Mise au point de vecteurs permettant une expression fiable de gènes codant pour des ARN interférent et des microRNA

Ashraf Sawafta

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ECOLE DOCTORALE LOGIQUE DU VIVANT
2008

THESIS

In Partial Fulfillment of the Requirements for the degree of

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Ashraf Sawafta

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Design of Vector for the Expression of shRNA in Transgenic Animals

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الاهداء

الى من عاش منه كي يرى كيف اكون

الى من غرس في الرجال طموحا وفي الارض زيتون

الى من كان يدعوني الدكتور قبل ان اكون ... الى روح جدي رحمه الله

الى الشمعه التي احترقت كي تضيء لنا الطريق

الى امي المتفائلة التي زرعت في الطموح والعنفوان

الى ابي صانع الأجيال الذي علمنا المثابرة والكفاح

الى إخوتي النجباء الذين يعتبرون نجاحي نجاحا لهم

الى زهرة الياسمين الشامخة بشموخ اخوتها المحبين لها الناجعين.. شقيقتي الغالية

ولا انسى اساتذتي المبجلين حانيا لهم الرأس شكرا وعرفانا بالجميل

ولا انسى اهداء نجاحي الى ابناء شعبي من كان منهم شهيدا أو وراء القضبان

والى كل من ساندني ودعمني من الاصدقاء الاحباء

DEDICATION

This thesis is dedicated to my wonderful parents, who have raised me to be the person I am today. You have been with me every step of the way, through good times and bad. Thank you for all the unconditional love, guidance, and support that you have always given me, helping me to succeed and instilling in me the confidence that I am capable of doing anything I put my mind to. Thank you for everything. I love you! Also this thesis is dedicated to my brothers, my wonderful sister,also to Dana the most speacial person in my life, and in loving memory of my grandparents finally, this thesis is dedicated to all my friends those who supported me all the way since the beginning of my studies.

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Abreviations:

ATP: adenosine 5′-triphosphate

β-gal: β-galactosidase

BAC: bacterial artificial chromosome; biospecific affinity chromatography

BLAST: Basic Local Alignment Research Tool

bp: base pair

BSA: bovine serum albumin

cDNA: complementary deoxyribonucleic acid

CHO: Chinese hamster ovary (cells)

Da: Dalton

dATP: deoxyadenosine triphosphate

ddATP: dideoxyadenosine triphosphate

ddNTP: dideoxynucleoside triphosphate

ddTTP: dideoxythymidine triphosphate

DMEM: Dulbecco's modified Eagle (or minimum essential) medium

DNA: deoxyribonucleic acid

DNase: deoxyribonuclease

dNTP: deoxynucleoside triphosphate

DTT: dithiothreitol

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme-linked immunosorbent assay

ES: embryonic stem (cells)

GFP: green fluorescent protein

HBSS: Hanks' buffered salt solution

hCMV: human cytomegalovirus

hGH: human growth hormone

HIV: human immunodeficiency virus

HSV: herpes simplex virus

Ig: immunoglobulin

IPTG: isopropyl-1-thio-β-D-galactoside

IRES: internal ribosomal entry site

kb: kilobase

kDa: kilodalton

LTR: long terminal repeat

miRNA: microRNA

mRNA: messenger ribonucleic acid

nt: nucleotide

5 ' HS4: hypersensitive area in DNAse I in 5 ' of the locus of the globine β of chicken

Oligo: oligonucleotide, a short, single-stranded DNA or RNA.

Oligo(dT): oligodeoxythymidylic acid

ORC: origin recognition complex

ORF: open reading frame

Ori: origin of replication

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

RNA: ribonucleic acid

RNAi: RNA interference

RNase: ribonuclease

rRNA: ribosomal ribonucleic acid

RT: reverse transcriptase

RT-PCR: reverse transcription/polymerase chain reaction

SDS: sodium dodecyl sulfate

siRNA: short interfering RNA

ss: single stranded

SSC: sodium chloride/sodium citrate (buffer); side (light) scatter (in flow cytometry)

T: thymine or thymidine; one-letter code for threonine

TAE: Tris/acetate (buffer)

Taq: Thermus aquaticus DNA (polymerase)

TBE: Tris/borate (buffer)

TE: Tris/EDTA (buffer)

tRNA: transfer ribonucleic acid

UTR: untranslated leader region

Sense strand: The coding sequence of mRNA.

Antisense strand: The noncoding strand complementary to the coding sequence of mRNA.

Summary:

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CHAPTER 1

BIOLOGY OF RNAi

1. RNA interference (RNAi)

In 1998, it was demonstrated that injection of double stranded RNA (dsRNA) into nematodes induces the post-transcriptional silencing of gene encoding homologous mRNA, a process called 'RNA interference' (RNAi) (Fire et al., 1998). RNAi was first described in *C. elegans* in a process in which small double-stranded RNAs induce homology dependant degradation of mRNA (Sharp, 2001). RNAi has been linked to many previously described silencing phenomena such as post-transcriptional gene silencing (PTGS) in plants and quelling in fungi (reviewed by Dykxhoorn et al., 2003). RNAi is an evolutionarily conserved phenomenon (see table 1 for more details). It is a multi-step process that involves generation of active small interfering RNA (siRNA) in vivo through the action of an RNase III endonuclease, named Dicer. The resulting 21- to 23-nt siRNA mediates degradation of the complementary homologous RNA (Sharp, 2001).

Table 1 : Eukaryotic organisms and RNAi phenomena
Examples of eukaryotic organisms exhibiting RNAi- related phenomena from (Agrawal et al., 2003)

Kingdom	Species	Stage tested	Delivery method
Protozoans	*Trypanosoma brucei*	Procyclic forms	Transfection
	Plasmodium falcipamm	Blood stage	Electroporation and soaking
	Toxoplasma gondii	Mature forms in fibroblast	Transfection
	Paramecium	Mature form	Transfection and feeding
	Leishmania donovanii	Larval stage and adult stage	Tried but not working
Invertebrates	*Caenorhabditis elegans*	Adult	Transfection, feeding bacteria carrying dsRNA, soaking
	Caenorhabditis briggsae	Adult worm	Injection
	Brugia malayi (filarial worm)	Sporocysts	Soaking
	Schistosoma mansoni	Adult	Soaking

	Hydra	Adult	Delivered by micropipette
	Planaria	Adult	Soaking
	Lymnea stagnalis (snail)	Cell lines, adult, embryo	Injection
	Drosophila melanogaster	Early embryonic stages	Injection for adult and embryonic stages, soaking and transfection for cell lines
	Cyclorrphan (fly)	Early embryonic stages	Injection
	Milkweed bug	Early embryonic stages	Injection
	Beetle	Larval stage	Injection
	Cockroach	Adult and cell line	Injection
	Spodoptera frugiperda		Injection and soaking
Vertebrates	Zebra fish	Embryo	Microinjection
	Xenopus laevis	Embryo	Injection
	Mice	Prenatal, embryonic stages, and adult	Injection
	Humans	Human cell lines	Transfection
Plants	Monocots/dicots	Plant	Particle bombardment with siRNA/transgenics
Fungi	*Neurospora crassa*	Filamentous fungi	Transfection
	Schizosaccharomyces pombe	Filamentous fungi	Transgene
	Dictyostelium discoideum		Transgene
Algae	*Chlamydomonas reinhardtii*		Transfection

RNAi and related phenomena protect the organisms from invasion by both exogenous (eg., viruses) and endogenous (eg., mobile genetic elements) genetic parasites (Bernstein et al., 2001b). Eukaryotic genomes are largely composed of repetitive DNA sequences. Among them, transposable elements have the potential to perform replication cycles involving DNA or RNA intermediates. These repeated and mobile sequences have been found in all living organisms, and can comprise up to 40% of the genome, as is the case in humans. Tight control of these invaders is thus an important feature of their regulation to prevent eukaryotic genomes from their mutational threat. During the last few years, evidence has emerged that the host has developed mechanisms to silence such repeated sequences or destroy any RNA foreign genetic material detected in a cell. One of the main actors of this regulation is the RNAi pathway, which acts as a sequence-specific RNA degradation mechanism (Tabara et al., 1999; Martinez & Tuschl, 2004; Buchon & Vaury, 2006). In addition to a mechanism for degrading transcripts, several observations suggest that RNAi is involved in other active processes. For example, dsRNA delivered by microinjection into the intestine exerts interference effects in tissues throughout both the injected animal and its progeny suggesting the existence of activities that transport and perhaps amplify the interfering agent (Fire et al., 1998).

The process by which specific mRNA are targeted for degradation by complementary short-interfering RNAs (siRNA) has increasingly become a powerful tool for genetic analysis and is likely to become a powerful therapeutic approach for gene silencing and a possible approach to the *in vivo* inactivation of gene products linked to human disease progression and pathology (Bernstein et al., 2001a; McManus et al., 2002; Heidersbach et al., 2006). For the last 6 years, scientists have learned much about the general mechanisms underlying RNAi, but the detailed mechanism of action of RNAi remains to be elucidated. Consequently, understanding the mechanism of RNAi has become critical for developing the most effective RNAi methodologies for both laboratory and clinical applications (Chiu & Rana, 2003).

Both biochemical and genetic analysis have participated in increasing our understanding of how RNAi works and lead to the recognition of an early step in RNAi mechanism in which the dsRNA is recognized and is targeted for a RNAase-dependent digestion, and a late step that leads to the silencing of the target mRNA. The general mechanism of RNAi involves the cleavage of double-stranded RNA (dsRNA) to short 21-23-nt siRNAs (see Figure 1& Figure 2 for more details). This

processing event is catalyzed by Dicer, a highly conserved, dsRNA-specific endonuclease that is a member of the RNase III family (Hammond et al., 2000; Zamore et al., 2000; Bernstein et al., 2001b; Hamilton et al., 2002; Provost et al., 2002; Zhang & Doudna, 2002). Processing by Dicer results in siRNA duplexes that have 5'-phosphate and 3'-hydroxyl termini, and subsequently, these siRNA are recognized by the RNA-Induced Silencing Complex (RISC) (Hammond et al., 2000).
Active RISC complexes promote the unwinding of the siRNA through an ATP-dependant process and the unwind antisense strand guides RISC to the complementary mRNA (Nykanen et al., 2001). The targeted mRNA is then cleaved by RISC at a single site that is defined with regard to where the 5'-end of the antisense is bound to the mRNA target sequence. The cleavage site is located near the center of the region spanned by the guiding siRNA as shown in Figure 3 (Hammond et al., 2000; Elbashir et al., 2001b).
It was first discovered that in plants, RNAi can suppress gene expression via two distinct pathways: post-transcriptional (PTGS) and transcriptional (TGS) gene silencing (Manika Pal-Bhadra et al., 2002). PTGS involves siRNAs targeted to mRNA or pre-mRNA whereas TGS involves siRNAs targeted to gene promoters (i.e. PTGS= mRNA targeting, TGS = DNA targeting) (Kawasaki & Taira, 2004). TGS was only recently reported to be operable in some mammalian cells. The observed TGS in mammalian cells appears to involve both histone and DNA methylation (Morris et al., 2004; Morris & Rossi, 2006).
SiRNA/miRNA duplexes are proceeds from long dsRNA and miRNA precursors by RNase type III enzyme called Dicer. The produced dsRNA then unwound and assembled into RISC, RITS (RNA-induced transcriptional silencing) or miRNP. mRNA-target degradation is mediated by RISC, while target mRNAs translation repression is guided by miRNPs and the RITS complex guides the condensation of heterochromatin. rasiRNA and RITS which was founded in certain groups (Yeast, *A. thaliana, D. melanogaster*) then it has been shown that they exist also in mammals. In animals, complementary target RNAs is cleaved by siRNAs, whereas miRNAs mediate translational repression of mRNA targets. Chromatin modifications are guided by rasiRNAs. C. *elegans* and mammals carry only one Dicer gene. In *D. melanogaster* and *A. thaliana*, specialized Dicer or DLC proteins preferentially process long dsRNA or miRNA precursors (see Figure 1).

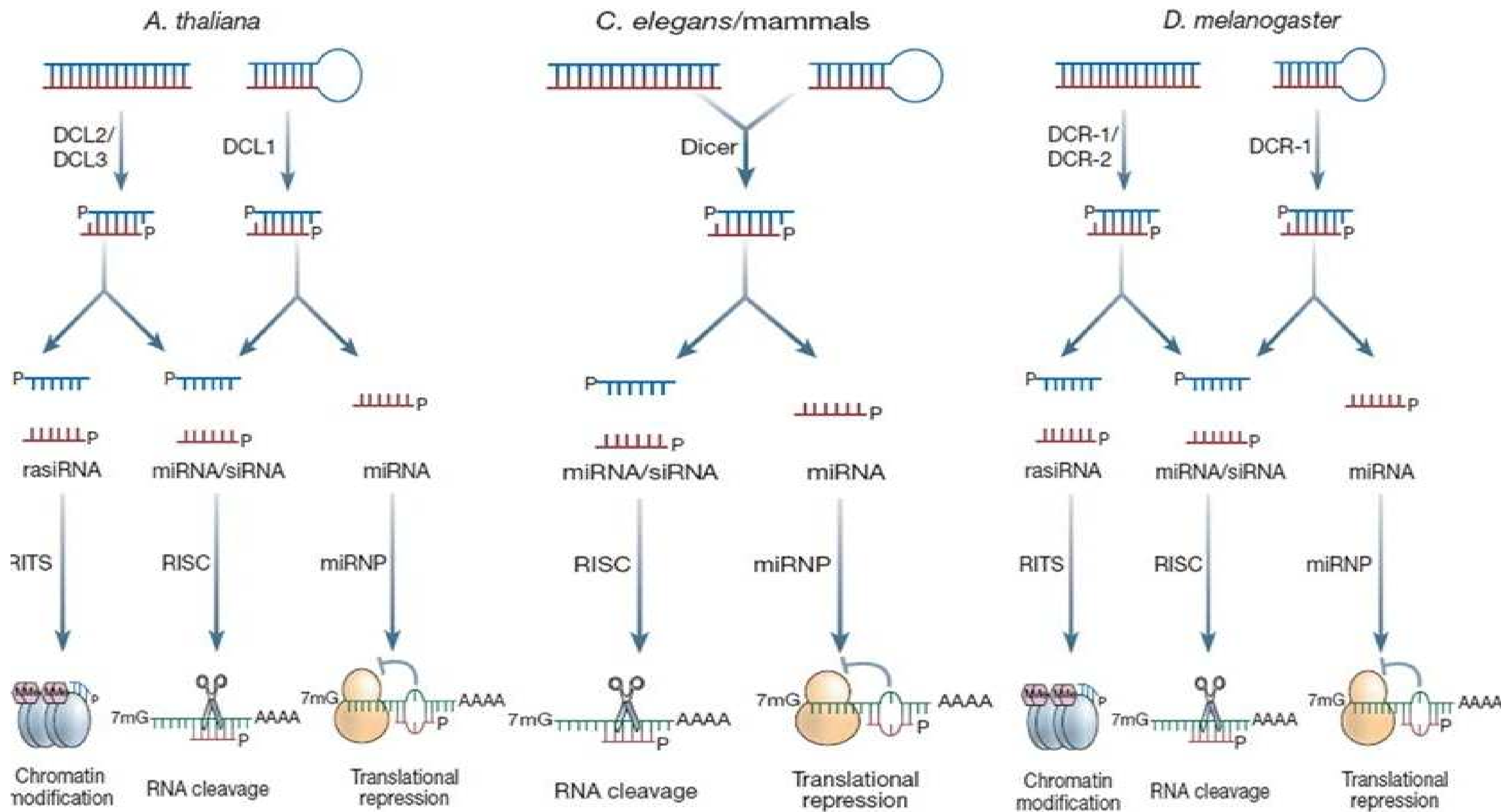


Figure 1 : RNA silencing pathways in different organisms.

Long dsRNA and miRNA precursors are processed to siRNA/miRNA duplexes by the RNase-III-like enzyme Dicer. The short dsRNAs are subsequently unwound and assembled into effector complex: RITS (RNA-induced transcriptional silencing) or miRNP. RISC mediates mRNA-target degradation; miRNPs guide translational repression of target mRNAs, and the RITS complex guides the condensation of chromatin. In animals, siRNAs guide cleavage of complementary target RNAs, whereas miRNA mediates translational repression of mRNA targets, rasiRNAs guide chromatin modification. (From Meister & Tuschl, 2004)

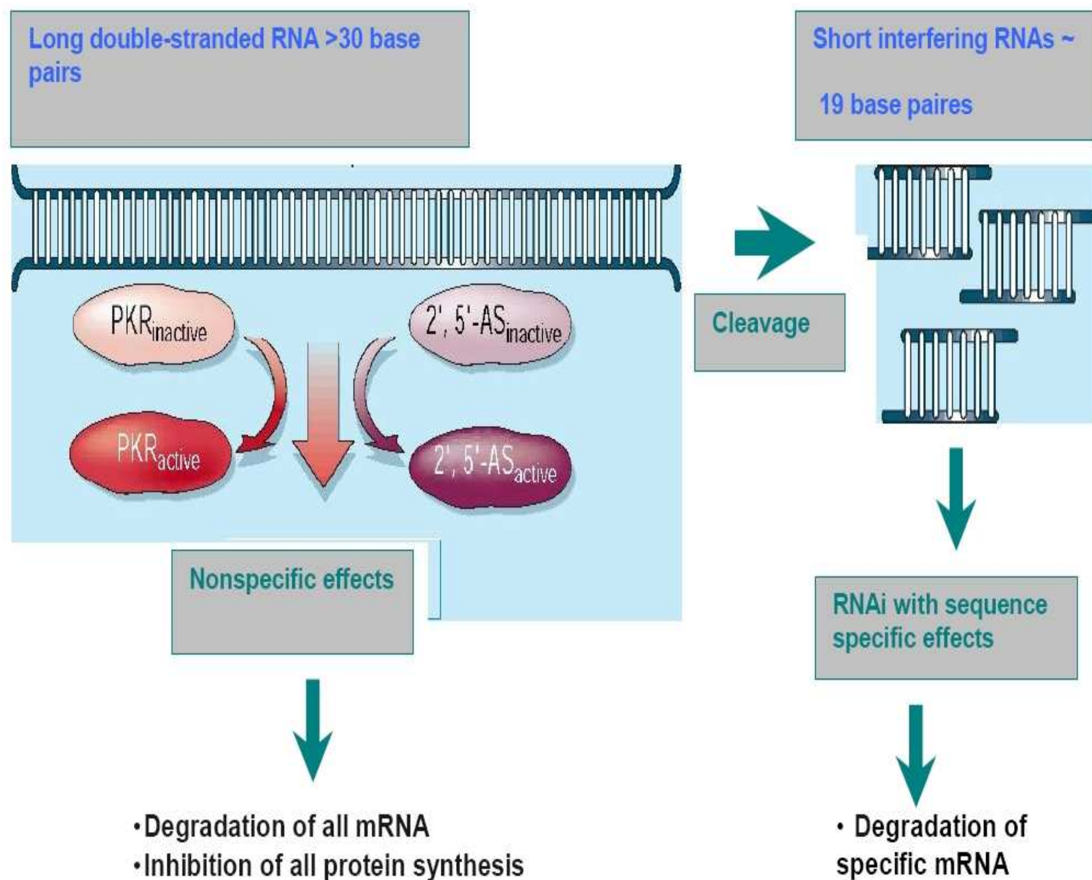


Figure 2 : Mammalian cells dsRNA pathways.
Fig.2 shows the two pathways of dsRNA in mammalian cells, the nonspecific as well as the specific pathway are shown.

Mammalian cells have at least two pathways that compete for double-stranded RNA (dsRNA). The RNAi nonspecific pathway (red arrows) is triggered by dsRNA of any sequence larger than 30nt. The nonspecific effect triggers an interferon response which leads to cell death and apoptosis. The nonspecific effects occur because dsRNA activates two enzymes: PKR, which in its active form phosphorylates the translation initiation factor eIF2α to shut down all protein synthesis, and 2', 5' oligoadenylate synthetase (2', 5'-AS), which synthesizes a molecule that activates RNase L, a nonspecific enzyme that targets all mRNAs. The nonspecific pathway represents a

host response to stress or viral infection (From Bass, 2001). The second pathway is the sequence-specific pathway (green arrows), in which the initiating dsRNA is first broken into short interfering siRNAs. SiRNAs have sense and antisense strands of about 21 nucleotides that form 19 base pairs to leave overhangs of two nucleotides at each 3' end. SiRNAs are though to provide the sequence information that allows a specific messenger RNA to be targeted for degradation

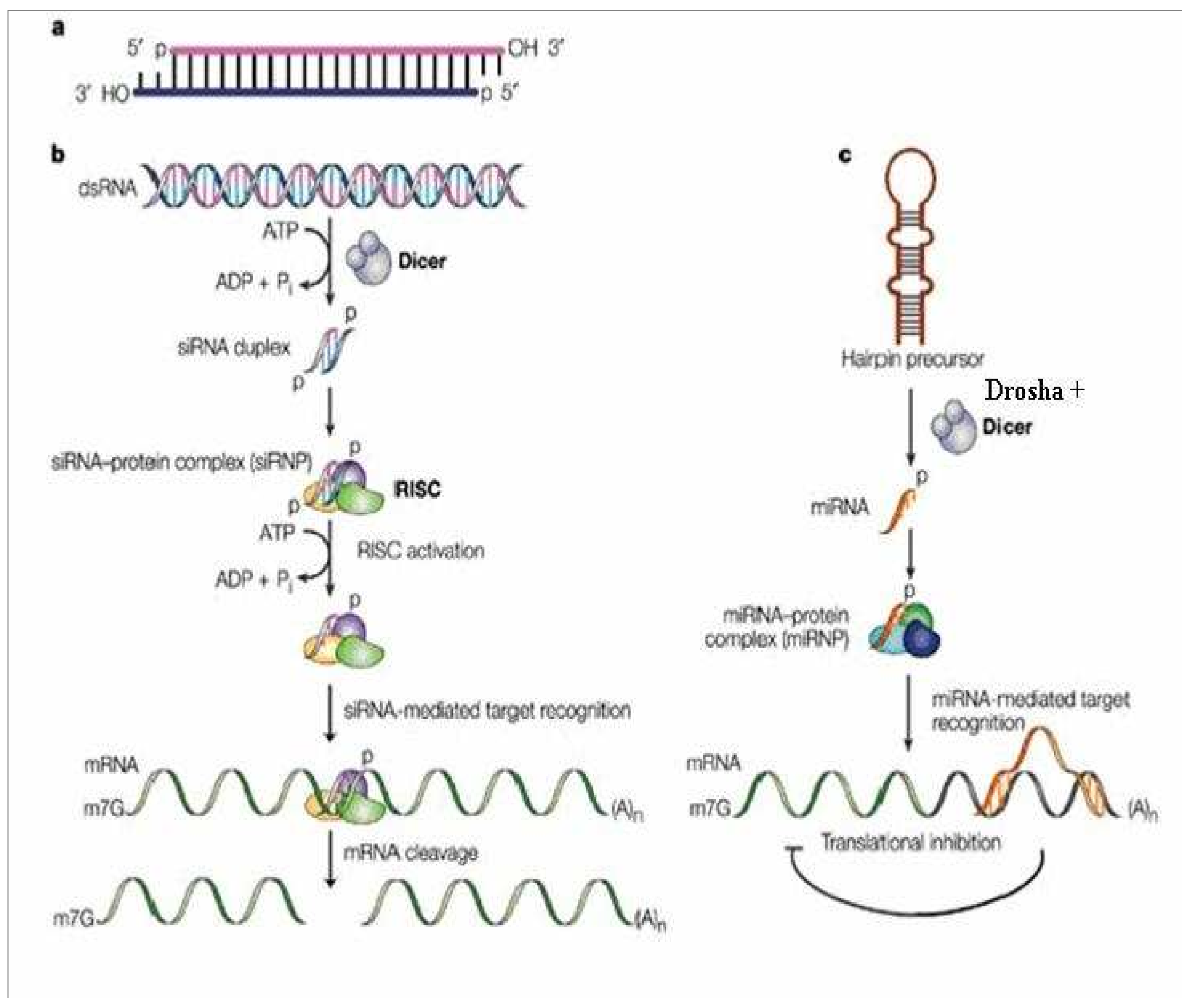


Figure 3 : RNAi post-transcriptional gene silencing mechanisms.
(A) Short interfering RNAs (siRNAs) are generally between 19 and 27nt in length with the characteristic 2-nt unpaired overhangs and 5'-phosphate and 3' hydroxyl groups. (B) The siRNA pathway to RNA interference. Long dsRNAs are processed by the RNAse-III-like enzyme Dicer into siRNAs. Processed siRNAs are then to target the available RNA with a complementary sequence. The target RNA will then be cut at the centre of the newly formed duplex between target RNA and the small antisense RNA. (C) The microRNA (miRNA) pathway. Long, imperfect hairpin structures are also processed by Dicer to form single-strand miRNAs that are incorporated in the miRNA-protein complex (miRNP). These miRNAs then pair with partial complementarity to their target mRNAs leading to translational repression. (From Dykxhoorn et al., 2003)

2. RNAi mechanism and mode of actions

A combination of results obtained from several *in vivo* and *in vitro* experiments led into a two-step mechanistic model for RNAi/PTGS. The first step, referred to as the RNAi initiating step, involves binding of the RNA nucleases to a large dsRNA and its cleavage into discrete ≈21- to ≈25-nucleotide RNA fragments (siRNA). In the second step, these siRNAs join a multinuclease complex, RISC, which degrades the homologous single-stranded mRNAs. At present, little is known about the RNAi intermediates, RNA-protein complexes, and mechanisms of formation of different complexes during RNAi. Although there is still several missing links in the process of RNAi and how RNAi works, the molecular basis of it is not fully known and need extensive research (Agrawal et al., 2003).

2.1. Processing of dsRNA into siRNAs

Hamilton & Baulcombe, (1999) reviewed that studies of PTGS in plants provided the first evidence that small RNA molecules are important intermediates of the RNAi process. This was observed during the course of a research on transgenic petunia flowers that were expected to be more purple (Napoli et al., 1990). Indeed surprisingly, some of the transgenic petunia plants harboring the chsA (chalcone synthase) coding region under the control of a 35S CaMV promoter lost both endogene and transgene chalcone synthase activity, and thus many of the flowers were variegated or developed white sectors (Napoli et al., 1990).

