/HC-Pro, a viral suppressor of RNA silencing, interferes with Arabidopsis development and miRNA unction. *Dev Cell* 4, 205-217

131. Stephenson, M. L., and Zamecnik, P. C. (1978) Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide. *Proc Natl Acad Sci U S A* 75, 285-288

132. Kurreck, J. (2003) Nucleic acids chemistry and biology. *Angew Chem Int Ed Engl* 42, 5384-5385

133. van der Krol, A. R., Mol, J. N., and Stuitje, A. R. (1988) Antisense genes in plants: an overview. *Gene* 72, 45-50

134. van der Krol, A. R., Mur, L. A., de Lange, P., Mol, J. N., and Stuitje, A. R. (1990) Inhibition of flower pigmentation by antisense CHS genes: promoter and minimal sequence requirements for the antisense effect. *Plant Mol Biol* 14, 457-466

135. Jorgensen, R. (1990) Altered gene expression in plants due to trans interactions between homologous genes. *Trends Biotechnol* 8, 340-344

136. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411, 494-498

137. Zamore, P. D., Tuschl, T., Sharp, P. A., and Bartel, D. P. (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* 101, 25-33

138. Elbashir, S. M., Lendeckel, W., and Tuschl, T. (2001) RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev* 15, 188-200

139. Bernstein, E., Caudy, A. A., Hammond, S. M., and Hannon, G. J. (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409, 363-366

140. Ketting, R. F., Fischer, S. E., Bernstein, E., Sijen, T., Hannon, G. J., and Plasterk, R. H. (2001) Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. *Genes Dev* 15, 2654-2659

141. Dykxhoorn, D. M., Novina, C. D., and Sharp, P. A. (2003) Killing the messenger: short RNAs that silence gene expression. *Nat Rev Mol Cell Biol* 4, 457-467

142. Volpe, T. A., Kidner, C., Hall, I. M., Teng, G., Grewal, S. I., and Martienssen, R. A. (2002) Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science* 297, 1833-1837

143. Dudley, N. R., and Goldstein, B. (2003) RNA interference: silencing in the cytoplasm and nucleus. *Curr Opin Mol Ther* 5, 113-117

144. Zeng, Y., and Cullen, B. R. (2002) RNA interference in human cells is restricted to the cytoplasm. *Rna* 8, 855-860

145. Paddison, P. J., Caudy, A. A., and Hannon, G. J. (2002) Stable suppression of gene expression by RNAi in mammalian cells. *Proc Natl Acad Sci U S A* 99, 1443-1448

146. Martinez, L. A., Naguibneva, I., Lehrmann, H., Vervisch, A., Tchenio, T., Lozano, G., and Harel-Bellan, A. (2002) Synthetic small inhibiting RNAs: efficient tools to inactivate oncogenic mutations and restore p53 pathways. *Proc Natl Acad Sci U S A* 99, 14849-14854

147. Grishok, A., Pasquinelli, A. E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D. L., Fire, A., Ruvkun, G., and Mello, C. C. (2001) Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. *Cell* 106, 23-34

148. Hutvagner, G., and Zamore, P. D. (2002) A microRNA in a multiple-turnover RNAi enzyme complex. *Science* 297, 2056-2060

149. Lagos-Quintana, M., Rauhut, R., Lendeckel, W., and Tuschl, T. (2001) Identification of novel genes coding for small expressed RNAs. *Science* 294, 853-858

150. Zeng, Y., Yi, R., and Cullen, B. R. (2003) MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms. *Proc Natl Acad Sci U S A* 100, 9779-9784

151. Zamore, P. D. (2002) Ancient pathways programmed by small RNAs. *Science* 296, 1265-1269

152. McManus, M. T., and Sharp, P. A. (2002) Gene silencing in mammals by small interfering RNAs. *Nat Rev Genet* 3, 737-747

153. Ambros, V. (2003) MicroRNA pathways in flies and worms: growth, death, fat, stress, and timing. *Cell* 113, 673-676

154. Pasquinelli, A. E., and Ruvkun, G. (2002) Control of developmental timing by microrna and their targets. *Annu Rev Cell Dev Biol* 18, 495-513

155. Storz, G. (2002) An expanding universe of noncoding RNAs. *Science* 296, 1260-1263

156. Hannon, G. J. (2002) RNA interference. *Nature* 418, 244-251

157. Elbashir, S. M., Harborth, J., Weber, K., and Tuschl, T. (2002) Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods* 26, 199-213

158. Lee, N. S., Dohjima, T., Bauer, G., Li, H., Li, M. J., Ehsani, A., Salvaterra, P., and Rossi, J. (2002) Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nat Biotechnol* 20, 500-505

159. Paul, C. P., Good, P. D., Winer, I., and Engelke, D. R. (2002) Effective expression of small interfering RNA in human cells. *Nat Biotechnol* 20, 505-508

160. Tuschl, T. (2002) Expanding small RNA interference. *Nat Biotechnol* 20, 446-448

161. Kennerdell, J. R., and Carthew, R. W. (2000) Heritable gene silencing in Drosophila using double-stranded RNA. *Nat Biotechnol* 18, 896-898

162. Saunders, L. R., and Barber, G. N. (2003) The dsRNA binding protein family: critical roles, diverse cellular functions. *FASEB J* 17, 961-983

163. Scadden, A. D., and Smith, C. W. (2001) Specific cleavage of hyper-edited dsRNAs. *EMBO J* 20, 4243-4252

164. Vickers, T. A., Koo, S., Bennett, C. F., Crooke, S. T., Dean, N. M., and Baker, B. F. (2003) Efficient reduction of target RNAs by small interfering RNA and RNase H-dependent antisense agents. A comparative analysis. *J Biol Chem* 278, 7108-7118

165. Sternberg, N., and Hamilton, D. (1981) Bacteriophage P1 site-specific recombination. I. Recombination between loxP sites. *J Mol Biol* 150, 467-486

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

167. Hoess, R. H., and Abremski, K. (1984) Interaction of the bacteriophage P1 recombinase Cre with the recombining site loxP. *Proc Natl Acad Sci U S A* 81, 1026-1029

168. Sauer, B. (1987) Functional expression of the cre-lox site-specific recombination system in the yeast Saccharomyces cerevisiae. *Mol Cell Biol* 7, 2087-2096

169. Odell, J., Caimi, P., Sauer, B., and Russell, S. (1990) Site-directed recombination in the genome of transgenic tobacco. *Mol Gen Genet* 223, 369-378

170. Sauer, B., and Henderson, N. (1988) Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc Natl Acad Sci U S A* 85, 5166-5170

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

172. Orban, P. C., Chui, D., and Marth, J. D. (1992) Tissue- and site-specific DNA recombination in transgenic mice. *Proc Natl Acad Sci U S A* 89, 6861-6865

173. Li, X., Zhao, X., Fang, Y., Jiang, X., Duong, T., Fan, C., Huang, C. C., and Kain, S. R. (1998) Generation of destabilized green fluorescent protein as a transcription reporter. *J Biol Chem* 273, 34970-34975

174. Maruyama, H., Higuchi, N., Nishikawa, Y., Kameda, S., Iino, N., Kazama, J. J., Takahashi, N., Sugawa, M., Hanawa, H., Tada, N., Miyazaki, J., and Gejyo, F. (2002) High-level expression of naked DNA delivered to rat liver via tail vein injection. *J Gene Med* 4, 333-341