In mammals a collaborative effort of Phil Zamore, Tom Tuschl, Phil Sharp and David Bartel gave the first evidence that RNAi could work in vitro (Zamore et al., 2000). The works that has been done by Tuschl et al., (1999) gave direct evidence that the generation of siRNAs in RNAi occurred in an *in vitro* cell-free system obtained from a *Drosophila* syncytial blastoderm embryo. They demonstrated that when dsRNAs radiolabeled within either the sense or the antisense strand were incubated with *Drosophila* lysate in a standard RNAi reaction, 21- to 23-nucleotide RNAs were generated with high efficiency, then incorporated into the RNA-induced silencing complex (RISC) after being unwound and separated in the siRNP (siRNA-protein complex). Protein components of RISC will use a single strand of the previous siRNA duplex. Single-stranded ^{32}P-labeled RNA of either the sense or antisense strand was

not efficiently converted to 21- to 23-nucleotide products which indicated that dsRNAs rather than single strand RNAs are responsible in generating the 21-23 nucleotides. The formation of the 21- to 23-nucleotide RNAs did not require the presence of corresponding mRNAs (Tuschl et al., 1999; Sharp & Zamore, 2000). Elbashir et al., (2001a) confirmed the role of the small RNAs in RNAi: in their works they showed that synthetic 21- to 23-nucleotide RNAs, when added to cell-free systems, were able to guide efficient degradation of homologous mRNAs.

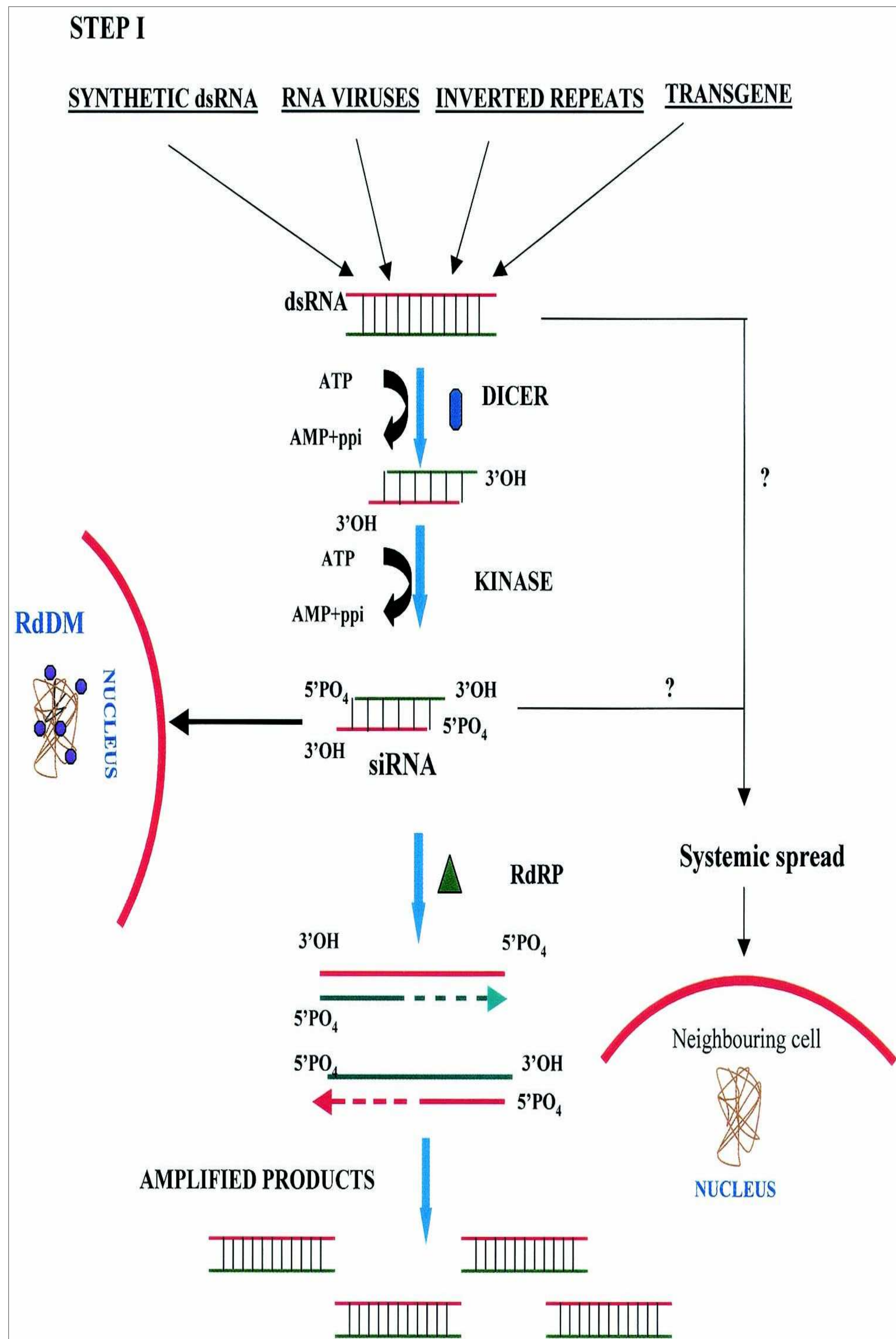
The involvement of RNase III-type endonucleases in the degradation of dsRNA to siRNAs was first predicted on the basis of homology with the binding and cleavage properties of *E. coli* RNase III enzymes (Bass, 2000). The RNase III enzymes work by cutting both strands of dsRNA, leaving a 3' overhang of 2 nucleotides. They chemically analyzed the sequences of the 21- to 23-nucleotide RNAs generated by the processing of dsRNA in the *Drosophila* cell-free system. They characterized the final product of this process and they showed the presence of 5'-phosphate, 3'-hydroxyl, and a 3' 2-nucleotide overhang and no modification of the sugar-phosphate backbone in the processed 21- to 23-nucleotide RNAs (Hutvagner et al., 2001).

Bernstein et al., (2001a) have performed both biochemical fractionation and candidate gene approaches to identify the enzymes that may play an important role in each step of RNAi. They demonstrated that RISC and the 22- nucleotide sequence generating activity may be separable because they were capable to clear RISC activity from extracts by high-speed centrifugation, whereas the activity that produces 22-nucleotide sequences remained in the supernatant. Ketting et al., (2001) showed that one of these identified genes in *Drosophila*, Dicer, codes for the RNA processing enzyme that fragments dsRNA into 22-nucleotide fragments in vitro and that this 22-nucleotide was similar to the 22-nucleotide which was produced by the RNAi system. They demonstrated by their work that this enzyme was responsible for the initiation of the RNAi, and is capable to digest the dsRNA into uniformly sized small RNAs (siRNAs) (Bernstein et al., 2001a). These types of nuclease are evolutionally conserved in worms, flies, fungi, plants, and mammals (Bernstein et al., 2001a). As reviewed in Agrawal et al., (2003) Dicer consists of four distinct domains, among which are an amino *terminal* helicase domain, and a PAZ domain (a 110-amino- acid domain present in proteins like Piwi, Argo, and Zwille/pinhead). The direct correspondence in size of these RNAs with those generated from dsRNA by cell extract suggested a role of this protein in dsRNA degradation. The role of Dicer in

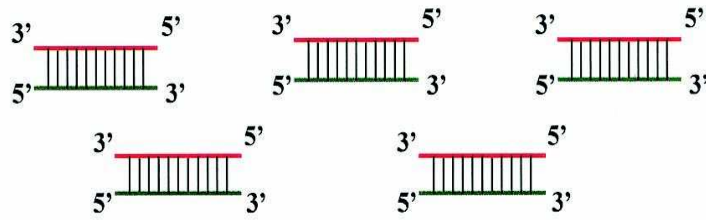
RNAi was further confirmed by the fact that the introduction of Dicer dsRNA into *Drosophila* cells diminished the ability of the transfected cells to carry out RNAi in vitro. Similar experimental studies were carried out with *C. elegans* extract, and an ortholog of Dicer named DCR1 was identified (Agrawal et al., 2003).

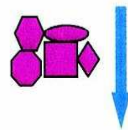
One of the many interesting features of RNA interference is the apparently catalytic nature of the phenomenon in some species. Sijen et al., (2001) have shown that a few molecules of dsRNA are sufficient to degrade a continuously transcribed target mRNA for a long period of time. Although the conversion of long dsRNA into many small siRNAs results in some degree of amplification, it is not sufficient to bring about such continuous mRNA degradation. Evidence that RNA-dependent RNA polymerase (RdRP) play a crucial roles in the RNAi systems comes from the facts that when genes encoding RdRP were mutated, it affected the RNAi system (Lipardi et al., 2001). It was proposed that this type of polymerase might replicate siRNAs as epigenetic agents, permitting their spread throughout plants and between generations in *C. elegans.* Studies by Lipardi et al., (2001) and Sijen et al., (2001), provided convincing biochemical and genetic evidence that RdRP indeed plays a critical role by amplifying RNAi effects. This amplification occurs in plants and *C. elegans* only but not in drosophila and vertebrates. This is expected to reduce the efficiency of the RNAi system in higher species.

Figure 4 illustrates the steps of gene silencing induced by double strand RNA (dsRNA) in *C. elegans*.

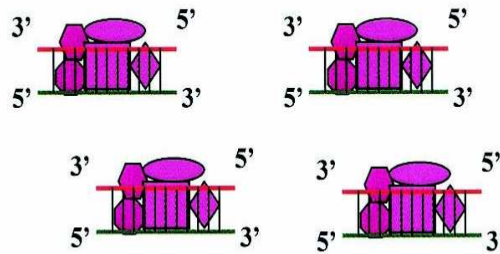

STEP I
SYNTHETIC dsRNA
RNA VIRUSES
INVERTED REPEATS
TRANSGENE
dsRNA
ATP
AMP+ppi
DICER
3'OH
3'OH
?
ATP
KINASE
AMP+ppi
RdDM
NUCLEUS
5'PO4
3'OH
?
5'PO4
3'OH
siRNA
RdRP
Systemic spread
3'OH
5'PO4
5'PO4
Neighbouring cell
5'PO4
3'OH
5'PO4
AMPLIFIED PRODUCTS
NUCLEUS

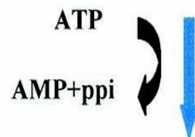
STEP II





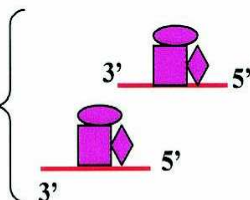
NUCLEASE COMPLEX

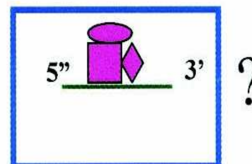




HELICASE FROM RISC COMPLEX

Activated RISC





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NUCLEASE COMPLEX BINDING TO SENSE STRAND IS NOT CERTAIN

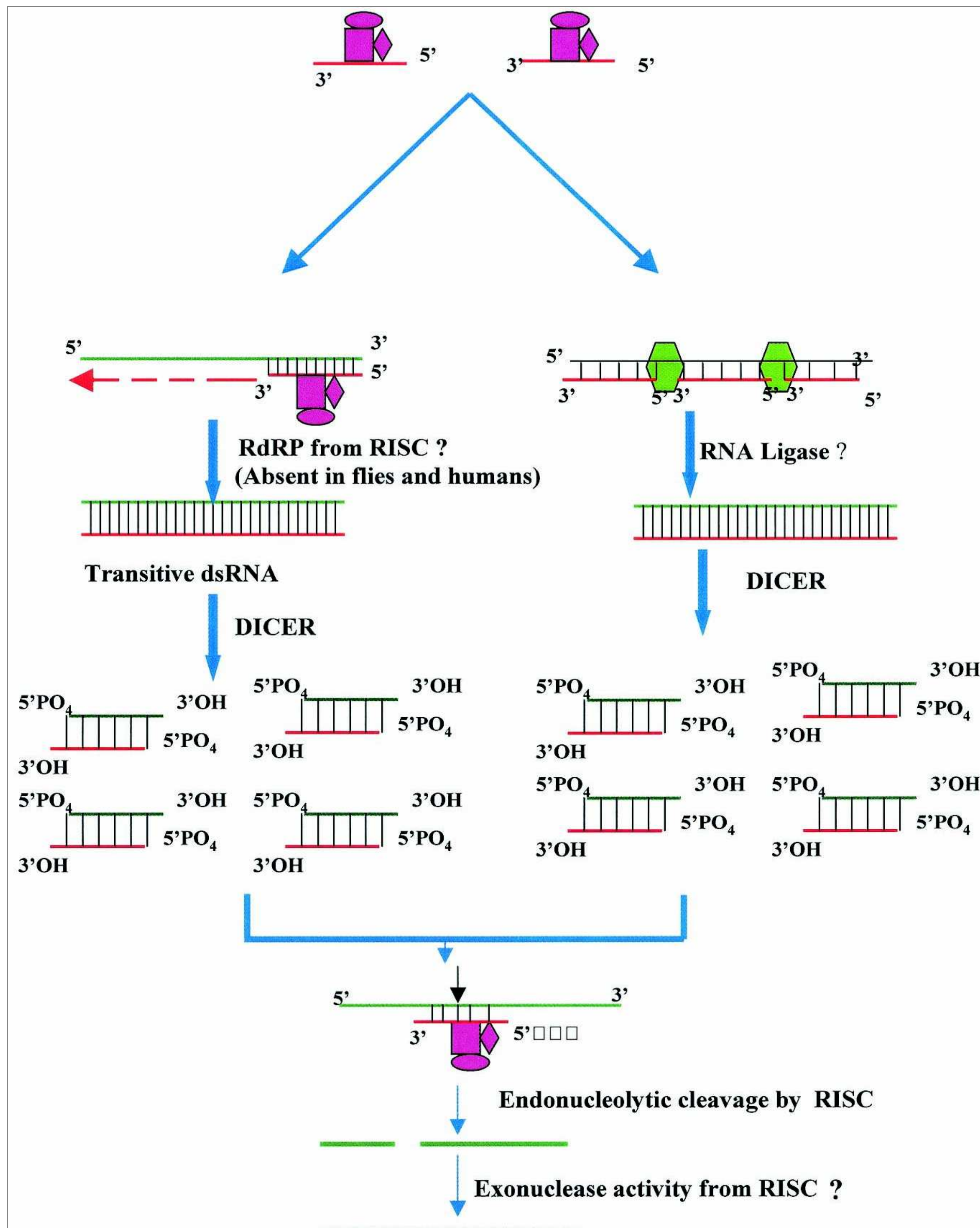


Figure 4 : The mechanism of gene silencing induced by double-stranded RNA.
Illustration of the proposed two-step model for the mechanism of gene silencing induced by double-stranded RNA. In step I, dsRNA is cleaved by the Dicer enzyme to produce siRNAs. A putative kinase seems to maintain 5' phosphorylation at this step. Systemic spread of silencing. Amplification might occur due to the presence of RdRP (▲). In step II, the siRNAs generated in step I bind to the nuclease complex (RISC). A helicase present in the complex might activate RISC by unwinding the siRNAs. The antisense component of siRNA in the RISC guides the complex towards the cognate mRNA (—), resulting in endonucleolytic cleavage (↓) of the mRNA. (RdDM: RNA-dependent DNA methylation). (From Agrawal et al., 2003).

2.2. MicroRNA processing

The genome of animals contains at least two hundreds of miRNA genes which encode for short regulatory RNA molecules. miRNAs repress the expression of protein-coding mRNA providing a previously unappreciated regulatory mechanism for gene expression. Upon binding of an individual miRNA, or a combination of several miRNAs to the 3′ untranslated region of a target mRNA, either translation repression or mRNA cleavage is induced through activation of the RNA-Induced Silencing Complex (RISC). Studies on multiple vertebrate genomes indicate that miRNA can repress more then a third of all genes. It is impossible to understand the RNAi in vertebrate cells without taking into consideration the biogenesis and function of microRNA (miRNA). Mature miRNAs are noncoding RNAs about 22 nucleotides in length expressed by all metazoan eukaryotes (Bartel, 2004). Gained evidence from several works indicate that the human genome encodes over 300 different miRNA molecules and these miRNAs are believed to play an important role in the post-transcriptional regulation of many aspects of cellular differentiation (Bartel, 2004).

As illustrated by Cai et al., (2004) miRNAs are initially transcribed as part of one arm of an RNA stem-loop structure of about 80 nucleotides that in turn forms part of a longer primary miRNA (pri-miRNA) transcript (Figure 5). The first step in miRNA processing occurs in the nucleus. It starts by the recognition of key elements of the secondary structure of the pri-miRNA stem-loop by the RNase III enzyme Drosha and its cofactor DGCR8 (Bohnsack et al., 2004; Denli et al., 2004; Zeng & Cullen, 2006). It is believed that Drosha-DGCR8 cleaves the pri-miRNA stem-loop about 22 nucleotides away from the junction of the stem and the terminal loop, leaving a characteristic two-nucleotide 3' overhang. Then the resulting precursor miRNA (pre-miRNA) hairpin of about 60 nucleotides is bound by the nuclear export factor exportin 5 (Exp5) (Lund et al., 2004) (Figure 5). This step and the recognition are highly dependent on RNA structure and optimally requires an RNA stem of 16 base pairs or more flanked by a short, approximately two-nucleotide 3' overhang (Zeng & Cullen, 2006). Then bound pre-miRNA is transported to the cytoplasm and released there.

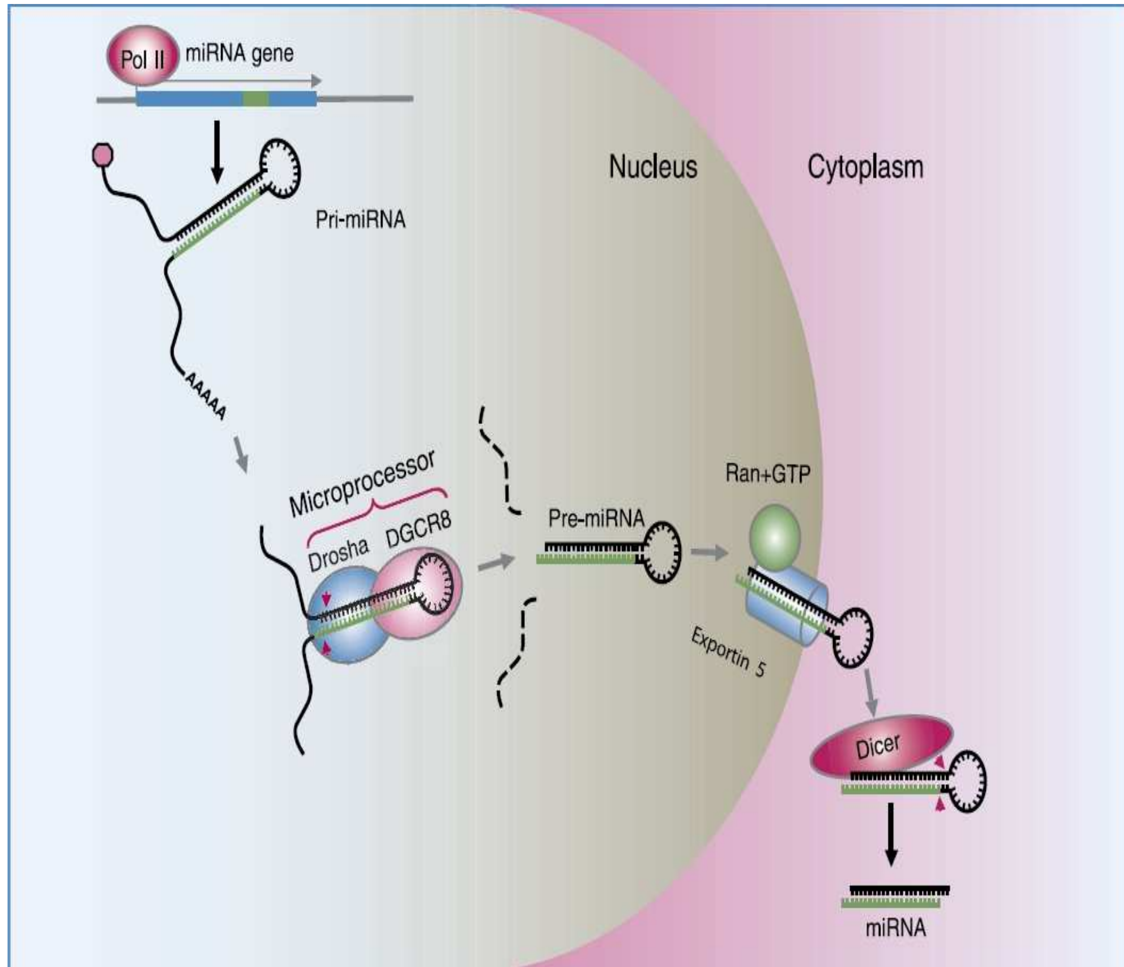


Figure 5 : miRNA biogenesis.
Long primary transcripts (pri-miRNAs) is encoded in the cellular DNA and transcribed in the nucleus, containing one to several miRNAs are generated by polymerase II. Processed by the recently identified microprocessor complex, comprising Drosha (RNase III endonuclease) and DGCR8 (double-stranded RNA binding proteins) recognize the distinct hairpin secondary structure of the pri-miRNA and specifically cleave at the base of the stem loop releasing a 60- to 70-nucleotide pre-miRNA are then transported by Exportin 5 to the cytoplasm where Dicer, a second RNase III endonuclease, cleaves 22-nucleotide from the Drosha cleavage site to yield the mature miRNA. After strand separation, the mature miRNA represses protein production either by blocking translation or causing transcript degradation (Gregory & Shiekhattar, 2005).

In the cytoplasm pre-miRNA is recognized by a heterodimer, consisting of the RNase III enzyme Dicer and its cofactor TRBP10 (see Figure 6 for more details). Once again, structure is important for recognition. It has been demonstrated (Macrae et al., 2006) that RNA stem of 19 base pairs or more and a two-nucleotide 3' overhang are crucial factors in the recognition by Dicer. Then Dicer-TRBP complex binds the base of the pre-miRNA hairpin and cleaves about 22 nucleotides away, leaving another two-nucleotide 3' overhang and removing the terminal loop. Hammond et al., (2000) found that Dicer and TRBP play an important role in facilitating the assembly of one strand of this miRNA duplex intermediate into a protein 'effector complex' called the

RNA-induced silencing complex (RISC). It acts as a 'guide RNA' to direct RISC to homologous mRNA species. This step is similar with what happens with shRNA.

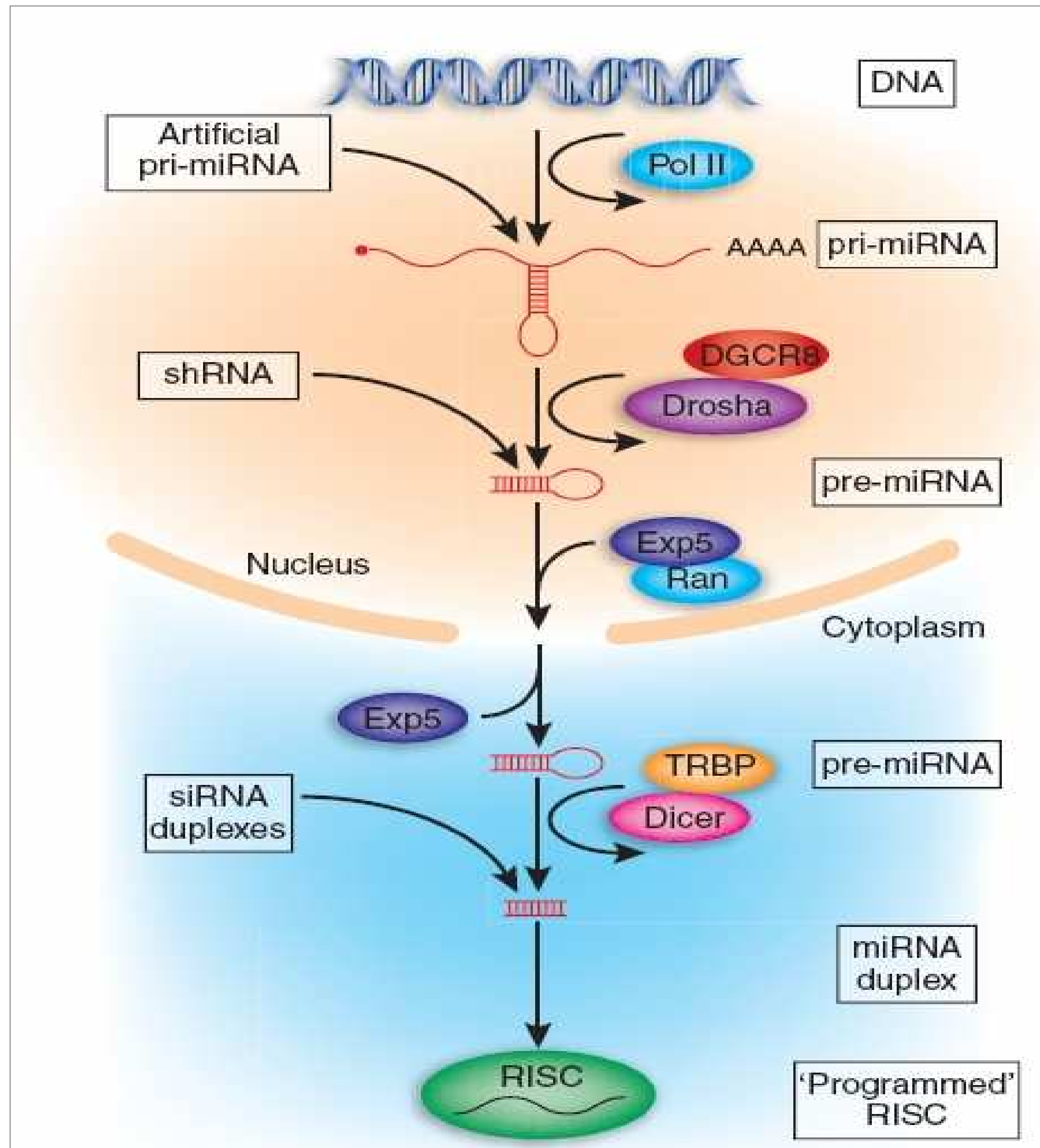


Figure 6 : The miRNA biogenesis pathway in vertebrate cells.
Fig. 6 illustrates the miRNAs biogenesis pathway in vertebrate. Pri-miRNA is first generated by polII, then recognized by Drosha and its cofactor DGCR8 which cleaves this pri-miRNA into pre-miRNA to be exported by exportin 5 from the nucleus into the cytoplasm where it is recognized by Dicer. The processing of pre-miRNA by dicer leads to the production of 21-23nt which will be incorporated to RISC complex.

2.3. Degradation of mRNA

As previously described the mRNA is cleaved only within the region of sequence identity with the dsRNA (Ngo & Bouck, 1998). Cleavage occurs at sites 21–23 nucleotides apart, the same interval observed for the dsRNA itself. The 21–23 nucleotide fragments from the dsRNA are guiding mRNA cleavage. Several works that has been done on this mechanism suggested that the double-stranded siRNAs produced in the first step are believed to bind an RNAi-specific protein complex RISC (Elbashir et al., 2001b; Ketting et al., 2001; Cullen, 2006a) (Figure 7). In an ATP dependent manner, this complex might undergo activation which permits for the antisense component of the unwound siRNA to become exposed and allow the RISC to perform the downstream RNAi reaction.(Zamore et al., 2000). It has been found by several works that the antisense siRNAs in the activated RISC pair with cognate mRNAs and the complex cuts this mRNA approximately in the middle of the duplex region (Agrawal et al., 2003).

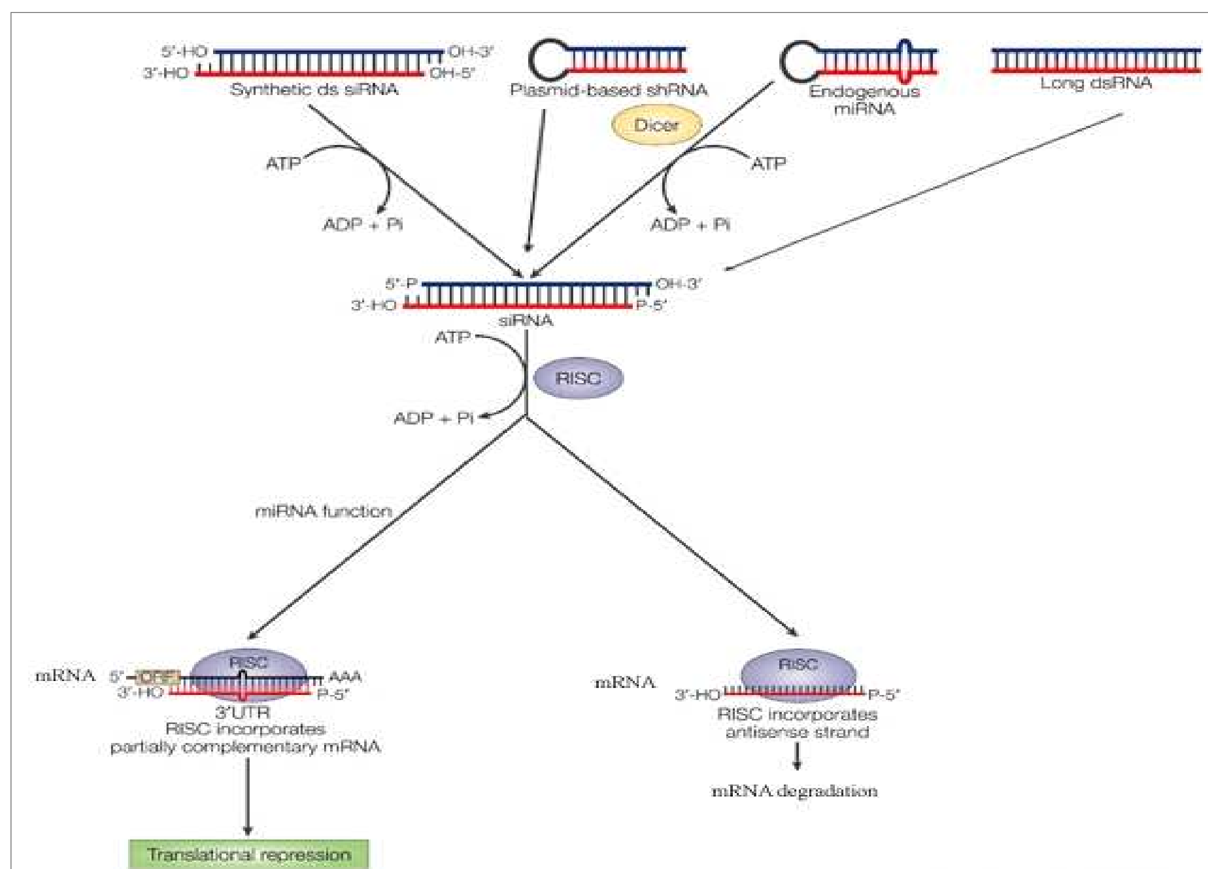


Figure 7 : shRNA mechanism and mode of action.

The importance of RISC complex has been the matter of several investigations. But few independent studies demonstrated the importance of the RISC complex in this part of RNAi reactions (Hammond et al., 2000). The target cleavage site has been mapped to 11 or 12 nucleotides downstream of 5' end of the guide siRNA, and then the cleaved mRNAs are subjected to degradation by exoribonucleases (Hammond et al., 2000).

Three models have been proposed to explain the mechanism by which siRNAs direct target RNA destruction. In the first model, the target destruction is achieved by RNA-dependent RNA polymerase (RdRP). The RdRP is required to convert the target mRNA into dsRNA (Lipardi et al., 2001). As proposed by Tomari et al., (2004), the RdRP is believed to implement single-stranded siRNAs as primers for the target RNA-templated synthesis of complementary RNA (cRNA). Dicer then cleaves the resulting cRNA/target RNA hybrid. This leads to the destruction of the mRNA and to the generation of new siRNAs in the process (Figure 8). This process is ATP-dependent. The second model proposes that single-stranded siRNAs do not act as primers for an RdRP, but they work by assembling along the length of the target RNA and are then ligated together by an RNA ligase to generate cRNA (Lipardi et al., 2001). Dicer destroys the cRNA/target RNA hybrid. Again this model suggests that target recognition and destruction require ATP to catalyze ligation process, and to support Dicer cleavage. Like the first model, Lipardi and coworkers demonstrated that it is necessary for siRNA to have 3' hydroxyl group for the RNAi. As illustrated in Figure 7, two distinct enzyme complexes act in the RNAi pathway. Dicer generates siRNAs from dsRNA. These siRNAs are then incorporated into a second enzyme complex, the RNA-induced silencing complex (RISC), in an ATP-dependent step or series of steps during which the siRNA duplex is unwound into single strands. One of the resulting single-stranded siRNA is proposed to guide the RISC to recognize and cleave the target RNA. Nowadays it is well known that the third model is the correct pathway.

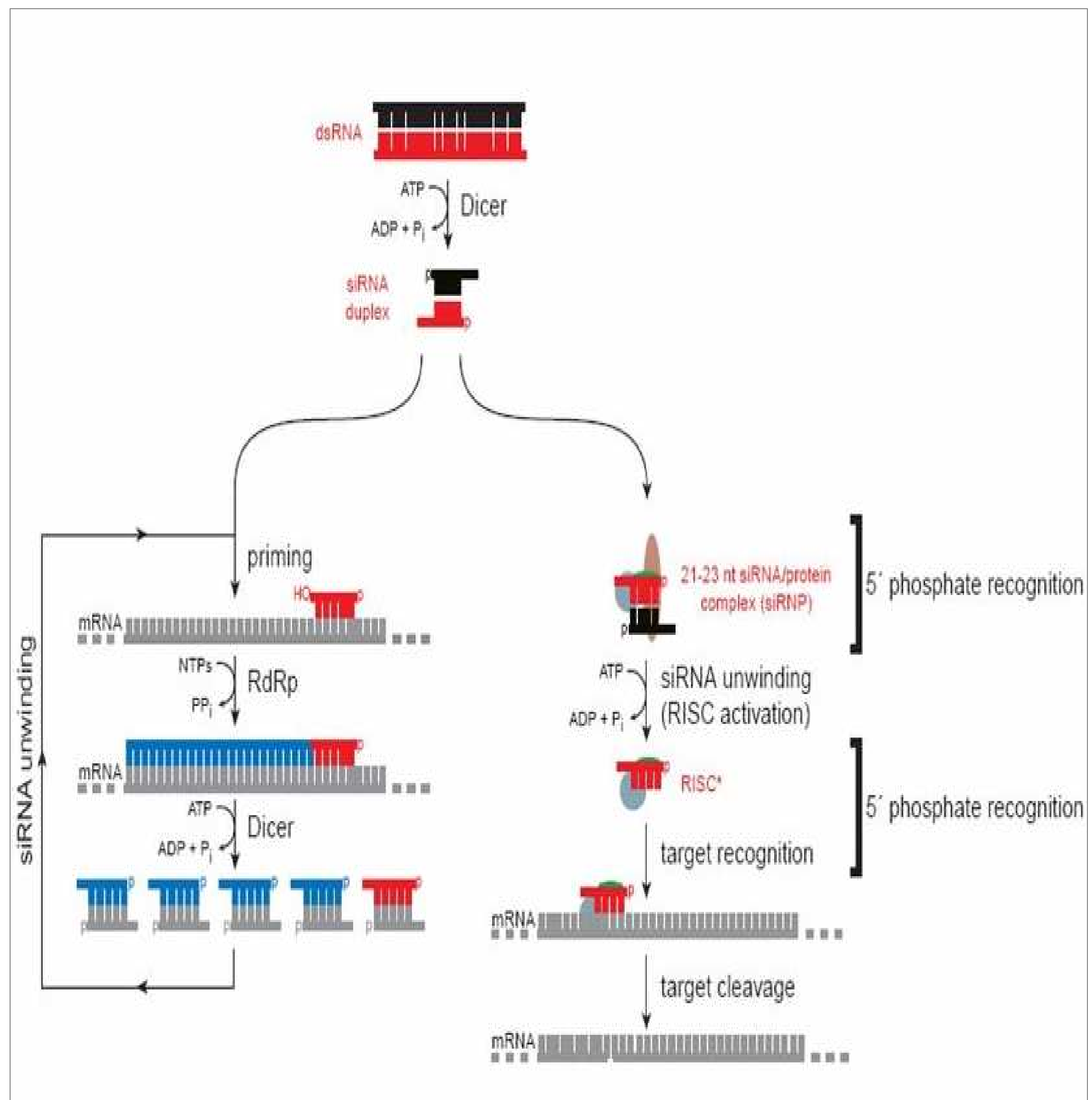


Figure 8: Proposed models for RNAi pathway in Drosophila.

In both models, dsRNA is converted to siRNA by an ATP dependant endoribonuclease Dicer. The models differ in the subsequent function of siRNAs. In the left side of the above figure, siRNAs are postulated to function as primers for the target RNA-templated synthesis of cRNA by an RdRP. The final product of this process which is dsRNA is cleaved by Dicer into a new crop of siRNAs, which can prime the conversion of additional target RNAs into dsRNA. In the right, siRNAs are proposed to be incorporated into an endonuclease complex distinct from Dicer, the RISC, and again according to an ATP dependant mechanism, whereas endonucleolytic cleavages of the target RNA appear to require no high energy cofactors.

2.4 Transcriptional gene silencing (TGS)

Transcriptional gene silencing (TGS) is induced by the same molecules that induced post-transcriptional gene silencing (PTGS) but results in activation of the gene for transcription rather than by RNA destruction (Sijen et al, 2001). In plants, double-stranded RNA induces a transcriptional gene silencing accompanied by *de novo* methylation of a target promoter. This effect can be triggered by a double-stranded RNA containing promoter sequences. The promoter dsRNA is synthesized in the nucleus, is partially cleaved into small RNAs ~23 nucleotides in length. Mette et al. (2000) were able to induce transcriptional gene silencing in tobacco and *Arabidopsis* by using constructs designed to produce double-stranded promoter RNA

Until recently, there was no evidence that a similar pathway operated in mammals. Two new studies suggest that small RNAs can direct DNA methylation and chromatin modification in human cells. Morris et al. (2004) proved that promoter-directed siRNA inhibits transcription of an integrated, proviral, elongation factor 1 alpha (EF1α) promoter-green fluorescent protein reporter gene and of endogenous EF1α. Silencing was associated with DNA methylation of the target sequence.

2.4.1 Proposed mechanisms for TGS in S. Pombe

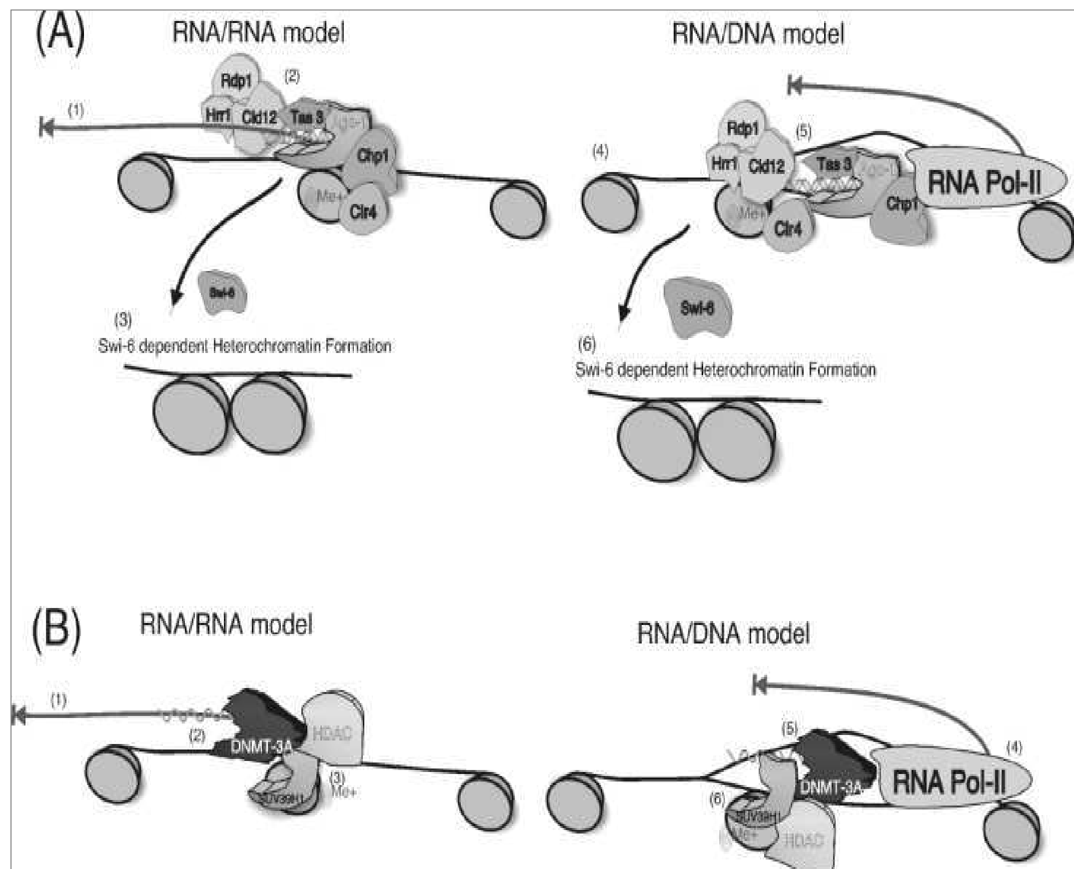


Figure 9 : Proposed mechanisms for TGS in S. pombe
Two models for siRNA mediated TGS have been proposed, either an RNA/RNA or a RNA/DNA mediated mode of silencing. In S. pombe (A) siRNAs may interact with a long non-coding transcript which spans the targeted chromatin (1) subsequently allowing the RITS/RdRP complex to localize to the targeted region (2) resulting in gene silencing (3) reviewed by Agrawal et al, (2003). Alternatively, siRNA mediated silencing may function through an RNA/DNA intermediate. The siRNAs may gain access to the targeted DNA by the effects of RNA Pol-II opening up the targeted region (4) for the siRNA/RITS/RdRP complex to gain access (5) leading to gene silencing (6). (See Fig 9 A&B for more details).

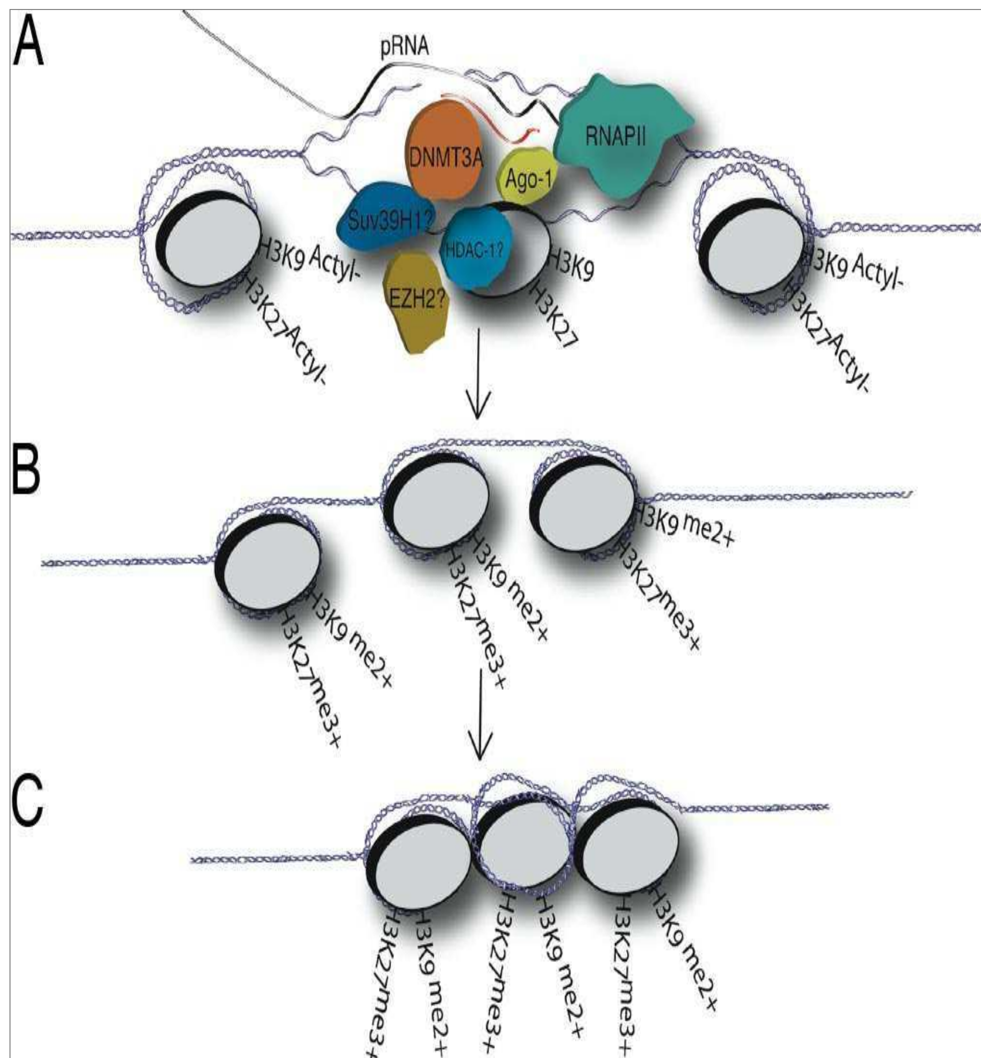


Figure 10 : Model for RNA-directed TGS in human cells

(A) The promoter associated RNA model of RNA-mediated TGS proposes that a variant species of mRNA, a promoter-associated mRNA, essentially containing an extended 5_UTR, is recognized by the antisense strand of siRNAs or possibly endogenous antisense RNAs during RNAPII-mediated transcription of the RNA-targeted promoter. (B) The antisense strand of the siRNA might then guide a putative transcriptional silencing complex (possibly composed of DNMT3A, Ago-1, HDAC-1, and/or EZH2) to the targeted promoter where histone modifications result and the initial gene-silencing event. (C) The initial silencing event or prolonged suppression of the siRNA-targeted promoter may result in heterchromatization of the local siRNA-targeted genomic region and is not, based on these data, thought to be the result of slicing of the low-copy promoter associated RNA but rather due to a recruitment of chromatin remodeling factors or complexes to the targeted promoter that result in the gene silencing (Han et al, 2007).

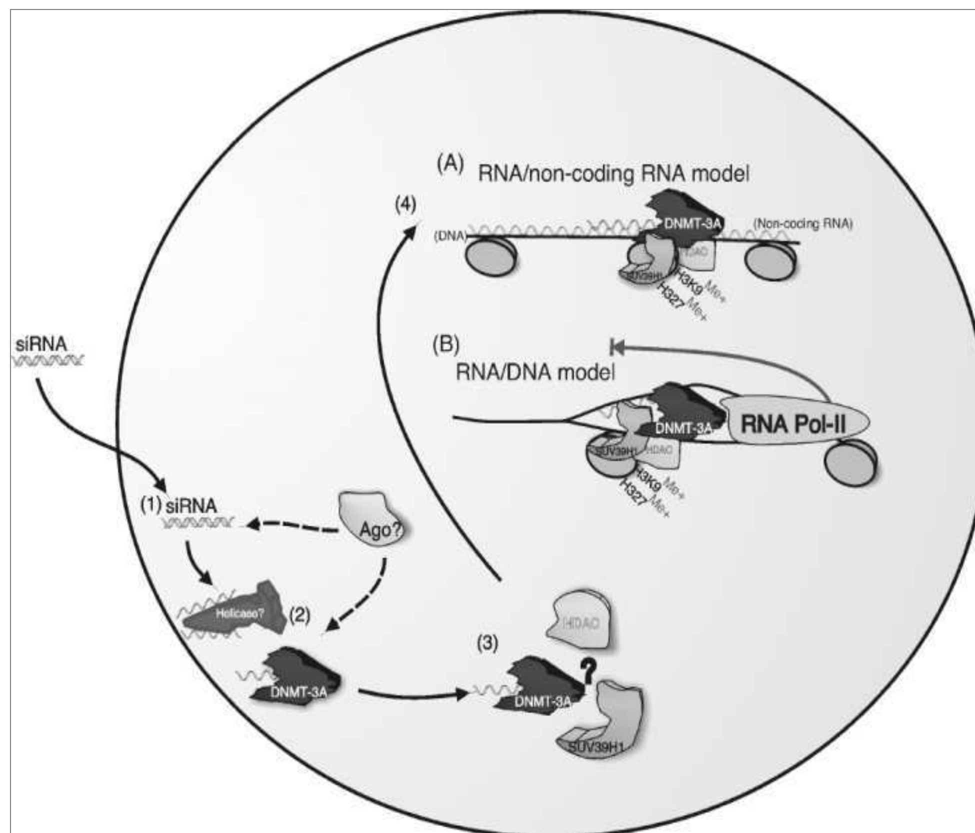


Figure 11 : proposed mechanism for TGS in human.

2.4.2 Proposed mechanisms for TGS in human.

A. Model one for siRNA mediated TGS in Human Cells

In a transcriptionally active gene (1) promoters are transcribed by RNA Polymerase II (RNA Pol II) to produce a low copy promoter specific transcript (2). Then the siRNAs may associate with argonaute 1 (Ago-1) and possibly a complex containing DNMT3A (2). The forming complex might interact directly or be bound by HDACs and/or Suv39H1 (3). At this step or prior to this step Ago-1 may also be active to unwind the siRNA, resulting in that the antisense strand from the siRNA probably directs the siRNA/DNMT3a complex with or without the HDACs and/or Suv39H1 or EZH2 to the targeted low copy promoter RNA (3). The promoter RNA corresponds in location to the targeted promoter and the siRNA provides the specificity in targeting a chromatin remodeling complex probably containing HDAC-1 which could deacetylate histone 3 Lysines 9 and/or 27. The deacetylated histones would then permit histone methlytransferases to methylate H3K9 and H3K27 (3). The result of the above mentioned steps would be the initial silencing of transcription at the RNA

Pol II targeted promoter (4). If the silencing is reinforced, the gene may become methylated and permanently silenced (Figure 11B).

B. The second model for siRNA mediated TGS in Human Cells.

In model two a non-coding RNA might be produced by RNA Pol II (1) the produced RNA could be interwoven with the DNA in chromatin (2). This RNA could then act as scaffolding for the antisense strand of promoter specific siRNAs to recognize the corresponding targeted promoter (3). The antisense strand of the promoter specific siRNA is probably associated with a chromatin remodeling complex containing Ago-1, DNMT3A, HDAC1, Suv39H1, and possibly EZH2 (as described previously in Model 1). The promoter siRNA targeted complex could then specifically remodel the targeted promoter local histones, i.e. deacetylate histone 3 lysines 9 and/or 27, which would then allow methylation of the promoter associated histones via histone methyltransferases. The result would be the initial silencing of transcription at the RNA Pol II targeted promoter (4). If the silencing is reinforced the gene may become methylated and permanently silenced (Figure 11A).

3. Natural role of RNAi

In 1990, Napoli et al., (1990) was trying to change the color of petunias by inserting a supercharged copy of the gene that controls production of purple pigment. Unexpectedly he got instead white petunias. Nowadays it is clear that Jorgensen had stumbled across a natural mechanism of gene silencing a process which is well known now as RNA interference (RNAi). In this process a double strand RNA is capable of inducing gene turn off (Napoli et al., 1990).

It remained so until 1998 when Fire and coworkers (Fire et al., 1998) showed for the first time that double-stranded RNAs (dsRNAs) were able to trigger sequence-specific gene silencing in a wide variety of organisms, including nematodes, plants, trypanosomes, fruit flies and planaria. For the first time this work opened a promising window towards an effective set of tools to interfere selectively with gene function (Fire et al., 1998). RNA interference (RNAi) is considered to be an effective genome defense mechanism. For the genome defense system to be effective, it meets two main requirements: it needs to be gene-specific and it asks an amplification step to fight off multiplying parasites. RNAi meets these requirements as it specifically recognizes

dsRNA and sometimes like in plants and *c elegans* it contains an RNA amplification step.

In plants, RNAi appears to be a defense mechanism against molecular parasites such as viruses and transposons. Motley et al., (2000) and Waterhouse et al.,(2001) demonstrated that as a consequence of virus infection or transposable elements, plants have developed adaptive mechanism against this infection. In plants, viral genomes can be targeted by the RNAi machinery and probably as a response to it. Viruses on their own have developed strategies to inhibit RNAi, as plant viruses were found to carry silencing-suppresser genes that act to limit the efficacy of RNAi in various ways (Waterhouse & Fusaro, 2006). It is known that RNA viruses replicate via an RNA intermediate of opposite polarity: thus viral genomes and replicative intermediates can form dsRNA which can trigger RNAi formation.

Tabara et al., (1999) proved that *C. elegans* mutants which are deficient in RNAi were able to activate several transposons. This finding supports the idea that one of the natural roles of RNAi system is transposon silencing. Apparently, transposons are normally silenced in *C. elegans* (which is a germline-specific process) and this process is dependent on the RNAi pathway. These *C. elegans* transposons have terminal inverted repeats and transcripts having both terminal inverted repeats can base pair and form dsRNA which could trigger RNAi. Indeed, such dsRNA was detected.

Despite the intense studies of RNAi, there has been no evidence that RNAi work as a system of defense against viral infection in vertebrates, while in plants it is clear that RNAi controls viral infection (Voinnet et al, 1999). Vertebrate viruses recruit several genes to the battle with the host interferon and adaptive immune systems, but still no genes are yet known in vertebrate viruses that antagonize the RNAi system.

Sequencing of the human genome has shown that 45% consists of remnants of transposon as separated sequences and it is conceivable that RNAi plays a role (or played a role in previous time) in defending genome against these molecular parasites, a mechanism probably not strictly necessary in vertebrates.

In worms, parts of the RNAi machinery are important not just for suppressing replication of transposons but also for forming small RNAs—microRNAs (miRNAs). MicroRNA can also function as reverse regulators of disease. So, when certain microRNAs have low levels of expression, their target genes are not suppressed and aggressive. Recently it has been shown that two microRNAs regulate the most

common human leukemia (B-cell lymphocytic). These two microRNAs are miR-29 and miR-181.

RNAi besides its natural function has several and various possible applications. RNAi are being used: (1) to induce virus resistance in transgenic organisms, (2) to improve the quality or production of crops by suppressing unwanted traits (like the softening of tomato fruits or the content in caffeine and nicotine) (3) to eliminate gene transcript (knock-down strategy) acting by reverse genetics strategies known to make it possible (4) to perform certain types of gene-therapy by cleavage of disease-derived transcripts (viral RNAs or mRNAs of cell proliferation genes in cancer cells) (http://www.niob.knaw.nl/researchpages/ketting/publications.html).

CHAPTER 2

THERAPEUTIC GENE SILENCING AND INHIBITION OF PATHOGENS BY RNAi

Gene therapy is the process of inserting nucleic acids (e.g. usually DNA/gene) into cells or tissues to correct or prevent a pathological process. Examples include the gene addition for the treatment of genetic disorders as well as therapeutic nucleic acids to stimulate new cell growth for tissue regeneration, demise of cancerous and virus infected cells (as defined by the American society for gene therapy) (http://www.asgt.org/about_asgt/index.shtml#3).

Nowadays, RNAi is routinely used in laboratories for loss-of-function analyses and increasingly for the rescue of phenotypes caused by dominant acting mutant genes. Scientists have succeeded in applying RNAi in vitro and in vivo to block the effects of disease genes. One of the most important promising features of the RNAi is the ability to generate gene knockdown for studying gene functions by reverse genetics (Haasnoot et al., 2003).

1. Inhibition of pathogens by RNAi

It is now apparent that the mechanisms that mediate RNAi have been evolutionarily conserved in all multicellular eukaryotes, thus indicating that this unique form of homology-dependant gene silencing is a key to one or more aspects of eukaryotic biology. One obvious potential function for RNAi machinery would be to defend cells against viruses that express dsRNA as part of their life cycle.

The role of RNAi during viral infection in mammals is still under investigations. Some reports suggest that mammalian cells have strong-nonspecific responses to viral dsRNA through the direct interaction of dsRNA with the cellular proteins, such as protein kinase R, retinoic acid- inducible gene I (RIG-1) or toll-like receptor (TLR) 3, which is considered to be an important factor triggering signaling pathways that lead to the expression of type one interferon and the activation of non-specific RNases. The expression of interferon leads to the expression of a large number of genes that creates an antiviral state in the host cell as reviewed by Elbashir et al., (2001a). As it was cited by Elbashir et al., (2001a), it has been demonstrated that the introduction of dsRNA longer than 30bp into mammalian cells were responsible for an induction of interferon pathway via dsRNA-dependant protein kinase (PKR) and for apoptosis of infected cells.

Besides, the introduction of chemically synthesized siRNAs or short hairpin RNA (shRNAs) generating siRNA less than 23 bp could be efficient to induce RNAi mediated gene silencing pathway when transfected into cells.

Moreover, it can be readily demonstrated that the artificial induction of an antiviral RNAi response in mammalian cells can confer strong protection against a wide range of pathogenic viruses (Table 2) (Dykxhoorn et al., 2003; Cullen, 2006b). It has been reviewed by Plaskert (2006) that there are some unanticipated interaction between RNAi machinery and mammalian viruses. The interactions of virus–specific small interfering RNAs (siRNAs) into cell provoke the RNAi system to target viruses, resulting in an effective therapeutic approach to inhibit virus replication *in vitro* and in animal models. For example Franz et al., (2006) have demonstrated that using RNAi generate Dengue virus resistance in genetically modified *Aedes aegypti*.

It has been well demonstrated that RNAi is a powerful tool for the inhibition of numerous viruses, including several important human pathogens such as human immunodeficiency virus type 1, hepatitis C virus, hepatitis B virus, dengue virus, poliovirus and influenza virus A (Haasnoot et al., 2003).

Table 2 : Examples of disease-related genes that have been targeted in mammals using siRNA

Gene/mRNA targeted	Type of gene	Method	Phenotype
p24	HIV-1 capsid protein	siRNA transfection; siRNA transfection of *in vitro* transcribed RNA	Decreased viral protein expression, decreased virus production; inhibition of HIV replication after fusion and before reverse transcription and transcription from integrated provirus
Rev	HIV-1 regulatory protein	siRNA transfection; plasmid-vector-mediated siRNA expression (tandem U6 promoters)	Decreased viral protein expression, decreased virus production
Vif	HIV-1 regulatory protein	siRNA transfection; plasmid-vector-mediated siRNA expression	Inhibition of HIV replication, degradation of preintegrated genomic HIV RNA
Tat	HIV-1 regulatory protein	siRNA transfection	Decreased viral protein expression, decreased virus production
LTR mRNA	HIV-1 long terminal repeat	siRNA transfection, *in vitro* transcribed siRNA	Inhibition of HIV replication after fusion and before reverse transription and transcription from integrated provirus

Poliovirus capsid	Capsid structural protein	siRNA transfection	Reduced viral titer, clearance of virus from infected cells
Poliovirus RNAP	RNAP	siRNA transfection	Reduced viral titer, clearance of virus from infected cells
HPV E6 mRNA	Viral transcript E6	siRNA transfection	Selective degradation of *E6* mRNA, accumulation of cellular p53, reduced cell growth
HPV E7 mRNA	Viral transcript E7	siRNA transfection	Selective degradation of *E7* mRNA, induced apoptotic cell death
RSV P protein	Phosphoprotein, smaller subunit of the RNA-dependent RNAP	siRNA transfection	Inhibition of P protein expression, reduced amounts of all viral proteins, no syncytia formation
RSV F protein	Fusion protein	siRNA transfection	No detectable F protein, no effect on other viral proteins, no syncytia formation
Hepatitis C virus NS5B	Non-structural protein 5B, viral polymerase mRNA	Hydrodynamic' siRNA injection	Decreased levels of the NS5B–luciferase fusion protein in mouse hepatocytes
Ras(V12)	Constitutively active oncogenic ras mutant	Moloney-based retroviral-vector-mediated siRNA expression	CAPAN-1 cells failed to form colonies in soft agar and failed to form tumours in nude mice when injected subcutaneously
bcr-abl	Oncogene, fusion of abl and bcr	siRNA transfection	Specifically decreased the *bcr–abl* mRNA without targeting either the *c-abl* or *c-bcr* mRNA, inhibited bcr–abl-dependent cellular proliferation
p53	Tumour suppressor gene	Plasmid-vector-mediated siRNA expression, Moloney-based retroviral-vector-mediated siRNA expression	Selection of cells stably knocked down in p53 expression; different p53 shRNAs produced different degrees of silencing, which was directly correlated with the severity of Myc-induced lymphomagenesis; loss of ras-induced senescence, growth in soft agar
53bp1	p53-binding-protein-1, mediator of DNA damage checkpoint	siRNA transfection	Decreased p53 accumulation, disruption of G2–M checkpoint arrest, intra-S-phase checkpoint in response to ionizing radiation
p73Dn	Tumour suppressor gene	siRNA transfection	Increased activity of p53-responsive promoter
Fas receptor	Proapototic Fas receptor	'Hydrodynamic' siRNA injection	Decreased levels of Fas receptor in murine hepatocytes *in vivo*, increased resistance to Fas-mediated apoptosis
CD4	Cell surface receptor, HIV-1 coreceptor	siRNA transfection	Decreased HIV-1 infection, decreased free viral titers
CCR5	Cell surface receptor; HIV-1	siRNA transfection; lentiviral-vector-	Decreased cell surface expression of receptors, inhibition of CCR5 tropic HIV-1 virus replication

	coreceptor	mediated siRNA expression	
CXCR4	Cell surface receptors, HIV-1 coreceptors	siRNA transfection	Decreased cell surface expression of receptors, inhibition of CXCR4 tropic HIV-1 virus replication
CD25	IL2 receptor α	Lentiviral-vector-mediated siRNA expression	Reduced cell surface expression of CD25, decreased proliferation of T cells when challenged with IL-2

Abreviation : **HPV**, human papilloma virus; **mRNA**, messenger RNA; **siRNA**, short interfering RNA; **shRNA**, short hairpin RNA; **RNAP**, RNA polymerase; **RSV**, respiratory syncytial virus. **(From Dykxhoorn et al., 2003).**

In recent years, RNAi has therefore been welcomed by the scientific community as a potentially powerful new tool to target diseases. Joost Hassnoot et al., (2007) demonstrated that results from *in vitro* studies and animal models indicate that RNAi can be highly effective at low dosage. Several RNAi-based antiviral drugs are currently being tested in clinical trials (See Table 3 for more details). The development of RNAi therapeutics is taking place at an unprecedented speed, moving from an obscure phenomenon reported in plant and *c. elegans* to therapeutic compound in clinical trials in the last few years.

Table 3 : Nucleic acid–based antiviral therapeutics that have entered clinical trials.

Virus	Inhibitor (name)	Target gene	Stage	Sponsor
CMV	Antisense oligonucleotide[a] (Vitravene; formivirsen/ISIS 2922	*IE2*	Approved	Isis Pharmaceutials (Carlsbad, CA, USA)
HIV-1	Ribozyme[b] (Rz2, OZ-1)	*tat*	Phase 1 complete, Phase 2 ongoing	Johnson & Johnson (New Brunswick, NJ, USA) subsidiary Tibotec Therapeutics (Bridgewater, NJ, USA)
	937-nt antisense gene[b] (*VRX496)*		Phase 1 complete	Gene Shears and Johnson Research
		env	Phase 1 complete	VIRxSYS (Gaithersburg, MD, USA)
	Dominant-negative anti-HIV-1 gene[b] (*RevM10*)	*rev*	Phase 1/2 ongoing	Systemix (Palo Alto, CA, USA) and National Cancer Institute (Bethesda, MD, USA)
			Phase 1 complete	The Saban Research Institute/USC Keck
				School of Medicine (Los Angeles)

	Decoy RNA[b]	*RRE*	Phase 1 complete	Childrens Hospital Los Angeles, University of Southern California School of Medicine (Los Angeles, CA) and Baylor College of Medicine (Houston)
	Short-hairpin RNA, ribozyme and RNA decoy[b] (Triple-R vector)	*tat/rev*, *CCR5*, *TAR*	Phase 1 complete	Colorado State University (Fort Collins, CO, USA) and Beckman Research Institute (Duarte, CA, USA)
	Antisense *TAR* and *RevM10*[b]	*TAR, rev*	Phase 1 complete	National Human Genome Research Institute (Bethesda, MD, USA)
	Antisense oligonucleotide[a] (*Gem92*)	*Gag*	Phase 2 discontinued	Hybridon (now Idera Pharmaceuticals, Cambridge, MA, USA)
	Antisense[b] *(HGTV43)*	ND	Phase 1/2 ongoing	Enzo Biochem(Farmingdale, NY, USA)
	Peptide nucleic acid (AVR-118)	ND	Phase 1/2 completed	Advanced Viral Research (Yonkers, NY, USA)
RSV	Small interfering RNA[a] (*ALN-RSV01*)	*Nucleocapsid*	Phase 1 ongoing and phase 2 planned for 2008	Alnylam Pharmaceuticals (Cambridge, MA, USA)
HCV	Ribozyme[a] (Heptazyme)	*IRES*	Phase 2 studies discontinued	Ribozyme Pharmaceuticals (Boulder, CO, USA; renamed Sirna, now part of Merck)
	Antisense oligonucleotide[a] (AVI-4065)	ND	Phase 2 studies discontinued	AVI BioPharma (Portland, OR, USA)
	Antisense oligonucleotide[a] (ISIS14803)	*IRES*	Phase 2 studies discontinued	Isis
HBV	Short-hairpin RNA[b] (*Nuc B1000*)		Phase 1 completed	Isis
		Pre-gen./pre-C, Pre-Sl, Pre-S2/S, X	Phase 1 ongoing	Nucleonics (Horsham, PA, USA)
HPV[c]	Antisense oligonucleotide[a] (MBI1121)	***El***	Phase 1 discontinued	Migenix (formerly Micrologix Biotech, Vancouver, BC, Canada)
	Peptide nucleic acid (AVR-118)	ND	Phase 1 discontinued	Advanced Viral Research (Yonkers, NY, USA)

Abbreviations
[a]Chemically synthesized. [b]Gene construct. [c]Human papillomavirus. ND, not disclosed. NA, not available

The RNAi therapy is moving toward clinical applications in humans and animals (Table 2). Its success for some diseases has not yet been definitely met and it needs further investigation. There is huge potential for crossing the obstacles and for using this technology in gene therapy. Yet the off-target effect as well as the delivery system are considered to be the most challenging problems facing this promising technology (Ralph et al., 2005).

Morris, (2006) showed that the *in vitro* HIV-1 multiplication was suppressed through siRNAs directed against HIV-1 tat and rev transcripts. Other viruses have also been successfully targeted by siRNAs *in vitro* with some success including Semliki Forest Virus (SFV), poliovirus, dengue virus, influenza virus, and hepatitis C virus and others.

It has been shown by Castanotto et al., (2002) and Lee et al., (2002) that nematodes and insects use RNAi to reduce the infection by flock house virus (FHV), a member of the nodavirus family. This virus is able to cause infection for both insects and vertebrate cells, and the infection of drosophila cells results in the increase of FHV specific siRNAs. It has also been reviewed by Haasnoot et al., (2003) that insect viruses SFV and FHV were strongly inhibited by the introduction of dsRNA.

Several studies were performed to determine whether RNAi targets incoming genomic RNA, the newly synthesized transcripts or both. It has been reported by several groups (Capodici et al., 2002; Jacque et al., 2002; Novina et al., 2002) that the incoming genomic RNA is indeed the target of siRNA leading to its destruction. Other reports by Coburn & Cullen, (2002), Hu et al., (2002) and Verma et al., (2003) showed no or only modest reduction in the level of proviral RNA. From those reports it remains possible that different siRNAs produce different effects. Some incoming RNA is protected from RNAi–mediated degradation by nucleocapsid (Hu et al., 2002). In contrast to RNA viruses, it has been shown that DNA viruses targeted by RNAi can result in degradation of the viral mRNAs (Jia & Sun, 2003).

McCaffrey et al., (2003) were the first to demonstrate that virus inhibition by RNAi in vivo is possible by a co-transfection of Hepatitis B Virus (HBV) DNA and shRNA expressing plasmid targeting the HBV sequence. The HBV multiplication was highly inhibited in mice liver.

There are now several reports on the in vitro inhibition of human and animal viruses (Table 4 and 5 for RNA and DNA viruses respectively).

Table 4 : Inhibition of RNA viruses by RNAi as illustrated in (Haasnoot et al., 2003).

Virus	Target gene	RNAi inducer	Cell type	Fold inhibition of virus replication
Dengue virus	capsid, PrM, NS5	~250-ntssRNA[a]	mosquitoes, BHK, C6/36	>50
	PrM	290-nt dsRNA plasmid derived[b]	C6/36	100
	PrM,E,NSl,NS5	77-nt dsRNA	C6/36	10
HCV	NS3, NS5B	21-bpsiRNA	Huh-7	10
	capsid, NS4B	21-bpsiRNA	Huh-7.5	-100
	5'UTR	21-bpsiRNA	Huh-7	6.7
	5'UTR, NS3, NS5b	21-bpsiRNA, intracellularly expressed siRNA[c]	Huh-7	-10
	5'UTR	21-bpsiRNA, intracellularly expressed siRNA[d]	Huh-7	~5
Polio virus	capsid, 3DP°'	21-bpsiRNA	HeLa S3, mouse embryonic fibroblasts	100
Influenza A viras	PB1, PB2,PA,NP,M,NS	21-bpsiRNA	MDCK, chicken embryos	200
SFV	Nsp-1,-2,-4	77-nt dsRNA	C6/36	2
RRV	VP4	21-bpsiRNA	MA104	-4
FHV	3TJTR	500ntdsRNA	S2	>100
RSV	Gag	21 bpsiRNA	chicken embryos, DF-1	5-10

All dsRNA fragments were transfected in the cells unless indicated otherwise. [a] Intracellular expressed dengue virus ssRNA using sindbis virus as a vector. [b] 290-bp hairpin RNA expressed from a transfected plasmid under the control of hsp 70 promoter.
[c] siRNAs expressed from a transfected plasmid under the control of two HI promoters for the sense and antisense fragment. [d] siRNAs expressed from a transfected plasmid under the control of two U6 promoters for the sense and antisense fragment, or as a 19-bp shRNA. The fold inhibition of virus production represents the result obtained with the most efficient siRNA

Table 5 : Inhibition of DNA viruses by RNAi as illustrated in (Haasnoot et al., 2003).

Virus	Target gene	RNAi inducer	Cell type	Fold inhibition of virus replication
HBV	X, core core, HBsAg/ Pol,X core	intracellularly expressed shRNA[a] intracellularly expressed shRNA[b]21-bpsiRNA	Huh7, HepG2 Huh-7, mice Huh-7, HepG2	20 >6 -4.7
HPV-16	E6, E7	21-bpsiRNA	CASKi, SiHa	No[c]
OpA/NPV	Op-iap3	511-ntdsRNA	Sf21,Ld652Y	No[c]
AcNPV	Gp64, Iel	619-,451-ntdsRNA	Sf21, *T. mollitor* larvae	>10
MHV-68	Rta,ORF45	21-bpsiRNA	293T	>43

All dsRNA fragments were transfected in the cells unless indicated otherwise. A 21 -bp shRNA stably expressed from pol III promoter, introduced via a retroviral vector. B 25-bp shRNA stably expressed from a plasmid under control of a U6 promoter. Q E6, E7 and Op-iap3 are non-essential viral genes, but virus production is negatively affected through apoptosis of the host cell.

From the wide range of viruses that can be successfully targeted by RNAi, we can conclude that these nucleic acid molecules can be used to target virtually any emerging or existing infectious agent. However, despite the excitement and the early proofs-of-principle in the literature, there are important issues and concerns about therapeutic applications of this technology.

These concerns include:

1. Difficulties to obtain efficient siRNA delivery
2. Uncertainty about potential siRNA toxicity
3. The emergence of siRNA resistant viruses (virus escape)
4. Off-targeting effect

It has been reported that viruses may escape the RNAi. This problem is true for viruses that exhibit genetic variation. This problem is considered being more severe for RNA viruses rather than DNA viruses (Haasnoot et al., 2003). Several reviews

(Llave et al., 2000; Hamilton et al., 2002; Lee et al., 2002; Mallory et al., 2002) suggest that certain viruses encode proteins that block one or more steps in the RNAi pathway. This resistance to siRNA may occur rather rapidly and could be due to a single nucleotide substitution

http://www.nature.com./gt/journal/v13/n6/fu11/3302688a.html-bib40

Recently HIV-1 has been shown to escape siRNA targeting through the evolution of alternative splice variants for the siRNA targeted transcripts (Morris, 2006).

There are several possible ways to circumvent RNAi resistance in therapeutic applications:

1. Designing siRNAs to best fit targets from an extensive data base of the variants of the particular virus.
2. To incorporate these best fit siRNAs into a multiple antiviral siRNA expressing transgene vector.
3. Alternatively, it could be better to design siRNAs targeting the conserved regions such as to target viral intron/exon splice junctions (Morris, 2006).

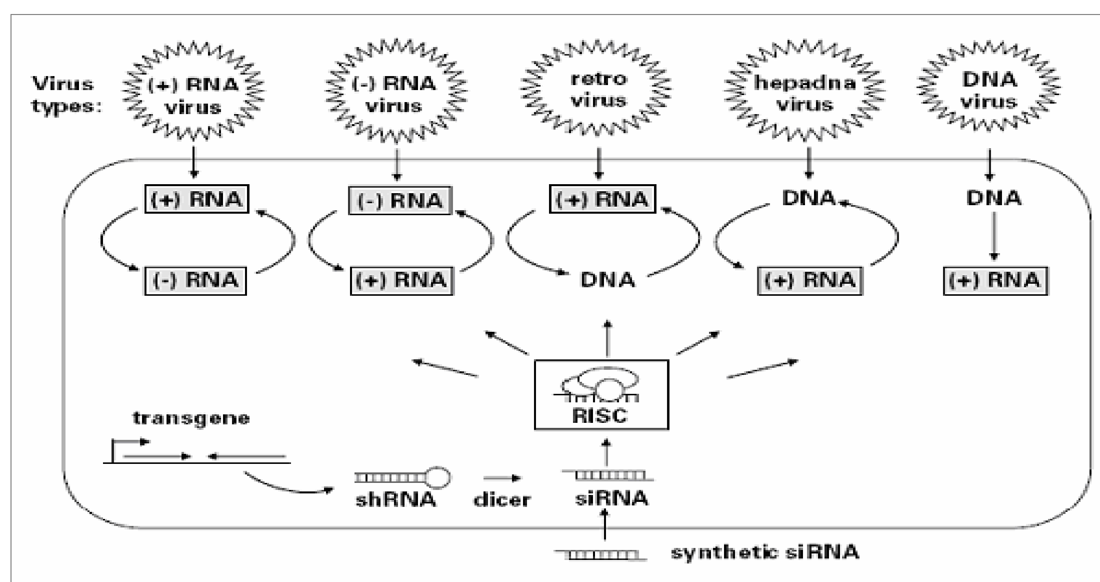


Figure 12 : Examples of some viruses that can be targeted by RNAi.
Synthetic siRNAs as well as short hairpin shRNA can lead to virus inhibition. All virus groups can potentially be targeted by RNAi. The viral RNA forms that are produced during replication and that can be targeted for destruction are boxed and marked grey. RNAi-mediated virus inhibition can be induced by transfection of synthetic siRNAs or by intracellularly expressed shRNA. Processing of shRNAs by dicer yields siRNAs that are incorporated into the RISC (From Haasnoot et al., 2003).

Thus far, the efficient inhibition of viral replication by RNAi technology has been described after transient transfection of siRNAs targeting the viral sequences into cells. But this transient transfection was proven not to be sufficient for long time interfering and can only provide temporary effects. Instead, researchers are interested in combining RNAi technology with gene therapy and focusing on intracellular production of siRNA or shRNAs which may provide long-term protection against the viral infection (Haasnoot et al., 2003).

One of the major problems limiting the use of RNAi as a tool for gene therapy and viral inhibition is the fact that the off target effect are still unclear. Moreover, the efficiency of siRNAs is highly variable, certain siRNAs being totally inefficient for no clear reasons.

2. RNAi methodology for the inhibition of virus genes

The RNAi can be used as direct medicine by the inhibition of virus genes (Boden et al., 2004). The advantage of gene knockdown over knockout is its relative simplicity, its flexibility and its reversibility (Houdebine, 2005). Vectors allowing an inducible and reversible induction of RNAi synthesis in transgenic animals are under investigation and should be available in the coming years.

Inhibition by the expression of certain genes by RNAi supposes that siRNA are present at the desirable moment in a desirable amount in the targeted cells.

SiRNAs can be obtained from various delivery processes:

1. Chemically synthesized siRNA,
2. Expression Vectors and Viral Vectors (including adenoviral, retroviral and lentiviral) containing siRNA or shRNA

   Expression vectors or viral vectors mostly used presently depend on RNA polymerase III (Pol III) promoters (Paddison et al., 2002). Normally, human or mouse U6 or human H1 are the most commonly used RNA polymerase III promoters. As Pol III enzyme initiates at known sites and terminates RNA transcripts at specific sites 5T, it is well suited for the synthesis of siRNAs or shRNAs (Abbas-Terki et al., 2002).
3. a group of siRNAs generated from Dicer or RNase III digested dsRNAs

siRNAs can be synthesized chemically and introduced into the cells by transfection. Another means consists of constructing vectors which express genes coding for of siRNA. siRNAs are of small size and they can be synthesized in a form directly usable only by vectors containing promoters of RNA polymerase III type. These vectors are very active when used at cell level but are not or poorly active in the case of transgenes. This type of gene construction is indeed very sensitive to the mechanisms of gene extinction. It has been shown that constructs containing genes for siRNA and a RNA polymerase III promoter are generally well expressed as transgenes when they are integrated into a lentiviral vector (Tiscornia et al., 2003). Other vectors must be developed to express siRNAs in an optimal way. One possibility is to construct vectors containing promoters of the type RNA polymerase II. These promoters which are responsible for the synthesis of the messenger RNAs are well known to work as transgenes and able to be active in various cell types in a specific and inducible way. This implies that the siRNAs generated by RNA polymerase II promoter are abundant and effectively released from the transcripts of the transgenes.

CHAPTER 3

THE MODEL: PSEUDORABIES VIRUS

1. Introduction and history

Pseudorabies Virus (PRV; also known as Suid Herpesvirus 1 or Aujeszky's Disease Virus) is the causative agent of Aujeszky's disease in pigs. This disease is characterized by illness affecting the nervous system as well as respiratory illness in older pigs. PRV can infect numerous other mammals and invariably leading to death in mice. Pigs can survive a productive PRV infection dependent on the age of the animal and the virulence of the virus. Therefore, they are considered the natural hosts of PRV (Granzow et al., 2001).

There has been huge works to potentially eradicate this virus and some success has been reported. While efforts to eradicate PRV in the USA and Europe have made great progress, it remains an endemic problem in many countries (Pomeranz et al., 2005).

1.1. Species affected

Pigs are the natural host for the pseudorabies virus and the only animal to become latently infected. However, the virus can infect nearly all mammals, including cattle, sheep, goats, cats, dogs and mice. It does not infect humans or most of the other primates and infections in horses are rare.

1.2. Herpesviruses

Herpesviruses are considered being large DNA-containing enveloped viruses that replicate in the nuclei of infected cells. According to the International Committee on Taxonomy of Viruses the family *Herpesviridae* is subdivided into the subfamilies *Alpha-, Beta-,* and *Gamma-herpesvirinae* based on their biological properties, genome content and organization, (Granzow et al., 2001). They have similar virion size (200 to 250 nm) and structure (capsid, tegument, and envelope), and undergo a latent phase in their life cycle (Pomeranz et al., 2005). There are some differences between the three major subfamilies in the cell types where latency is established and the length of their productive replication cycle.

Pseudorabies Virus (PRV) is classified as part of the alphaherpesviruses, which are considered as having the broadest host range among the three subfamilies. They tend to replicate rapidly with cytopathic effects and to produce viral particles in a matter of hours, establishing latency in the sensory ganglia.

It has been shown (Robbins et al., 1987) that humans harbor three alphaherpesviruses: varicella-zoster virus (VZV), herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2). Despite its significant homology to human alphaherpesviruses and its broad host range, there has been no evidence that PRV is transmitted to humans.

2. Molecular biology of PRV

The herpesvirus consists of virions particles which are all morphologically identical (Figure 13).

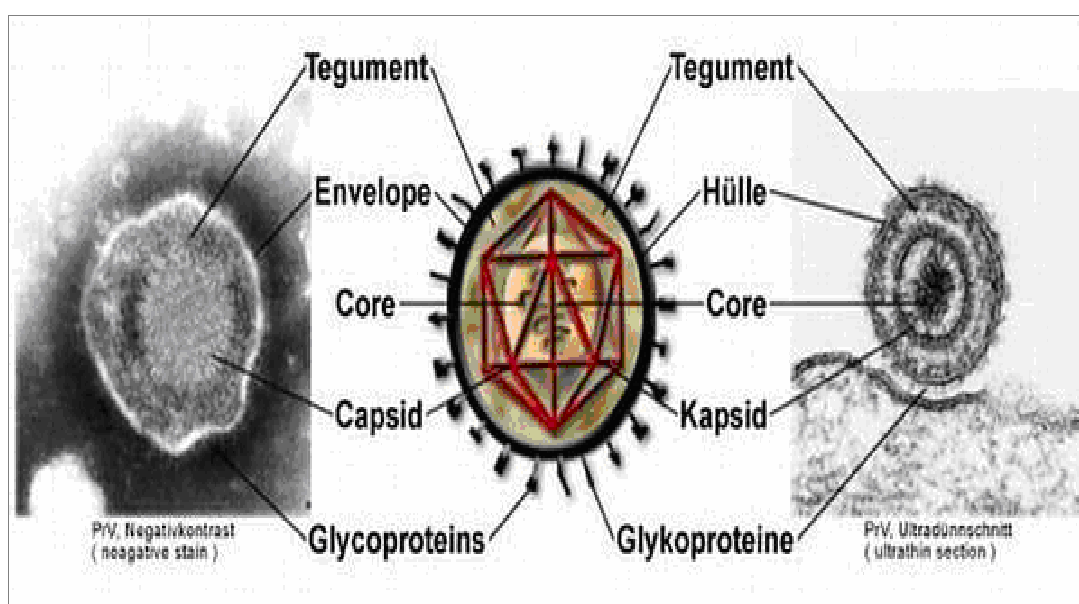


Figure 13 : Electron micrograph of Herpesvirus.
The Herpesvirus particle on the left is an electron micrograph of a thin- sectioned virion. On the right is a schematic view of the virion. As illustrated by the Friedrich- Loeffler- Institut.
http://www.fli.bund.de/589+M52087573ab0.html?&0=

As illustrated in Figure 13 and Figure 14 core contains the linear double-stranded DNA genome, which in the case of PrV encompasses 143.461 base pairs. The complete genomic sequence of PRV has been recently published (Klupp et al., 2004). The PRV genome is enclosed in an icosahedral capsid, which together form the nucleocapsid. This is surrounded by a proteinaceous material, the tegument. The outer envelope is derived from intracytoplasmic membranes and contains virally encoded proteins, mostly glycosylated proteins. These glycoproteins are not only major targets for the immune response of the host, they also play important functions during herpesvirus infection (Mettenleiter, 2003).

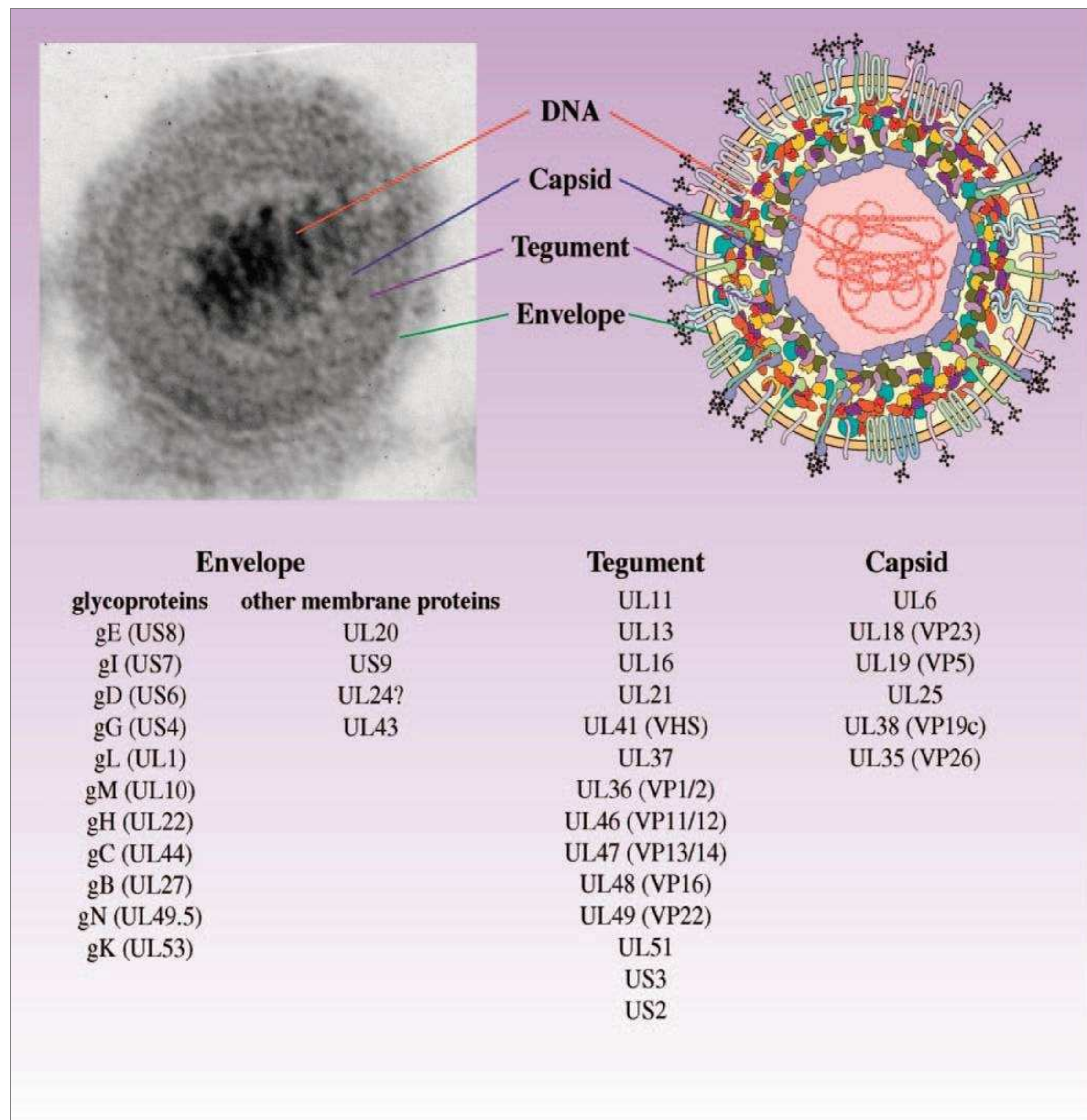


Figure 14 : The herpesvirus virion structure.
The central core contains the linear double-stranded DNA genome of the virus. The DNA is enclosed by protective icosahedral capsid forming the nucleocapsid. The capsid is embedded in a protein matrix known as the tegument; finally, the tegument is surrounded by the envelope, a lipid membrane containing several viral glycoproteins. Nearly half of all the PRV gene products are structural components of the mature virion (Pomeranz et al., 2005).

When the herpevirus virions attach to the cell membrane, the penetration process starts by direct fusion between the viral envelope and the plasma membrane. This leads to the generation of de-enveloped nucleocapsids which continue their way to reach the nucleopores by transport along cellular microtubules. The genomic DNA is then released into the nucleus, (Brittle et al., 2004).

2.1. Genome and gene content

The genome of the subfamily Alphaherpesvirus has several genes arranged in a partial collinear manner and encoding similar functions. The herpesvirus genomes can be divided into six classes depending on the overall arrangement of repeat sequences and unique regions.

It has been found that the PRV genome is characterized by two unique regions (U_L and U_S). The U_S region is flanked by the internal and terminal repeat sequences (IRS and TRS as illustrated in Figure 15). The sequence and gene arrangement of the entire PRV genome are known as well as a map of the likely transcript organization (Klupp et al., 2004).

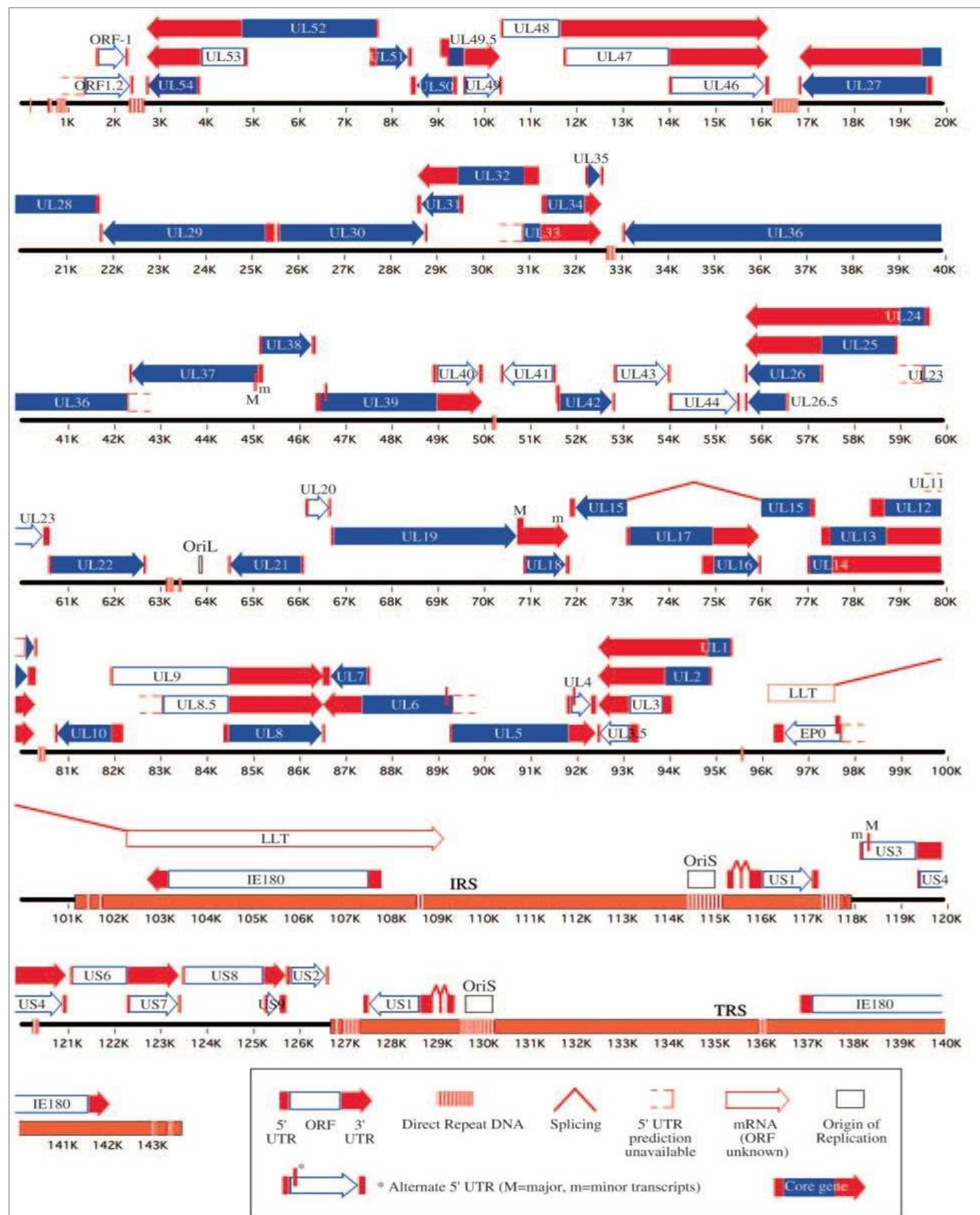


Figure 15 : The linear map of the PRV genome.
The linear map of the PRV genome in which the predicted gene and transcript organization are illustrated. As described earlier, the PRV genome consists of long and short unique segments, named UL and US, respectively. The US region is flanked by the inverted repeats IRS and TRS. Also the predicted locations of core and accessory genes, transcripts, DNA repeats, splice sites, and the origin of replication are indicated (Klupp et al., 2004)**.**

There have been around 70 different genes identified in PRV the functions of which are shown in table 6.

Table 6 : PRV gene functions.

Gene	Size (kDa)	Common name	Proposed function(s)[a]	Structural role	Core
ORF1.2	35.3		Unknown	Virion	No
ORF1	21.8		Unknown	Virion	No
UL54	40.4	ICP27	Transcription modulation; cell-cell spread; RNA-binding protein	Nonstructural	Yes
UL53	33.8	gK	Viral egress (secondary envelopment); glycoprotein K; type III membrane protein; gK/UL20 together inhibit glycoprotein-mediated membrane fusion	Virion (envelope)	No
UL52	103.3		DNA replication; primase subunit of UL5/UL8/UL52 complex	Nonstructural	Yes
UL51	25		Viral egress (secondary envelopment); tegument protein, potentially palmytoilated	Virion (tegument)	Yes
UL50	28.6	dUTPase	dUTPase	Nonstructural	Yes
UL49.5	10.1	gN	Immune evasion (TAP inhibitor); glycoprotein N; type I membrane protein; complexed with gM	Virion (envelope)	Yes
UL49	25.9	VP22	Interacts with C-terminal domains of gE & gM; tegument protein	Virion (tegument)	No
UL48	45.1	VP16, a-TIF	Gene regulation (transactivator); viral egress (secondary envelopment); tegument protein	Virion (tegument)	No
UL47	80.4	VP13/14	Viral egress (secondary envelopment); tegument protein	Virion (tegument)	No
UL46	75.5	VP11/12	Unknown; tegument protein	Virion (tegument)	No
UL27	100.2	gB	Viral entry (fusion); cell-cell spread; glycoprotein B; type I membrane protein	Virion (envelope)	Yes
UL28	78.9	ICP18.5	DNA cleavage and packaging; *component of the UL15/UL28 terminase*	Capsid precursor	Yes
UL29	125.3	ICP8	DNA replication and recombination; binds single stranded DNA	Nonstructural	Yes
UL30	115.3		DNA replication; DNA polymerase subunit of UL30/UL42 holoenzyme	Nonstructural	Yes
UL31	30.4		Viral egress (nuclear egress); present only in primary enveloped virion; interacts with UL34	Primary virion (tegument)	Yes
UL32	51.6		*DNA packaging; efficient localization of capsids to replication compartments*	Capsid precursor	Yes

UL33	12.7		*DNA cleavage and packaging; associates with UL28 and UL15*	Nonstructural	Yes
UL34	28.1		Viral egress (nuclear egress); present only in primary enveloped virion; tail-anchored type II nuclear membrane protein; interacts with UL31 Surface capsid protein	Primary virion (envelope)	Yes
UL35	11.5	VP26	Viral egress (capsid tegumentation); large tegument protein; interacts with UL37 and capsid	Virion (capsid)	Yes
UL36	324.4	VP1/2	Viral egress (capsid tegumentation); interacts with UL36	Virion (tegument)	Yes
UL37	98.2		*Minor capsid protein; UL38/UL18/UL18 triplex component*	Virion (tegument)	Yes
UL38	40	VP19c	Nucleotide synthesis; large subunit of ribonucleotide reductase	Virion (capsid)	Yes
UL39	91.1	RR1	Nucleotide synthesis; small subunit of ribonucleotide reductase	Nonstructural	Yes
UL40	34.4	RR2	Gene regulation, RNAse, degrades host and viral mRNAs	Nonstructural	No
UL41	40.1	VHS	DNA replication; polymerase accessory subunit of UL30/UL42 Holoenzyme	Virion (tegument)	No
UL42	40.3		Inhibits glycoprotein-mediated membrane fusion; type III membrane protein	Nonstructural	Yes
UL43	38.1		Viral entry (virion attachment); glycoprotein C; type I membrane protein; binds to heparan sulfate	Virion (envelope)	No
UL44	51.2	gC	*Major scaffold protein*; substrate for UL26 protease; capsid formation and maturation	Virion (envelope)	No
UL26.5	28.2	pre-VP22a	*Minor scaffold protein*; capsid maturation protease Capsid-associated protein; *required for capsid assembly*	Capsid precursor	Yes
UL26	54.6	VP24	Unknown; type III membrane protein	Capsid precursor	Yes
UL25	57.4		Nucleotide synthesis; thymidine kinase; selectively activates acyclovir	Virion (capsid)	Yes
UL24	19.1		Viral entry (fusion); cell-cell spread; glycoprotein H; type I membrane protein; complexed with gL	?	Yes
UL23	35	TK	Unknown, capsid-associated tegument protein; interacts with UL16	Nonstructural	No
UL22	71.9	gH	Viral egress; type III membrane protein; required	Virion (envelope)	Yes

			for gKprocessing; gK/UL20 together inhibit glycoprotein-mediated membrane fusion		
UL21	55.2		Major capsid protein; forms hexons and pentons	Virion (tegument)	Yes
UL20	16.7		*Minor capsid protein; UL38/UL18/UL18 triplex component*	?	
UL19	146	VP5	DNA cleavage and encapsidation	Virion (capsid)	Yes
UL18	31.6	VP23	Unknown; tegument protein; interacts with UL21	Virion (capsid)	Yes
UL17	64.2		*DNA cleavage/encapsidation; terminase subunit of the UL15/UL28 terminase; interacts with UL33, UL28 & UL6*	Virion (inner capsid)	Yes
UL16	34.8		Unknown	Virion (tegument)	Yes
UL15	79.1		Unknown; protein-serine/threonine kinase	Capsid precursor	Yes
UL14	17.9		DNA recombination; alkaline exonuclease	?	Yes
UL13	41.1	VP18.8	Viral egress (secondary envelopment); membrane-associated tegument protein	Virion (tegument)	Yes
UL12	51.3	AN		?	Yes
UL11	7	gM	Inhibits glycoprotein-mediated membrane fusion; glycoprotein M; type III membrane protein; C terminus interacts with tegument protein UL49; complexed with gN	Virion	Yes
UL1	41.5	OBP		Virion (envelope)	Yes
UL9	90.5	OPBC	*Sequence-specific ori-binding protein*, ATP-dependent helicase motif	Nonstructural	No
UL8.5	51		C-terminal domain of UL9	?	No
UL8	71.2		*DNA replication; part of UL5/UL8/UL52 helicase/primase complex*	Nonstructural	Yes
UL7	29		Unknown	?	Yes
UL6	70.3		*Capsid protein; portal protein; docking site for terminase*	Virion (capsid)	Yes
UL5	92.1		*DNA replication; part of UL5/UL8/UL52 helicase/primase complex*; helicase motif	Non-structural	Yes
UL4	15.8		Unknown	?	No
UL3.5	24		Viral egress (secondary envelopment); membrane-associated protein	?	No
UL3	25.6	UNG	Unkown	Nonstructural	Yes

UL2	33	gL	*DNA repair*; Uracil-DNA glycosylase	Nonstructural	Yes
UL1	16.5	ICP0	Viral entry (fusion); cell-cell spread; glycoprotein L; membrane anchored via complex with gH	Virion (envelope)	No
EPO	43.8	ICP4	Gene regulation (transactivator); early protein; ND10 structure modulation; contains RING finger motif	Virion	No
IE180	148.6	RSp40/ICP22	Gene regulation (transactivator); immediate-early protein	Nonstructural	
US1	39.6	PK	Unknown; *HSV-1 homolog (ICP22) acts as regulator of gene expression*	?	No
US3 (minor)	42.9	PK	Minor form of protein kinase (53-kDa mobility); inhibits apoptosis; mitochondrial targeting motif	?	No
US3 (major)	36.9		Viral egress (nuclear egress); inhibits apoptosis; major form of protein kinase (41-kDa mobility); found in both primary and secondary enveloped virions	Virion (tegument)	No
US4	53.7	gG	Unknown; glycoprotein G (secreted)	Secreted Virion (envelope)	No
US6	44.3	gD	Viral entry (cellular receptor binding protein); glycoprotein D; type I membrane protein	Virion (envelope)	No
US7	38.7	gI	Cell-cell spread; glycoprotein I; type I membrane protein; complexed with gE	Virion (envelope)	No
US8	62.4	gE	Cell-cell spread; glycoprotein E; type I membrane protein; complexed with gI; C-terminus interacts with UL49; protein sorting in axons	Virion (envelope)	No
US9	11.3	11K	Protein sorting in axons; type II tail-anchored membrane protein	Virion (tegument)	
US2	27.7	28K	Tegument protein; membrane associated protein	?	

Identified PRV genes, the functions of the 70 identified PRV genes

There are two copies of the genes encoding IE180 and US1 proteins because of their location within the IRS and TRS. Gene degradations names were derived from their location order within the UL or US region

2.2. Transcriptional architecture

In alphaherpesviruses the immediate-early genes or latency transcripts are found to be spliced. In PRV most of the core transcription elements are considered to be shared between genes. The TATA boxes initiate divergent transcripts and they are also functioning as polyadenylation signals for upstream genes. As shown in Figure

15 PRV genome also contains multiple short DNA repeat elements, which are found in between converging transcripts, the function of which could be to prevent transcription from one gene into an oppositely transcribed gene (Klupp et al., 2004).

2.3. Core genes

Core genes are those which are highly conserved among *Alpha-*, *Beta-*, and *Gammaherpesvirinae*. There is a set of 40 herpesvirus genes which are highly conserved among the three subfamilies. It encodes proteins that perform steps considered as being crucial for the replication of herpesviruses. Almost all PRV core genes are found in the U_L region (Figure 14).

3. Immediate- early gene

It has been found that Pseudorabies virus (PRV) immediate-early (IE) gene product is required as a transactivator for expression of the viral early and late genes. During infection, the IE gene is the first gene to be expressed (Taharaguchi et al., 2003). Vlcek et al., (1989) were the first to report the complete DNA sequence of the PRV immediate-early (IE) gene, the surrounding genes and its flanking nucleotide sequences which represent all together around 5091 base pairs. This sequence consists of a coding region starting with an **ATG** codon in position 263 from the transcription-initiation site and ends with a **TGA** codon in position 4601, resulting in a protein of 1446 amino acids (150kDa). In this protein there are two regions of PRV IE protein extending from amino acids 482 to 659 and 959 to 1350 Vlcek et al., (1989). The replication of the herpesvirus is regulated in a cascade fashion. In this process, synthesis of the α-transcript does not require the prior production of proteins, but synthesis of β-transcripts does and the synthesis of γ-transcripts additionally requires viral DNA replication. Together these three phases α-β-γ form the immediate- early (IE) (Vlcek et al., 1989). After the infection of susceptible cells with PRV, the previously shown phases require 0-1 hr, 1-3 hr, and 3-9 hr, respectively. There are two proposed models for the production of IE transcripts after the infection with PRV. The first model suggests the production of only one unique IE transcripts of 5.1 kb giving a single (180 kDa) IE protein. The second model requires the synthesis of three structurally related proteins (Vlcek et al., 1989). The IE protein is required for the continuous transcription of early and late viral RNA and for shutting of the synthesis of its own RNA as well as of most but not all host-cell macromolecules. This IE

protein also has been proven to be able to transactivate other viral promoters, e.g. of adenovirus.

4. Veterinary impact of Pseudorabies (Aujeszky disease)

In 1813 and later, reports from the United States of America have pointed to the existence of an animal disease consistent with PRV. It was first called “mad itch” in cattle and was characterized by heavy itching. The name Aujeszky comes from Aládar Aujeszky a Hungarian veterinary surgeon, who was the first to demonstrate that PRV is the main agent of the disease pseudorabies, or Aujeszky's disease (Pomeranz et al., 2005).

By the 1960’s this disease started playing a dramatic role in swine production. Primary viral replication occurs in the nasal and oropharyngeal mucosa. PRV is tropic for both respiratory and nervous system tissues of swine and viral particles enter sensory nerve endings innervating the infected mucosal epithelium. Mortality that results from the infections with PRV varies with the age of the pigs, the overall health status of the animals, the viral strain, and the infectious dose. Normally, younger swines are more susceptible for the PRV infection and typically exhibit symptoms of central nervous infection whereas older swines exhibit symptoms of respiratory disease (Kluge et al., 1999).

For the infected piglets, it has been found that the incubation period of PRV is typically 2 to 4 days. Symptoms are that piglets are listless, febrile, and uninterested in nursing. Within 24 h of exhibiting these symptoms, the piglets progressively develop signs of central nervous system infection, including trembling, excessive salivation, in coordination, ataxia, and seizures. Infected piglets may sit on their haunches in a “dog-like” position because of hind limb paralysis, lay recumbent and paddle, or walk in circles. Once piglets develop central nervous system abnormalities, they die within 24 to 36 h. Mortality of suckling pigs with pseudorabies is extremely high, approaching 100%, (Pomeranz et al., 2005).

CHAPTER 4

AIMS OF THE THESIS

Aim of the thesis

This work aimed in the long term at gene knock down in transgenic animals by generating lines of pig resistant to Aujeszky disease. The purpose of the thesis was to construct reliable vectors which are capable to express RNAi in cells as in transgenic animals and to define the experimental conditions in vitro and vivo allowing a reliable use of siRNA to obtain transgenic animals resistant to the disease of Aujeszky.

When I started my thesis, there was not much information known about RNAi use. The U6-RNAI approach was first chosen to target the immediate early gene of PRV. Four major axes have been retained to choose effective shRNAs (siRNAs and miRNAs) against the PRV IE gene using the best known criteria at that time. This gene was targeted as its expression is mandatory for the replication of the virus.

The main aim of this work was to design and construct several expression vectors containing powerful promoter of RNA polymerase type II and III active in all the cell types. These vectors have to be able to resist to the mechanisms of transgene silencing. To reach this goal, these vectors may contain U6-RNAi constructs introduced into various sites or in the vicinity of others vectors. These assistant vectors are designed only to protect the U6-RNAi from the mechanisms of silencing of transgenes. In this case, the polymerase II vector is only one carrier for U6-RNAi construction but the RNAi is transcribed from Pol III promoter not a Pol II promoter. Alternatively, the RNA polymerase II vectors may themselves transcribe the genes coding for shRNAs from a Pol II promoter.

The following questions were addressed:

- How to find appropriate siRNA sequence and to design and to construct vectors to express these siRNA genes in transgenic animals?
- What are the siRNAs capable of having the best inhibiting effects on the IE mRNA?
- What are the factors that determine the efficiency of shRNA (siRNA, miRNA)?
- Can vectors depending on RNA polymerase II promoters be as efficient as those depending on polymerase III to induce a specific silencing effect both in vitro and *in vivo*?
- Would it be possible to design a transgenic vector depending on RNA polymerase III promoter U6 expressing shRNA and assisted by a polymerase II promoter?

- In animals, can we modulate the silencing effect by modifying the shRNA insertion site in the vector of expression?
- What is more efficient in gene silencing: siRNA or miRNA?
- What are the best tools to be used: siRNA or miRNA?
- What are the best mRNA target regions: translated region for mRNA degradation or 3'UTR (untranslated region) for inhibition of translation?
- Is a double and simultaneous targeting on both translated and untranslated region of the same IE mRNA more capable of inhibiting IE protein synthesis?
- What are the main reasons for the off-targeting effect?
- Is a success in inhibiting of IE gene expression by siRNA *in vitro* capable of reducing the pathogenicity of PRV virus?

More generally, it must be considered that the problems to solve for using siRNA in transgenic animals are the following:

To design siRNA efficiently targeting the RNA to be inhibited

To design siRNA having no off-targeting effects

To express shRNA at a sufficient level to obtain RNA interference

To express shRNA of a not too exceeding level to avoid unspecific cytotoxic effect

Main steps of the experimental works:

The different steps of the experimental work were the followings:

1) Construction of a gene containing targeted sequences of IE gene of the Aujeszky virus in the 5'UTR of the firefly luciferase reporter gene. The destruction of IE sequence involves a disappearance of the luciferase by the mechanism of PTGS (post transcriptional gene silencing). This tool facilitated the search and the determination of siRNAs having a powerful inhibitory effect on IE mRNA without the drawbacks of virus culture and manipulation (Figure 16).

Figure 16 : Luciferase reporter gene pLM24IELuc

2) Construction of vectors containing the promoter of the U6 gene (of type polymerase III) followed by a sequence coding for siRNAs specific of the IE genes. The co-transfection of this vector with target gene makes it possible the finding, in a simple way, of RNAi able to cause a strong inhibition of IE gene (Figure 17)

Figure 17 : The basic scheme for the shRNA.

3) Test of the efficiency of these pBS-IE siRNAs in transient transfection assay with various cell lines (PK15, CHO, and ST) and choice of the best sequence.

4) Construction of several vectors containing a powerful promoter of type RNA polymerase II active in all cell types, with the U6-RNAi construct introduced into various sites. The assistant vector was previously designed and constructed in our laboratory. It was chosen in the present work since it has a good capacity to resist to the mechanisms of transgene silencing. In this case, the polymerase II vector was only a carrier for the U6-ARNi construct (Figure 18). These constructs were prepared in

attempt to prevent the silencing of the transgene expression known to occur at very high frequency with transgenes under the control of U6 promoter possibly due the close vicinity of the promoter and terminator in concatemeric transgenes.

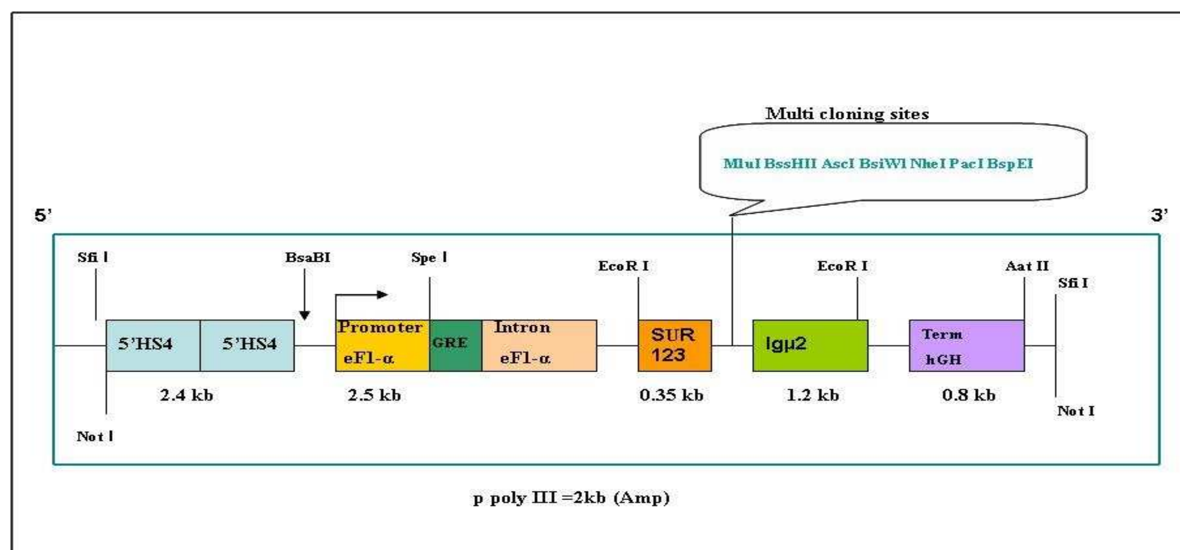


Figure 18: Vector of transgenesis pM10.

5) Construction of vectors containing the sequence of shRNAs introduced without promoter into an exon or an intron of the RNA polymerase II vector. The synthesis of shRNA was thus expected to take place in all the cells of the organisms, a strategy which may contribute to ensure a good protection of the animals against an infection by the virus (Figure 18). These constructions were done to avoid any problem of silencing due to the U6 promoter as we did not know at this stage if he construction described in 4 is able to express or not in transgenic animals.

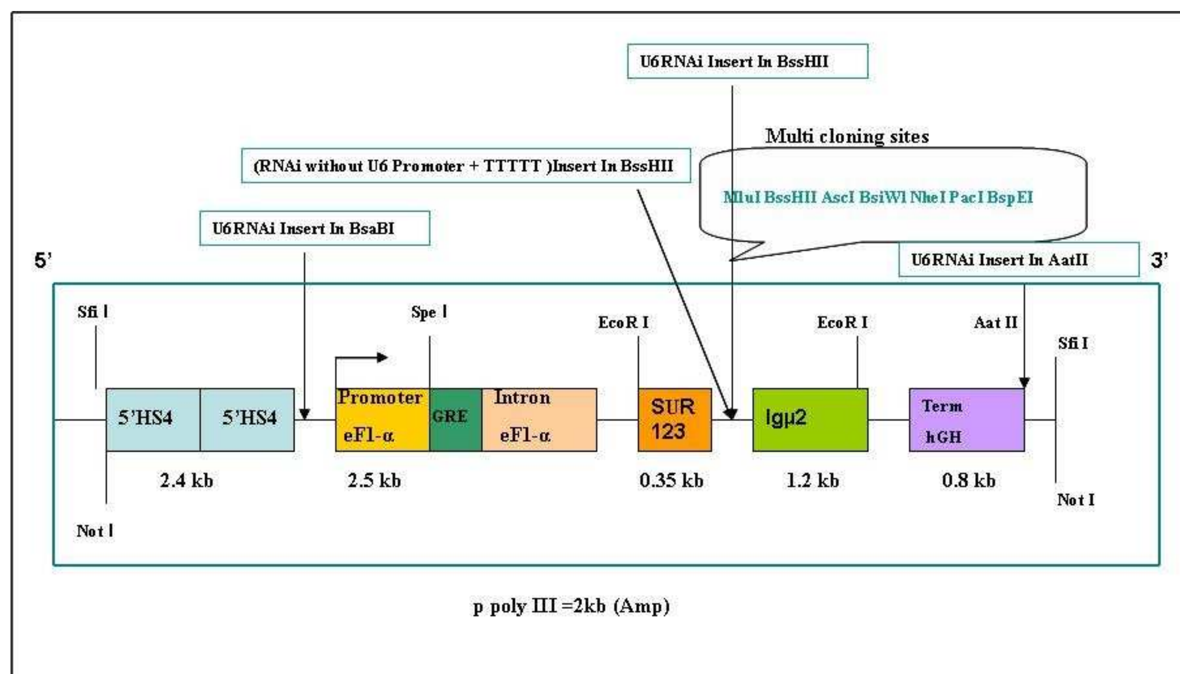


Figure 19 : M10 vector includes the multicloning sites.

6) Construction of vectors containing the elements allowing the synthesis of microRNAs containing sequences of the siRNAs described previously. These vectors were constructed to be independent of both the U6- promoter and the shRNA.

7) Evaluation of the effectiveness of these various constructions to inhibit IE gene expression in cell systems, in comparison with the pBS-U6IE shRNA of reference chosen in (3).

8) Generation of transgenic mice harboring the vectors which showed the greatest capacity to inhibit IE gene in cells.

9) Development and adaptation of an RT-PCR test allowing the measurement of the concentration of mature siRNA in the cells and the transgenic mice.

10) Evaluation of the resistance of the transgenic mice to an infection by the virus of Aujeszky's disease.

11) The search of additional shRNA genes targeting other translated regions of the IE mRNA and the 3UTR region of the IE mRNA.

12) Evaluation of the knockdown capacity of the new shRNA using the tools depicted above.

CHAPTER 5

MATERIALS AND METHODS

1. Preparation of the shRNA genes

Three different series of siRNA encoding genes were constructed in the first part of this work. They all were containing the same sequence targeting the same region of the IE mRNA. A series of genes expressing the siRNA under the activity of a RNA polymerase III (polIII) promoter (the U6 small gene promoter) was constructed. Two other series of genes expressing siRNAs under the activity of polII promoters were then constructed. One encompassed the siRNA sequence flanked on both sides by a 5T sequence. The second consisted in the miR30 skeleton where the endogenous miRNA sequence was replaced by the targeted IE siRNA sequence. Both 5T and miRNA constructs were placed downstream of the human EF-1α gene promoter.

<u>Introduction of shRNA gene sequence in pBS-U6 vector</u>

The preparations of the shRNAs required specific oligonucleotides. Throughout all the work that has been carried out the oligonucleotides were purchased from MWG-Biotech (Ebersberg) ready for hybridization and direct cloning into the opened vectors.
Two primers were ordered (forward and reverse). They were hybridized and cloned immediately downstream of the U6 gene promoter in the ApaI and EcoRI sites which had been added to the primers. After hybridization, the shRNA contained ApaI & EcoRI site at both ends for direct cloning downstream of U6 gene promoter.

Primers used for preparing the shRNA

1. 5′ CGCCGACGATCTCTTTGACTTTCAAGAGAAGTCAAAGAGATCGTCGGCTTTTTTACGCGTG 3′
3′ CCGGGCGGCTGCTAGAGAAACTGAAAGTTCTCTTCAGTTTCTCTAGCAGCCGAAAAAATGCGCACTTAA 5′

2. 5′ CGCCGCTCCCATCGGGTGAGAAAATTCAAGAGA TTTTCTCACCCGATGGGAG TTTTTTG 3′
3′ CCGGGCGGC GAGGGTAGCCCACTCTTTT AAGTTCTCT AAAAGAGTGGGCTACCCTC AAAAAACTTAA 5′

3. 5′ GGCGCGGACTCTGAAGA AAGCTT TCTTCAGAGTCCGCGCCGG CCC TTTTTT G 3′
3 ‘CC GGCCGCGCCTGAGACTTCT TTCGAA AGAAGTCTCAGGCGCGGCC GGG AAAAAACTTAA 5′

4. 5′ GGCTCTCCGGCGGCTATCA AAGCTT TGATAGCCGCCGGAGAGCCGG CCC TTTTTT G 3′

3 'CCGG CCGAGAGGCCGCCGATAGT TTCGAA ACTATCGGCGGCCTCTCGGCC GGG AAAAAACTTAA 5′

5. 5′ GGCCGCCTTCCTCCTTCTT AAGCTT AAGAAGGAGGAAGGCGGCCGG CCC TTTTTT G 3′

3 'CCGG CCGGCGGAAGGAGGAAGAA TTCGAA TTCTTCCTCCTTCCGCCGGCCGGG AAAAAACTTAA 5′

6. 5′ GCCTTCCTCCTTCTTCT AAGCTT AGAAGAAGGAGGAAGGCGG CCC TTTTTT G 3′

3 'CCGGCGGAAGGAGGAAGAAGATTCGAA TCTTCTTCCTCCTTCCGCC GGGAAAAAACTTAA 5′

7. 5′ GGCCTCGCTCAGGCAGAAA AAGCTT TTTCTGCCTGAGCGAGGCCGG CCC TTTTTT G 3′

3 'CCGG CCGGAGCGAGTCCGTCTTT TTCGAA AAAGACGGACTCGCTCCGGCC AAAAAACTTAA 5′

8. 5′ GGCCTCGCTCAGGCAGAAATTCGAATTTCTGCCTGAGCGAGGCCGGCCC TTTTTT G 3′

3 'CCGGCCGGAGCGAGTCCGTCTTTAAGCTTAAAGACGGACTCGCTCCGGCC GGGAAAAAACTTAA 5′

9. 5′ CTCCCATCGGGTGAGAAATTGCAA TTTCTCACCCCGGATGGGAGGGCCC TTTTTT G 3′

3 'CCGGGAGGGTAGCCCACTCAAAAACGTTAAAGAGTGGGAGCCTACCCTCCCGGGAAAAAACTTAA 5′

10. 5′ CGGTCCCCTTCTCCTCCTTCTAAGCTTAGAAGGAGGAACCGAAGGGGACCGGGCCCTTTTTT G 3′

3′ CCGGGCCAGGGGAAGAGGAGGAAGATTCGAATCTTCCTCCTTGGCTTCCCCTGGCCCGGGAAAAAACTTAA 5′

11. 5′ CCGGTCCCCTTCTCCTCCTTCTAAGCTTAGAAGGAGGAACCGAAGGGGACCGGCCCTTTTTT G 3′

3 'CCGGGGCCAGGGGAAGAGGAGGAAGATTCGAATCTTCCTCCTTGGCTTCCCCTGGCCGGGAAAAAACTTAA 5′

12. 5′GATCGTCCCGGTCCCCTTCT AAGCTTAGAAGGGGAC GAACGGGACGATCGG CCCTTTTTT G 3′

3′CCGGCTAGCAGGGCCAGGGGAAGATTCGAATCTTCCCCTGCTTGCCCTGCTAGCCGGGAAAAAACTTAA 5′

13. 5′GCCCTCCTCCTCTCCTTCTAAGCTTAGAAGGAGAGACAGAGGAGGGCGGCCCTTTTTT G 3′

3 CCGGCGGGAGGAGGAGAGGAAGATTCGAATCTTCCTCTCTGTCTCCTCCCGCCGGGAAAAAACTTAA 5′

14. 5′GGCCCTTCTCCTCCGTCTTAAGCTTAAGACGGAGG TCCAGAAGGGCCGGCCCTTTTTT G 3′

3'CCGGCCGGGAAGAGGAGGCAGAATTCGAATTCTGCCTCCAGGTCTTCCCGGCCGGGAAAAAACTTAA 5′

Annealing of the oligonucleotides

To anneal the two oligonucleotides, we mixed the following in an Eppendrof tube: H_2O:180 µl, oligo 1 a (250pmol/ µl in our stock): 10 µl, oligo 1b (250 pmol/ µl in our stock): 10 µl. The two oligonucleotides were incubated for 10 min in boiling water.

Water was moved to the bench for 30 min to 1 hr until it reaches room temperature. The concentration of the annealed oligonucleotides normally falled between 3000~4000 ng/µl as determined by Nanodrop. To avoid getting multiple insertions in the vectors, the oligonucleotides were phosphorylated by T4 polynucleotide kinase. Mixing 1 or 5 µl of this hybridized shRNA in the ligation to 50 ng of the pBS-U6 vector.

Vector preparations and oligonucleotide subcloning:

1st step subcloning:

The pBS/ U6 plasmid was digested by ApaI at 25 Cº or room temperature for 5 hrs or overnight. Klenow and 4 dNTPs (10mM each 500 µl) were added to blunt the 3-protruding end of ApaI. This was done at 37 ºC for 1 hr or at RT. DNA was purified to remove the Klenow and dNTPs (10mM each 500 µl) using Qiagen spin column. DNA for ligation was digested by HindIII. For the ligation reaction, a molar ration of 1:3 to 1:5 between the vector and the annealed oligos was used.

2nd step subcloning:

The prepared construct in 1st step, was used in the 2nd step subcloning. The construct from the 1st step was digested by EcoRI and HindIII and the second pairs of oligonucleotides were subcloned.

pBS-U6 digestion and preparation.

ApaI and EcoRI digestion.

5 µg of DNA (pBS-U6) were added in 30 µl H_2O final volume, 3 µl buffer A (suitable for both enzymes) and 2 µ l ApaI and 2 µl EcoRI. The mixture was incubated at 37 ºC for 15 minutes.

Digest purification

The digestion mixture was then purified and concentrated by using MSB Spin PCR from Invitek. 200 µl binding buffer were added to the digestion mixture and vortexed. The mixture was transferred onto a spin filter and centrifugate for 1 minute at 12.000 rpm, the filtrate was diluted and centrifuged for 2 minutes. The spin filter was put into

a new 1.5 ml receiver tube. DNA was eluted by 20 µl H2O and centrifuged for 1 minute at 10.000 rpm.

Digested vector dephosphorylation (when required).

The mixture of 100 ng of the digested vector, 1.5 µl buffer, 1.5 µl phosphatase enzyme and H_2O up to 10 µl was incubated at 37 º C for 40 minutes. The reaction was stopped by heating at 65 º C for 15 minutes.

Subcloning of shRNA into the pBS-U6.

Ligations were carried out by using T4 DNA ligase, (Roche cat No. 10 481 220 001). The ligation protocol was as followed: The mixture containing 50 ng of the digested vector (pBS-U6), 1 µl of hybridized shRNA or 5 µl depending on the concentration, dilution buffer (prepared from 5X buffer (Roche rapid ligation kit)) to make a total volume of 10 µl, 10 µl ligation buffer 2 X and 1 µl ligase. The mixture was incubated for 20 minutes at 25ºC. The ligation mixtures were then purified by MSB Spin PCR 50 from Invitek according to the manufactures instructions.
Bacterial transformation and electrocompetent cell electroporation: for each transformation 20 µl of competent cells were used. The SOC medium was brought to room temperature. The cuvettes and microcentrifuge tubes were placed on ice. The appropriate numbers of Electrocomp™ GeneHogs® E. coli were thawed on ice. The electroporator for bacterial transformation was adjusted according to the manufacturer's instructions. 1- 2 µl of each ligation reaction were added to 20 µl of competent cells and was transferred to chilled electroporation cuvette that was placed on ice. 450 µl of SOC was added immediately to the cuvette after heat shock, the mixture was transferred to a 1.5 ml microcentrifuge tube and it was incubated at 300 rpm for 45 minutes at 37°C. The transformation reaction was diluted 100-fold with SOC, 20 to 150 µl from each transformation was plated on LB plates containing the appropriate antibiotic and the plates was incubated overnight at 37°C. Positive colonies were screened by plasmid isolation, PCR, and sequencing.
Screening for positive clones was carried out by sequencing analysis. One positive clone from each construct with the unmutated shRNA sequence was selected for further analysis. Constructs were validated in vitro. The best shRNA were used for cloning in M10. By using the (T7 mod and T3 mod) primers, the amplification

process by PCR allowed to have only the U6-RNAi with the suitable restriction sites at both ends. This facilitated the cloning process to M10.

PCR amplifications

The mixture 2 µl (~200 ng) U6RNAi construct DNA, 0.5 µl dNTP (10mM each 500 µl) 1 µl from primers (T7 mod&T3 mod), 0.3 µl Taq polymerase (high fidelity), 5 µl PCR amplification buffer and up to 50 µl H2O.

PCR reaction: the PCR was carried as 94 º C for 5 minutes, 94 º C for 1 minute, 55 º C for 1 minute, and 72 º C for 1 minute. This was repeated for 30 cycles, 72 º C 10 minutes. PCR added the three restriction sites BsaBI, BssHII and AatII at the end of U6-shRNA.

Cloning the PCR product in PGEM T Easy

The PCR product was purified on a low melting point Agarose gel (1%), the 550 bp band corresponding to the U6-siRNA were purified using the Qiaquick kit (Qiagen) according to the manufacture instructions. The purified U6-RNAi was then cloned in PGEM T Easy. One positive clone of PGEM T Easy was replicated for DNA maxi preparation and sequencing.

DNA Extraction and purification

DNA Maxi preparation was done by HiSpeed Plasmid Maxi Kit from Qiagen, while mini preparation was done by the alkaline lysis protocol. SiRNA sequencing: When sequencing was needed, it was carried out in MWG DNA Sequencing Service, Eurofins MWG GmbH Anzinger Strasse 7a D-85560 Ebersberg.

<u>Cloning of U6-ShRNA in M10 plasmid</u>

As it was described above, after validating the shRNA constructs by transient co-transfection in CHO cells, the most effective shRNA sequences were chosen for further work and the shRNA-U6 construct was cloned in M10 vector. The fragments containing the U6 gene promoter and the shRNA which were amplified by the couple of primers (T3 mod, T7 mod) were extracted and purified from the gel as described above. This fragment (~550 bp) was then cloned in pGEM T Easy.

T3 mod

BsaB1 BssHII AatII Pbs-US

5'ACGATAGCCATC GCGCGC GACGTC AATTAACCCTCACTAAAGGG 3'

T7 mod

Pbs-US BsaB1 BssHII AatII

3' GGGATATCACTCAGCATAAT CTATCGGTAG CGCGCG CTGCAGTA 5'

BsaBI construct preparation

BsaBI construct preparation: preparations of BsaBI U6-shRNA insert, digestion, 5 µg DNA of the modified U6RNAi in pGEM T easy, 5 µl digestion buffer, 2 µl BsaBI restriction enzyme and up to 50 µl H_2O. The mixture was incubated at 37 ºC for 2 hours.

Gel purification: after digestion the U6-shRNA purified on 1% low melting point Agarose gel as described previously using QiaQuick kit (according to supplier instructions).

Insert quantification: the insert concentration was measured by NanoDrop Spectrophotometer from NanoDrop Technologies.

ShRNA cloning in plasmid M10 (BsaBI site): insert subcloning into pM10, 50 ng of the dephosphorylated digested vector (M10), 1 µl of hybridized shRNA or 5 µl depending on the concentration, up to 10 µl by 1X ligation buffer, 10 µl ligation bufer 2 X and 1 µl ligase. Ligation was carried out at 25ºC for 20 minutes. The ligated plasmid was cultured on Ampicillin plates and positive clones were selected, sequenced and tested. The same protocol was used to prepare the other constructs.

<u>ShRNA gene for microRNAs strategy</u>

Primer design for miRNAs

The miRNA is of 125nt in the final design. It was ordered synthetically this relatively long DNA fragments, two primers capable to hybridize in their ends were ordered (Figure 20). Amplification of these hybridized strands by PCR gave the expected miRNAs.

Primer for miRNA preparation

Sense primer
5'GCGCGCTGCTGTTGACAGTGAGCGACTGGGCCGACGATCTCTTTGTCAC TTCAAAGCTGTGAAGCCACAGATGGGCTTTGAAGTCA **3'**

Antisense primer

5'GCTAGCCCCTTGAAGTCCGAGGCAGTAGGCAGCTGGGCCGACGATCTCT TTGACTTCAAAGCCCATCTGT **3'**

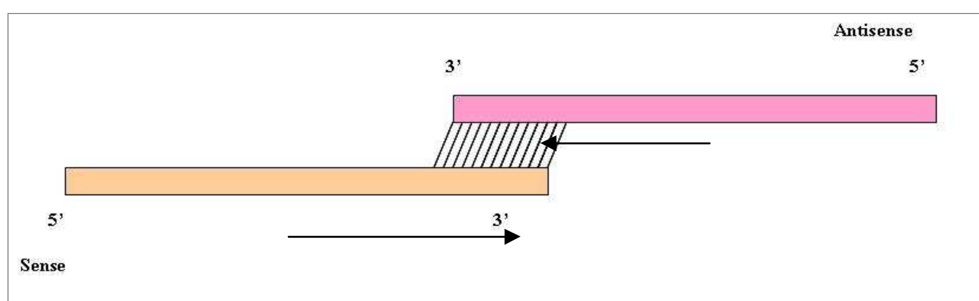


Figure 20 : miRNA preparation.
Basic scheme showing the preparation of the miRNAs. Two primers were designed to hybridize in their ends and the miRNA gene was generated from the PCR reaction.

Annealing

Mixing 10 µl (100pmol / µl in our stock) of the forward primer and 10 µl (100pmol / µl in our stock) of the reverse primer in final volume 50 µl H_2O. Heating 10 minutes at 100 º C. Cooling at room temperature. Mixing 10 or 15 µl of this hybridized miRNA the PCR reaction using high fidelity Taq polymerase.

PCR reaction

The PCR was carried as 94ºC for 5 minutes, 94ºC for 1 minute, 60ºC for 1 minute, and 72ºC for 30 seconds. This was repeated for 30 cycles, 72ºC 10 minutes. The PCR products which contained the miRNAs were purified and prepared for the subsequence cloning in the prepared M10 vector.

miRNA cloning in pM10

The above constructs were cloned in the multi-cloning sites of the pM10. All the miRNA constructs were cloned in BssHII and NheI sites as described above. One copy of miRNA sequence was cloned in pM10 vector except for one construct, the

cloned sequences miRNA targeted the translated region of IE gene, and one targeted the 3'UTR of IE gene (one copy in BssHII and NheI, and another in NheI and Pac I).

Sequencing

In order to identify possible mutations after cloning, all the selected clones were subjected to sequencing, carried out by MWG Biotech.

2. Preparation of the vectors containing the IE mRNA targets

2.1. The plasmids used as target were the following:

- pLM26 Luc. Reporter luciferase gene was added after a IRES (internal ribosomal entry site) which allows its efficient translation in all cases.
- pLM24 IE Luc. A part of the IE mRNA was added before the IRES. The targeted region of the IEmRNA was located at the 5' end of the mRNA. This plasmid was used to evaluate the mRNA degradation efficiency of different siRNA. A degradation of IE region was expected to prevent luciferase gene expression.
- pLM24 Luc 3'UTR. A part of the 3'UTR untranslated region of the IE mRNA was added after the luciferase gene. This plasmid was used to evaluate a single siRNA effect on mRNA translation. The siRNA binding to the IE was expected to inhibit luciferase gene translation. Two different plasmids have been constructed: one encompassed a 19nt long fragment of IE mRNA 3'UTR encompassing the exact sequence of the chosen siRNA; another encompassed a 900nt long fragment corresponding to a longest 3'UTR region of the IE mRNA.
- pLM24 IE Luc 3'UTR IE. A part of the IE mRNA was added before the IRES, followed by luciferase gene and the part of the 3'UTR untranslated region of IE mRNA. This plasmid was used to evaluate the effect of siRNA on mRNA degradation or inhibition of translation.

2.2. IE mRNA targets

2.2.1. Part of IE mRNA sequence (5'UTR and translated region)

```
tctctgccaa cccgagggga tccgaccgtc tccgctccgg cgcggactct gaagactccg
gctctccggc ggctatcagc cctcgacgga cgcccgaccc accgaggctc tcggcccgcc
agagaagagt cttcttcttc tcctcctccg gccgccttcc tccttcttct ccgccgcccg
ctctccgcgc tcggcgcccg gcctcgctca ggcagaaaga ccccgatcga gaccatggcc
gacgatctct ttgacttcat cgagaccgag ggcaacttca gccagctcct ggcggccgcc
gccgcggccg aggaagaggg catcgcgtcc ggccccgacg gcggcagcca gggctcccgg
cgccgcggct cctccggcga ggatctcctc ttcggcccgg gcggcctctt ctccgacgac
```

Figure 21 : 5’UTR and translated region.

The chosen 5’UTR region of the IE gene has been designed following the commonly used criteria for the determination of siRNA sequence. In red, the ATG codon.

2.2.2. The 3’UTR untranslated region of IE mRNA sequence

```
ctgctgctcc gctgagcggg gcgcccctc ggcccggccg gactctgact ctgactctcc
ggcccctcca ccggctcctc gaggcccttc tcctccgtct tctctcccct cgccctcggc
ccggtcctcg tcctcgtcct cgtccccgtc cccgtcctcc tcctcctctg cgtccgcggc
ggcggcctcg gtcccctcgg cccggcggcg cttgcctccc cggcgcctgc ctccccggcc
cggtggccct cctcctctcc ttctcctccg cgcggatccc ccggccggag gtggctgcgg
cggcggcgga ggtggcggcg gtggtggaag cggcggcggc ggccgcggcg gagggctcgg
cggcggagga tcgtcccggt ccccttctcc tcctcccgcg gtcccccggt ccccttctcc
tccttctccc atcgggtgag aaaagagttt gttttcagag tgagaaaata aagtttgtgc
tgtattttct gaaccagctc gagtctctga gatttttgg ggagatggag gcggccatct
tggcggtggt ctctggggtg gaggtggtct tgtggatggg ggtccctggt gggaggaaga
agaagaggtg gagggtcttg gtgggggtga cgggggtcct cctcctggag ggtcttggtg
gtggtgatgg gaagaagtgg atgggggtcc tcctcctgga gggtcttggt ggtggtgggt
cttagcagat gggggtccc tggtgggtct tagcagatgg gggtcctcct cctggagggt
cttggtggga agaagtagag ggtcttgggg atgttggggg tccttgatgg tggtggtggt
ggtggtggga ggtggacggt gttggtggtc ccggcgggtc ctggtgggag gtagatggtc
```

Figure 22 : 3’UTR untranslated region of IE mRNA sequence.

This figure illustrates part of the 3’UTR region of the IE gene of Pseudorabies virus, letters marked blue is the beginning of the stop codon.

Target plasmid construction

A chimeric gene containing the luciferase gene and the targeted 3’UTR region (19nt or 900nt) was first constructed in pBlueScript vector, excised from pBS by BssHII digest then subcloned to replace the Luciferase gene in pML24-IE plasmid. The final pML24-IE-luc-3’UTR IE plasmid thus encompassed both targeted regions of the IE gene (transcribed and non transcribed).

2.3.1. pLM24 IE Luc untranslated region of IE (19 nt)

. Primers used to prepare this construct

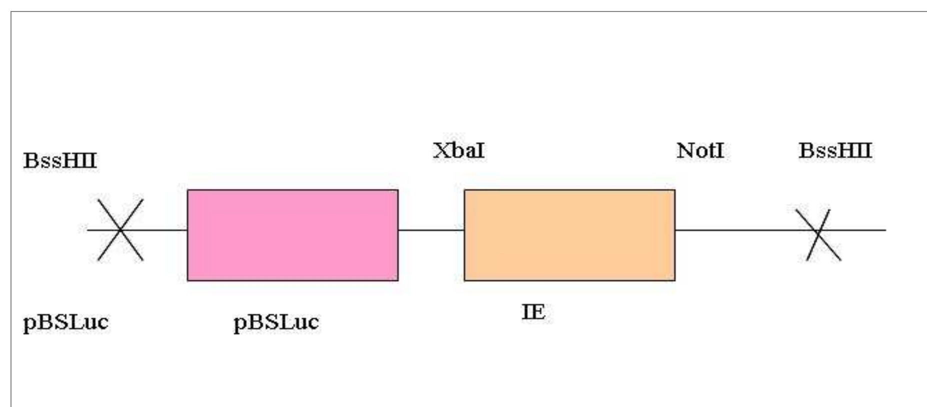
Luc Trad IE R (XbaI and NotI)

5’GGCCGCTTTTCTCACCCGATGGGAGT 3’

Luc Trad IE F (XbaI and NotI)

5’ CTAGACTCCCATCGGGTGAGAAAAGC 3’

Hybridization of these primers was carried out as described above. It was cloned in pBSLuc (XbaI and NotI).



The plasmid was digested by BssHII, and the insert was cloned in PLM 24 BssHII

Target plasmid

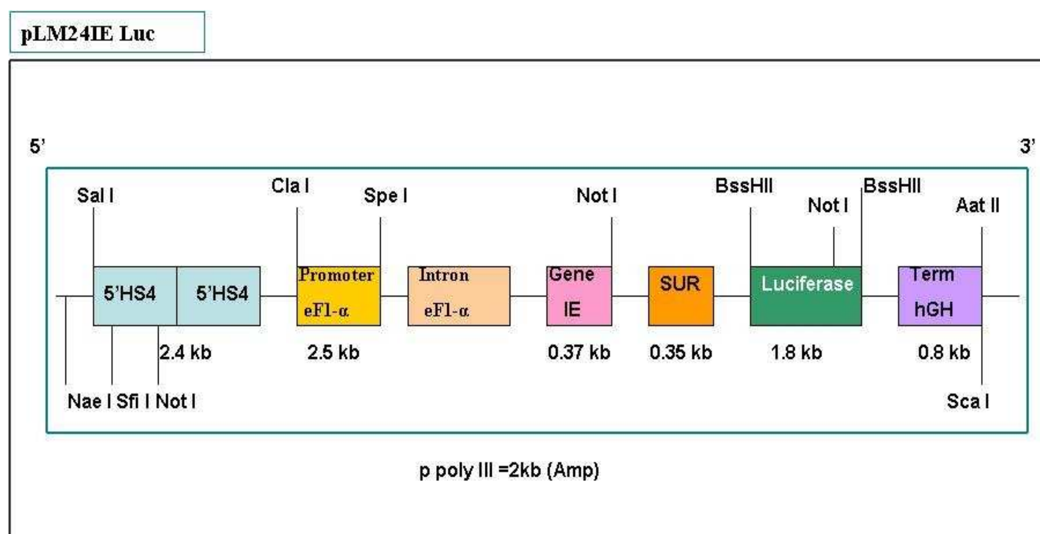


Figure 23 : Target plasmid pLM24 IE Luc.
This plasmid contained part of the IE sequence that contained the chosen targets in translated IE sequence. Luciferase gene was added into the pLM24 IE plasmid after an IRES (intraribosomal entry site) allowing an efficient translation of the reporter gene irrespectively of the reading frame of the first cistron added before the IRES).

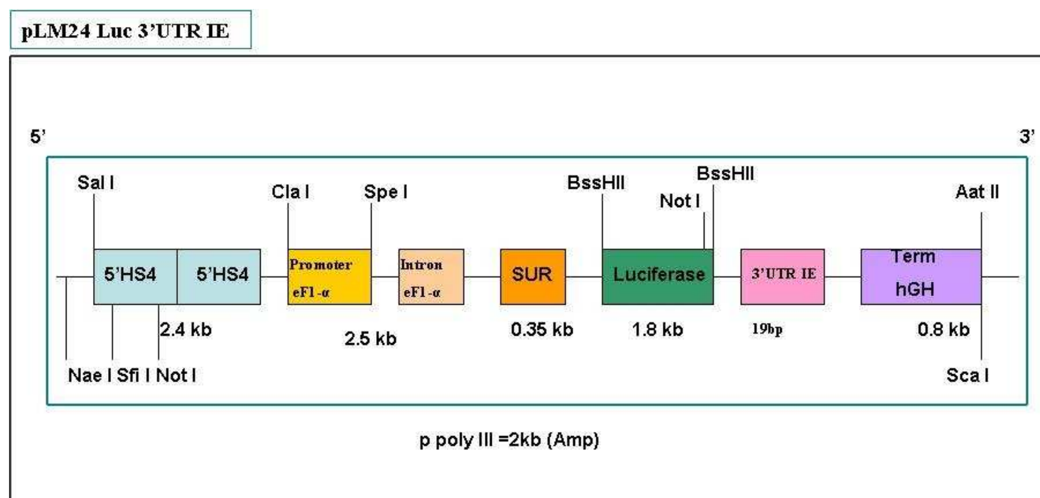


Figure 24 : pLM24 Luc 3'UTR plasmid.
Contained a short sequence 19nt of IE 3'UTR added after the luciferase gene. This plasmid may evaluate the efficiency of a single siRNA IE target plasmid acting on the IE 3'UTR untranslated region.

Preparation of new target containing the complete 3'UTR of IE gene

The 3'UTR region of the IE gene of the pseudorabies virus were amplified using the PCR. Genomic DNA of the virus was obtained from François Lefèvre (INRA, VIM).

3'UTR IE F primer

5' GCTCTAGACGGA CTC TGA CTC TGA CTC T 3'

3'UTR IE R primer

5' CGAGCT CGT GAA AACAAACTCTCTTTC 3'

Two restriction sites (XbaI at 5' and SacI at 3') were added to these primers for cloning to the vector.

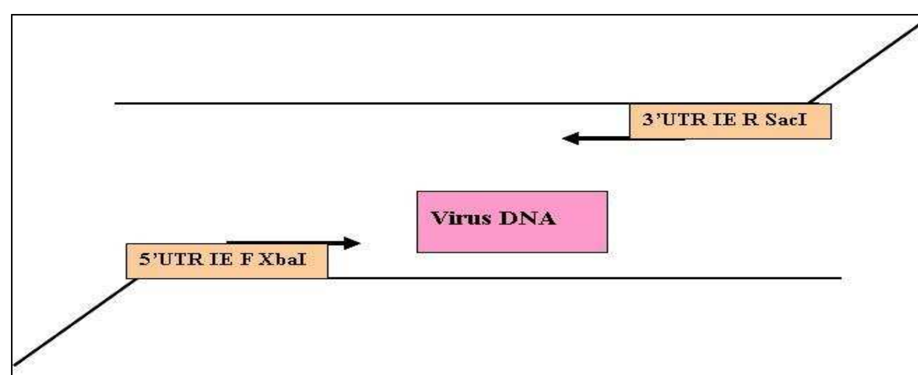
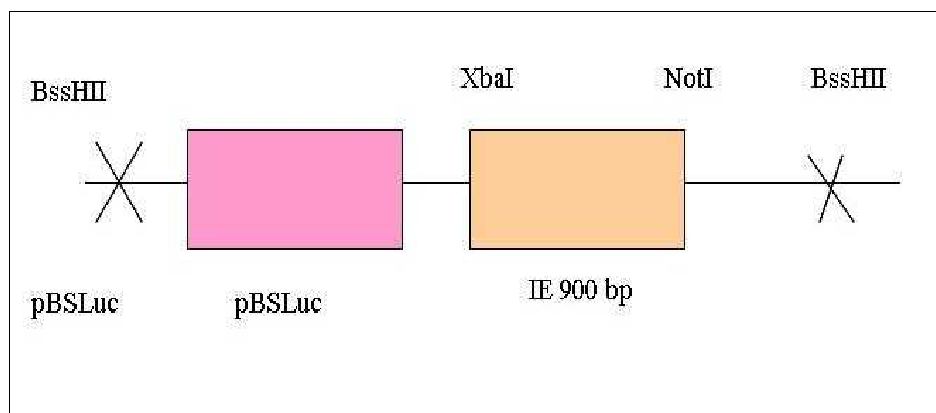


Figure 25 : PRV genomic DNA PCR.
Scheme proposed the amplification of genomic DNA of IE viral gene by PCR for 3'UTR target region preparations 900pb insert resulted from PCR amplification was cloned into pBsLuc (XbaI and SacI)



Digestion by BssHII, then it was cloned in PLM 24 BssHII

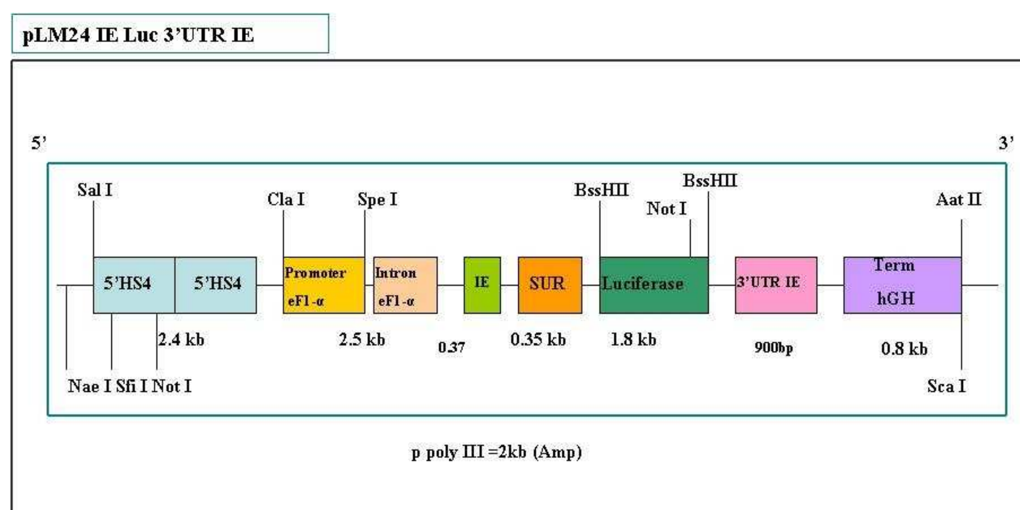


Figure 26 : pLM24 IE Luc 3'UTR IE plasmid.
Contained part of the IE sequence that contained the chosen targets in translated IE sequence as well as the complete sequence of IE 3'UTR added after the luciferase gene. This plasmid was used to evaluate the effect of siRNA on mRNA degradation or inhibition of translation

RNAi in the intron

It has been demonstrated by several works, that shRNA in an intron may have a high effect on the inhibition of gene expression (Lin et al., 2006; Nishikura, 2006; Xiao et al., 2006; Kim & Kim, 2007). ShRNA gene for cloning in the β-globin intron of pM10

RNAi intron

5'NruI TTTTTTGGCCGACGATCTCTTTGACTTCAAAGCTTTGAAGTCAAAGA GATCGTCGGCCTTTTTTG3'

RNAi intron

5'NruIAAAAAAGGCCGACGATCTCTTTGACTTCAAAGCTTTGAAGTCAAAG AGATCGTCGGCCAAAAAG 3'

shRNA sequence was prepared to be introduced into the β-globin intron, in the unique NruI site of pM10 expression vector.

Transcription gene silencing (TGS)

For preparing this TGS construct, the skeleton of the natural occurring human miRNAs (M30) was used. The target sequence was inserted to replace the original M30 sequence.

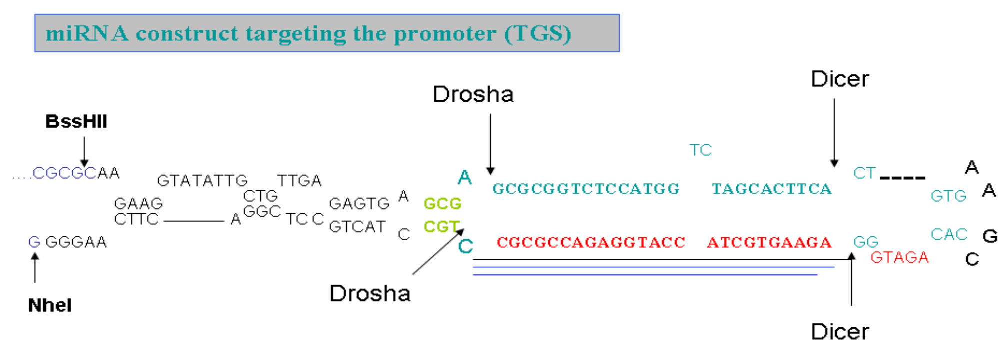


Figure 27 : scheme represent the TGS construct
Figure 27 represents the constructed TGS, Drosha as well as Dicer site of action are indicated.

3. Construct validation

3.1.1. Cell lines

Three types of cells were used in my works

CHO.K1 (Chinese Hamster Ovary) cell lines, St- Iawa cell lines and PK-15 (pig kidney cell lines)

3.1.2. Cell cultures

PK-15 & ST cell lines were grown in DMEM (Dulbeco's modified eagles medium) containing 10% fetal calf serum, 2mM of L-glutamine, 100U/ml penicillin and 100

μg/ml of streptomycin. CHO.K1 cell lines were grown in DMEM/HAMF12 containing 10% fetal calf serum, 2mM of L-glutamine, 100U/ml penicillin and 100 μg/ml of streptomycin. Cells were incubated at 37 C in a humidified atmosphere gassed with 95% air, 5% CO_2 for two days before trypsine treatment for transfection assays. Cells were subjected to trypsination when they reached confluence as followed. Cells were washed by HBSS, 1 ml trypsine for each 100 mm plates was added, plates were incubated at 37 ºC for 10 minutes for PK-15 & ST cells while for CHO.K1 2 minutes were enough, cells were collected with 7ml of complete medium, centrifuged and seed in new plates. Plates were incubated at 37 ºC
Cryotubes preparation. After trypsine treatment, cells from the three different cell lines were resuspended in a freezing medium composed of 90% of SVF and 10% of DMSO and dispatched in cryotube. Each cryotube contains 2 or $3*10^6$ cells in 700 μl of freezing medium. Cryotubes were then stored at -80 ºC or liquid nitrogen.

3.2. Cell tests and in vitro assays.

Cells were prepared for co-transfection by target plasmid and the RNAi construct 24-48 hours before transfection depending on cell density. Cells were treated with trypsine. Two 100 mm plates for each constructs assay were prepared. DNA from all the constructs was prepared by PhoenIX™ plasmid purification systems from Qbiogene, the OD were determined by NanoDrop Spectrophotometer from NanoDrop Technologies. Cells were cotransfected with three plasmids: the reporter plasmid, the plasmid containing the shRNA gene and the control gene containing the β-galactosidase gene under the dependency of a RNA PolII promoter active in all cell types.

In Vitro bioassays in transiently transfected CHO.K1 cells

The transfections were carried out in CHO.K1 cells using ExGen 500 (Euromedex, Souffelweyersheim, France) according to the manufacturer's protocol. CHO.K1 cells were seeded in six-well plates grown in DMEM/HAM-F12 medium containing 10% fetal calf serum and maintained at 37 °C in a humidified atmosphere gassed with 95% air, 5% CO2. When cells reached 80% confluence, after 24-48 h, they were transfected with PLM24-IE-luc, a plasmid bearing IE sequence downstream Ef1α promoter linked to a luciferase reporter gene, and pCH110, a plasmid encoding β-

galactosidase activity (Amersham Pharmacia Biotech) and different constructs for RNAi. Each well was transfected with a mix of 1µgof PCH110, 0.75 µg of PLM24-IE-luc and 0.75 µg of a RNAi construct with 5 µl of Exgen 500 in 100µl of NaCl 150 mM. The ratio of Exgen 500/DNA used was 2/1. The complex was incubated during 4h in 900 µl of OptiMEM by well, then the cells were washed and grown in complete medium during 48H. Cells were washed with phosphate-buffered saline and scrapped. Enzymatic activity was measured as described elsewhere (Bignon et al., 1993). The results were expressed as percentage of induction, and the ratio of stimulated to nonstimulated cells (presence or not of RNAi) after luciferase activity was normalized by correcting for β-galactosidase activity, to take into account the transfection efficiency.

Stable clone preparation

Stable clones were obtained by co-transfecting PK-15 cells with U6-RNAi plasmid and plasmid containing the puromycin resistance gene (1:9) with fugene 6 transfectant agent. Puromycin was used for the selection of positive clones, because cell death is fast and efficient. Cells were grown in complete medium containing 5 ng/ml of puromycin, after one week resistant clones were collected. These clones were tested by further analysis of RNAi expression level and for virus infection assay.

In Vitro Bioassays in Stably Transfected PK15 Cell clones

The transfections were carried out in stably transfected clones of PK15 cells using Lipofectamine 2000 (Life technologies) according mainly to the manufacturer's protocol. PK15 cells were seeded in six-well plates grown in DMEM medium containing 10% fetal calf serum and maintained at 37 ºC in a humidified atmosphere gassed with 95% air, 5% CO_2. When cells reached 80% confluence, after 24-48 h, they were transfected with PLM24-IE-luc, a plasmid bearing IE sequence downstream eF1-α promoter linked to a luciferase reporter gene, and pCH110, a plasmid encoding β-galactosidase. Lipofectamine 2000 was used as transfectant agent. Each well is transfected with 1 µg of PCH110 and 1 µg of PLM24-IE-luc, mixed with 10 µl of Lipofectamine 2000 in 500 µl of optiMEM (Life technologies). The ratio of Lipofectamine 2000/DNA used is 5/1. The complex is incubated overnight in OptiMEM, then cells were washed and grown in complete medium during 48H. Cells

were washed with phosphate-buffered saline and scrapped. Enzymatic activity was measured as described elsewhere (Bignon et al., 1993). The results were expressed as pourcentage of induction, and the ratio of stimulated to nonstimulated cells (presence or not of RNAi) after luciferase activity was normalized by correcting for β-galactosidase activity, to take into consideration the transfection efficiency.

4. Extraction of RNA from cells or tissues (Chomczynski & Sacchi, 1987)

For extraction of RNAi the protocol from (Chomczynski & Sacchi, 1987) was used. Total RNA was extracted from transfected cells for RT-PCR analysis. This method of extraction was also used for extraction of total RNA from transgenic mouse tissues. The quality of RNA was verified on gel.

4.1. Extraction solutions

Guanidin thiocyanate solution: 250 g guanidine thiocyanate (final concentration 4 M), 293 ml H_2O, 17.6 ml sodium citrate 0.75 pH 7 (final concentration 0.025 M), and 26.4 ml of 10% sarcosyl (final concentration 0.5%). Filtration on Millipore filter: just before use, 0.36 ml beta-mercaptoethanol per 50 ml thiocyanate solution (final concentration 0.1 M) was added. This last solution was kept for no more than one month at 20 ºC. Sodium citrate solution 0.75 M: 22 g trisodium citrate, up to 100 ml with water, pH was adjusted to 7 by HCl. Sarcosyl 10%: 10 g sarcosyl was adjusted to 100 ml with water and solved at 65 ºC. Chloroform- Isoamyl alcool: 49v:1v.
TE SDS: Tris 10mM pH 7.5, EDTA 1 mM was autoclavated, and up to 0.5 % of the final concentration SDS (2.5 ml of 20% plus 97.5 ml TE) was added.
TNES: Tris 10mM pH 7.5, NaCl 0.12 M, and EDTA 0.1mM was autoclavated, and up to 0.5 % of the final concentration SDS was added. Sodium Acetate 2 M pH 4.

4.2. Procedure

The tissues were homogenized or cells in the thiocyanate solution containing β-mercaptoethanol (1-3 ml per p100 plate). The cells were rapidly scraped, pipetted up and passed through a needle if the solution was too viscous. 100µl NaAc 2M /ml of homogenized material were added then vortexed carefully. 1 ml of water saturated phenol/ml of homogenized material was added and vortexed. 200 µl of chloroform-

isoamyl alcool were added and vortexed for 1 minute and immediately placed on ice for 15 minutes. The mixture was centrifuged 20 minutes 10 000 g (for 30 minutes, 3-4000 rpm). The upper aqueous phase was collected and 1 volume of isopropanol was added. DNA precipitated overnight at -20 ºC and centrifuged 30 minutes maximum speed. The pellet was washed by ethanol 70% and the pellet was dissolved by 200 µl distilled water. RNA concentration was measured from 1 µl using Nanodrop. RNA was aliquoted in separated tubes each contain the following: 10 µg RNA, 10 µl NaOH 2 M, 1 µl glycogene, 250 µl EtOH 100%. The aliquoted RNA was stored at -20 ºC.

5. Quantification of si/miRNA by real time PCR

Principle of RNAi quantification:

SiRNAs are small RNA of 23-30nt so their size is too small for being quantified directly by Quantitative PCR. So for quantification, it is necessary to elongate siRNAs. For this purpose, the method described by Shi and Chiang was used (Shi et al., 2005). First of all, the small RNAs were not polyadenylated. To perform the reverse transcription step, they must be polyadenylated. This step was carried out with the kit of polyadenylation from Ambion. Once polyadenylated, the siRNAs were reverse transcribed with a poly-T adapter. Precisely, the primer used for reverse is composed of 2 parts: first, a polyT (12) for matching with all polyA-RNA (red colour) and secondly of a universal oligonucleotide (blue colour) which will serve for ulterior PCR quantification (Figure 28).

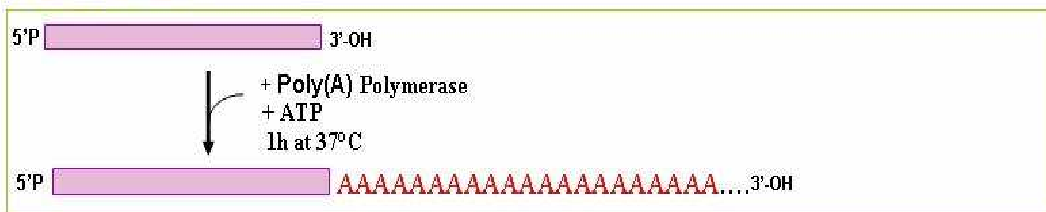
Universal Poly-T-adapter:

5'GCGAGCACAGAATTAATAACGACTCACTATAGGTTTTTTTTTTTT (AGC) (AGCT)-3'

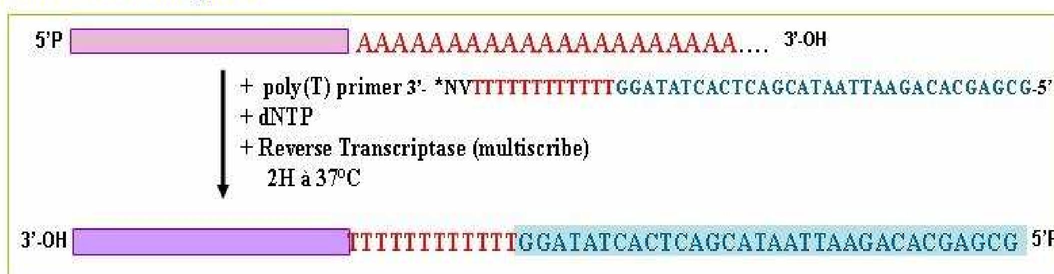
After reverse transcription, we obtained a cDNA of around 65 pb which is longer enough to be quantified by PCR quantitative with SYBR Green technique. For this quantification, a couple of primers composed of a universal primer complementary of polyT-adapter and a primer specific of RNAi sequence were used. This technique allowed us to determine with precision the level of expression of RNAi with the different constructs.

Detection of RNA interference by real time PCR

1 Polyadenylation



2 Reverse Transcription



3 Real time PCR

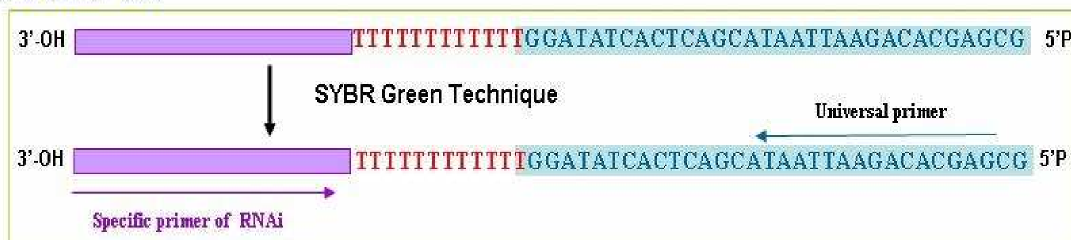


Figure 28 : Detection of RNA Interference by real time PCR.

Adaptation of the technique for our purpose

Polyadenalytion step

Reactions were performed according to the manufacturer's protocol (Ambion). Different quantities of PAP enzyme, and different amount of RNA were tested. For this test, a synthetic RNAi to determine the different parameters was used. The choice of this RNAi was based on the sequence of the first RNAi designed.

Synthetic RNAi: 5'UGAAGUCAAAGAGAUCGUCGG-3'

Mouse RNA or CHO.K1 RNA was used as template to test the background obtained with mouse tissue or CHO.K1. After comparison, it was decided to work for future analysis with the ratio 1U of PAP for 5 µg of total RNA. This quantity of enzyme was not limitative.

After polyadenylation, RNA was extracted with phenol/chloroform, and then precipitated with Na acetate and EtOH in presence of glycogene. RNA was then redissolved in water.

Polyadenylation Protocol:
Ambion Kit: Poly Polymerase Cat #2030. The reaction was made as described in protocol with some modifications. Polyadenylation reaction was made in a final volume of 60 µl. The ratio 1U of PAP for 5 µg of total RNA was used. The incubation was 1h at 37 °C. After the reaction, to inactivate the enzyme the total RNA was extracted with the same volume of phenol/chloroforme mix. The supernatant was precipitated with 2, 5 fold of EtOH in the presence of Na acetate (200 mM) and glycogen (20 µg by point). After 1h in -80°C, the Eppendorf was centrifuged and pellet was dissolved in 50 µl of water.

Condition for Reverse transcription

The reverse transcription was performed with "High Capacity cDNA Archive kit" from Applied Biosystems. This kit contains a multiscribe as reverse transcriptase. For each reaction, 2 µg of RNA (20µl PAP product) were reverse transcribed in a volume of 50 µl. In parallel, 2 µg of RNA were used as control for RT - (without enzyme). The concentration of polyT-adapter was similar to that of the oligo T primer given in the kit. According to supplier instructions, the reaction was performed during 10 min at 25°C and then at 37°C during 2 hours. The sample was then frozen at -20°C.

PCR Quantitative with SYBRGreen

Principle: The SYBR Green technique employs a fluorophor which intercalates in the minor grove of DNA. This agent is fluorescent only when it is incorporated in DNA. So during the cycles of the Poly Chain Reaction (PCR), the SYBR green intercalates in newly synthesized DNA strand. At each cycle, the level of emitted fluorescence is recorded. This fluorescence is proportional to the number of DNA molecules synthesized. The quantitative PCR depends on the continuous measurement of fluorescence emission during cycles of PCR reflecting the accumulation of product PCR. The quantitative aspect relies on the determination of Ct which corresponds to the minimum number of necessary cycles so that fluorescence exceeds the threshold

(fixed at the top of the background noise-beginning of exponential phase of amplification) (see Figure 29).

Real time PCR quantification of RNAi by SYBR Green

Principle :

Detection of PCR products by emission of fluorescence of an intercalating agent

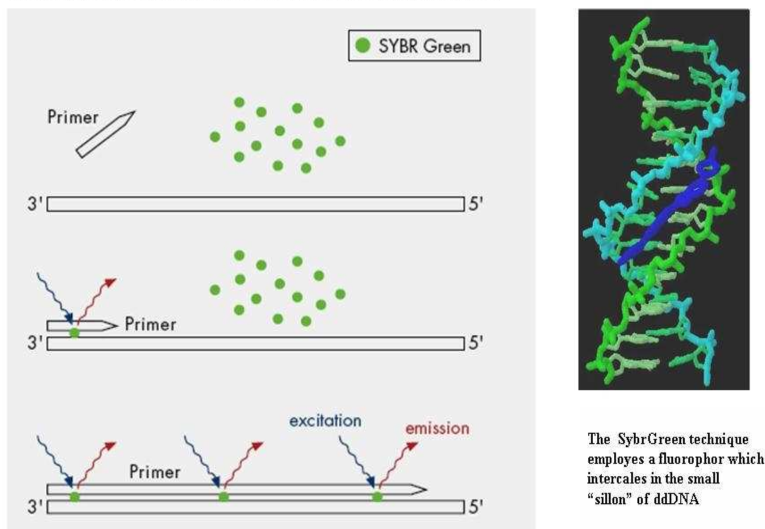


Figure 29 : Real time PCR quantification of RNAi by SYBR Green.
The quantitative PCR based on the continuous measurement of the emission of fluorescence during the cycles of PCR reflecting the accumulation of PCR product. The quantitative aspect lies in determining the Ct (cycle threshold "or" crossing point "), which corresponds to the number of cycles minimum necessary for the fluorescence exceeds the threshold (see above the background noise - early phase of exponential 'amplification).

For PCRQ detection, we used SYBR GREEN PCR Master MIX from Applied Biosystems according to manufacturer protocol. Except that the final volume reaction was 20 µl. The couple of primers used in PCRQ reaction were composed of a "universal" primer 5' GCGGAGCACAGAATTAATACGA 3' and a primer complementary to the sequence of the analyzed siRNA (for example: "RNAi sequence" 5' ugaagucaaagaucgucgg3'. Primer: 5' tgaagtcaaagagatcgtcgg3').

Transgenic mice

Mouse strain

FVB/N mouse strain was chosen to generate transgenic animals. The FVB/N mouse strain was developed in the early 1970's from a group of mice found to carry the *Fv1b* allele for sensitivity to the B strain of Friend leukemia virus. FVB/N is an inbred strains characterized by robust reproductive performance and consistently large litters. FVB/N mice offer a system suitable for most transgenic experiments and subsequent

genetic analyses. Moreover, fertilized FVB/N eggs contain large and prominent pronuclei, which facilitate microinjection of DNA. The phenotype of large pronuclei in the zygote is a dominant trait associated with the FVB/N oocyte but not the FVB/N sperm. FVB/N zygotes survived well after injection. Genetic markers of the FVB/N strain were analyzed for 44 loci that cover 15 chromosomes and were compared with those of commonly used inbred strains. In addition to the albino FVB/N strain, pigmented congenic strains of FVB/N are being established. These features make the FVB/N strain advantageous to use for research with transgenic mice (Taketo et al., 1991; Segovia et al., 1999).

Transgene preparation for microinjection.

For preparing the DNA for microinjection, a protocol optimized in our laboratory based on two purifications by Qiagene kit followed by Elutip D was used.

Solution preparation

TAE 1X Solution (4 times 500 ml): 1) 10 ml TAE 50 X in 500 ml bottle, 500 ml embryonic water was added and was kept at 4 ºC till utilization. 2) Low salt buffer (LS Buffer) for 200 ml solution: 200 mM NaCl; 20 mM Tris HCl; 1 mM EDTA pH 7.4. 8 ml NaCl 5 M, 4 ml Tris HCl 1 M pH 7.4, 400 µl EDTA 0.5 M and up to 200 ml embryonic water this solution was stored at 4 ºC after autoclaving. 3) High salt buffer (HS Buffer) for 200 ml solution: 1M NaCl; 20 mM Tris HCl; 1 mM EDTA pH 7.4. 40 ml NaCl 5 M, 4 ml Tris HCl 1 M pH 7.4, 400 µl EDTA 0.5 M and up to 200 ml embryonic water and it was stored at 4 ºC after autoclaving. 4) Ethanol 70%: 300 ml embryonic water was added to 700 ml ethanol 100% and was kept at 4 ºC. 5) Low melting point gel preparations: 1% low melting point GTG Agarose gel was used for preparations of the insert. 2 gm GTG Agarose in 200 ml TAE 1 X was heated by micro wave.

Procedure

1) Plasmid digestion: 20 µg high purity plasmid DNA was digested for 4 hours at 50 ºC by using 7µl Sfi restriction enzyme (50U/ 10 µl) in final volume of 100 µl followed by enzyme inactivation at 65 ºC for 20 minutes. 2) Digestion verification on gel: 2 µl of the digested plasmid was tested for the digestion efficiency on 0.7 %

Agarose gel in comparison with 300 ng of none digested plasmid with 10 µl 1kb ladder. 3) Low melting point Agarose gel: The digested DNA were loaded in the 1% gel, migration was carried out at 200 volts for 4 hours. 4) Transgene extraction from the gel: The DNA fragment was excised from the agarose gel with a clean, sharp scalpel. 5) Quick Gel Extraction Kit Protocol (using Qiagen ready to use kit): DNA was re-extracted from the gel following the supplier instructions. 6) Purification and measure of the concentration of DNA by Elutip D: Elutip D and DNA solution equilibration. The tip of an Elutip column was cut off about 2mm from the white plug in the narrow end. The cap was removed from the top of the Elutip. Two 5ml disposable syringes were labeled and marked one "low" and one "high". The plunger was removed from the "high" syringe and the Elutip was attached using a forceps for leverage. 2 ml "High" buffer was added by the pipettor, the buffer was pushed through the Elutip slowly. The Elutip was removed from the "high" syringe and was attached to the plungerless "low" syringe. 5 ml of "low" buffer (heated to 42 ºC) was loaded in the syringe and was forced through. The Elutip was ready at this point for binding the DNA. The Elutip was removed and then the "low" syringe plunger was removed, the Elutip prefilter was attached to the syringe, and then the Elutip was attached. 1 ml “low” solution was added to the DNA and it was kept at 42 ºC till utilization, the DNA/"low" solution was loaded into the syringe/prefilter/Elutip assembly. The DNA was slowly pushed (around 1 ml/min) through. The flowthrough was collected and the syringe was disassembled from the prefilter. The flowthrough was slowly pushed through.

3 ml "low" buffer was slowly added using the same syringe. The air was pushed through the Elutip assembly. 0.4 ml "high" buffer was added to the syringe. The plunger was replaced and the buffer was pushed through the Elutip slowly, the eluate was collected in a sterile Eppendorf tube. The Elutip was removed and the syringe was disassembled. The elution was repeated with 0.4 ml of "high" buffer. The pooled eluates were precipitated, 2 volumes of absolute ethanol were added and it was centrifuged in a cold-room at 25,000 g for at least one hour. The pulled was collected and it was dissolved in 40-80 µl injection buffers.

DNA microinjection to generate transgenic mice

Mice microinjection and transgenic animal preparations were carried out in cooperation between Bruno Passet and Sonia Prince. These constructs DNA fragments were diluted at 1 to 5ng/µl and microinjected into mouse pronuclei. Transgenic mice were identified by PCR analysis performed on genomic DNA extracted from the tail.

DNA extraction

DNA extraction was carried out as described by (Laird et al., 1991)
Proteinase K solution: Tris HCl pH 8 10 mM, EDTA pH 7.5 5 mM, SDS 1% and sodium acetate pH 8 300mM. The tissues were incubated overnight at 37ºC in the presence of 5µl proteinase K and 400 PK buffer, it was followed by 10 min centrifugation at 4 ºC. One volume phenol/ chlorophorme/ alcohol/ isoamyl was added to 300 µl of the supernatant, vortexed and it was centrifuged for 10 min at 4ºC. The aqueous phase was collected. One volume isopropanol was added to the collected phase and it was centrifuged. The pellet was washed by70% ethanol. The DNA was dissolved by 300 µl water

Transgenic animal screening

Transgenic animals were identified using PCR. Several sets of primer (SUR1, 2, 3, HGH and 5'HS4) were used for the screening of these animals.

SUR1, 2, 3 primers.

Sens: 5'-GGCCACAATTCGCCGGCG-3'

Anti-sens 5'-GCGGCAGAACGCGACTCA-3'

HGH primer

Sens : 5'-AAGTTCGACACAAACTCACA-3'

Anti-sens 5'-AGCAATTTGGGAGGCCAAGG-3'

5'HS4 primer

Sens : 5'-TCAAATCATGAAGGCTGGAA-3'

Anti-sens 5'-GAGTTGGATGAGAGATAAT-3'

CHAPTER 6
RESULTS

The use of siRNAs generated from shRNAs includes several steps: the design of shRNA sequence targeting the mRNA of interest, the construction of vectors expressing the shRNA, the validation of the shRNA and of vectors by transfection in cultured cells, the measurement of sh/siRNA in cells, the generation of transgenic mice expressing the shRNA genes, the measurement of sh/siRNA in mouse cells and the challenge of the animals with the PRV.

1. Selection of viral target gene and target sequence.

It was shown that the IE gene which is expressed very precociously is essential for the virus replication and multiplication. This gene can thus be a good target to block the viral cycle. The over-expression of an inactive mutant of gene IE in transgenic mice carried out in the laboratory of T. Ono (Japan) induced a resistance to the virus (personal communication) but the side effects of the transgene made this approach impossible to use as a therapeutic tool. The complete sequence of IE gene was reported by Klupp et al. (2004). Figure 30 illustrates the 5'P and 3'OH UTRs (untranslated regions) of this gene (Genbank accessions M34651 and X12904).

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GCTTATAAGC GCGGTCTCCA TCGTAGCACT TCACTGCGGT GCAGGTACGG ACAGCATCGT
TCTCTGCCAA CCCGAGGGGA TCCGACCGTC TCCGCTCCGG CGCGGACTCT GAAGACTCCG
GCTCTCCGGC GGCTATCAGC CCTCGACGGA CGCCCGACCC ACCGAGGCTC TCGGCCCGCC
AGAGAAGAGT CTTCTTCTTC TCCTCCTCCG GCCGCCTTCC TCCTTCTTCT CCGCCGCCCG
CTCTCCGCGC TCGGCGCCCG GCCTCGCTCA GGCAGAAAGA CCCCGATCGA GACCATGGCC
GACGATCTCT TTGACTTCAT CGAGACCGAG GGCAACTTCA GCCAGCTCCT GGCGGCCGCC
GCCGCGGCCG AGGAAGAGGG CATCGCGTCC GGCCCCGACG GCGGCAGCCA GGGCTCCCGG
CGCCGCGGCT CCTCCGGCGA GGATCTCCTC TTCGGCCCGG GCGGCCTCTT CTCCGACGAC

NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN
CTGCTGCTCC GCTGAGCGGG GCGCCCCCTC GGCCCGGCCG GACTCTGACT CTGACTCTCC
GGCCCCTCCA CCGGCTCCTC GAGGCCCTTC TCCTCCGTCT TCTCTCCCCT CGCCCTCGGC
CCGGTCCTCG TCCTCGTCCT CGTCCCCGTC CCCGTCCTCC TCCTCCTCTG CGTCCGCGGC
GGCGGCCTCG GTCCCCTCGG CCCGGCGGCG CTTGCCTCCC CGGCGCCTGC CTCCCCGGCC
CGGTGGCCCT CCTCCTCTCC TTCTCCTCCG CGCGGATCCC CCGGCCGGAG GTGGCTGCGG
CGGCGGCGGA GGTGGCGGCG GTGGTGGAAG CGGCGGCGGC GGCCGCGGCG GAGGGCTCGG
CGGCGGAGGA TCGTCCCGGT CCCCTTCTCC TCCTCCCGCG GTCCCCCGGT CCCCTTCTCC
TCCTTCTCCC ATCGGGTGAG AAAAGAGTTT GTTTTCAGAG TGAGAAAATA AAGTTTGTGC
TGTATTTTCT GAACCAGCTC GAGTCTCTGA GATTTTTTGG GGAGATGGAG GCGGCCATCT
TGGCGGTGGT CTCTGGGGTG GAGGTGGTCT TGTGGATGGG GGTCCCTGGT GGGAGGAAGA
AGAAGAGGTG GAGGGTCTTG GTGGGGGTGA CGGGGGTCCT CCTCCTGGAG GGTCTTGGTG
GTGGTGATGG GAAGAAGTGG ATGGGGGTCC TCCTCCTGGA GGGTCTTGGT GGTGGTGGGT
CTTAGCAGAT GGGGGGTCCC TGGTGGGTCT TAGCAGATGG GGGTCCTCCT CCTGGAGGGT
CTTGGTGGGA AGAAGTAGAG GGTCTTGGGG ATGTTGGGGG TCCTTGATGG TGGTGGTGGT
GGTGGTGGGA GGTGGACGGT GTTGGTGGTC CCGGCGGGTC CTGGTGGGAG GTAGATGGTC
CCGAGGGTCC CGGTGGTCCC GGGCGGGAGT TGGACGATGG TGGTCCTGCG GTGGTCGAGG
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Figure 30 : The IE gene of pseudorabies virus with 5'P and 3'OH UTRs (untranslated region)
ATG start codon is shown in red and bold as well as the stop codon TGA in blue and bold. The target sequence which is localized just after the start codon is marked in blue. The N stretch symbolizes the middle of the coding sequence.

The first shRNA was designed in the laboratory without referring to any consensus sequence as no model for shRNA designing was available at that time. ShRNA design and construction is considered to be an essential step in selecting an effective and efficient shRNA for gene silencing. Several works have been carried out to determine the best criteria for choosing the effective shRNA (Elbashir et al., 2001a; Taxman et al., 2006; Katoh & Suzuki, 2007; Li et al 2007). Throughout this work it was found that there are no clear and absolute rules for designing shRNA but some criteria which may help for obtaining the best shRNA have been proposed.

Table 7 : Proposed criteria for choosing the most effective siRNA and shRNA (from Li et al., 2007).

Functional siRNA	Functional shRNA
Strong preference for GC at positions 1-2	Starting with G or GG
Strong preference for AU at positions 3-7	Strong preference for AU at positions 3-7
Relatively GC-rich at positions 14-16	Relatively GC-rich at positions 14-16
Strong preference for AU at positions 17-19	Strong preference for AU at positions17-19
Strong preference for AU at positions 3, 6, 13, and 18	Strong preference for AU at positions 3, 6, 13, and 18
Preference for GC at position 9	Preference for AU at position 9
Weak preference for GC at position 11	Preference for GC at position 11

To be sure that the selected sequence has no significant homology in the genome with any sequence other that the gene to be targeted, BLAST tests must be achieved. However, due to the shortness of the target sequence (21-23 bp) and the size of the haploid genome ($3*10^9$ bp), it is rare to find no homology elsewhere in the genome. As $4^{15}<3*10^9<4^{16}$ and because of the non random use of di, tri or tetranucleotide sequences in the genome, it is a common result to find a perfect match of 17, 18 or even 19 nucleotides.

A blast search was thus performed on the Ensembl site version 49. In the case of the chosen shRNA sequence (GCCGACGATCTCTTTGACTTCAT), 2 transcripts and 4 genomic regions (Table 8) showed a partial homology with the chosen shRNA. This partial homology is not supposed to be sufficient to lead to the degradation of these transcripts. No off-target phenomenon is thus expected.

Table 8 : Sequences in the mouse genome and transcriptome showing significant homologies with the Sh target sequence.

Number of conserved siRNA nucleotides	Part of the siRNA conserved	Position in mouse genome	Comments
16	10-25	Chr3: 73 329 067 - 73 329 082	Between Slitrk3 (SLIT and NIRK-like family, member 3) and Bche (Butyrylcholinesterase) genes
16	10-25	Chr5: 121 936 185 - 1210936 200	In an intron of Tmem116 (Transmembrane protein 116) gene
16	10-25	Chr16: 57 659 885 - 57 659 900	In the second intron of Col8a (Collagen, type VIII, alpha) gene
16	10-25	Chr19: 54 086 239 - 54 086 254	In the third intron of the Shoc2 (Suppressor of clear homology) gene
15	10-25	Fucosyl transferase 9, Chr4, in 3' UTR	
15	10-25	Retinol dehydrogenase, Chr10 in third exon (coding)	

2. Designing of vectors expressing the shRNA gene

Construction and evaluation of the U6-shRNA vector

The first shRNA which corresponds to the IE sequence following the AUG codon was introduced into the pBS-U6 vector friendly given by Dr. Shi (Shi et al., 2002).

In the given pBS-U6 vector, the U6 promoter (RNA Polymerase III promoter) is cloned in pBluescript vector (pBS II KS +) between the sites Kpn I and Apa I. After cloning of the IE SH sequence in the ApaI site, the U6 promoter induces the transcription of a two repeat inverted motif leading to the production of a small hairpin RNA.

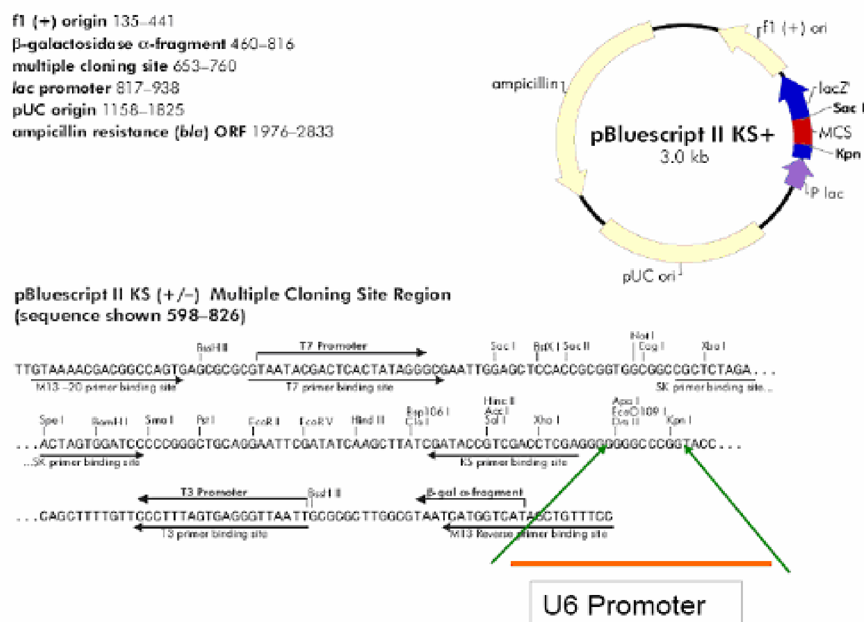


Figure 31 : Structure on the U6 gene promoter plasmid.
The pBluescript II KS+ map and the site of insertion of the U6 promoter in the mutiple cloning site (MCS) are indicated.

The cloning was performed in two steps. In the two-step cloning scheme, two pairs of oligonucleotides were designed to make the RNAi construct.

The native U6 promoter contains three Gs from which transcription was initiated. These three Gs were reformed in the RNAi construct and are part of the body of the siRNA. An ApaI site GGGCCC was located immediately after the U6 promoter in the pBS/U6. After the pBS/U6 vector was digested by ApaI and cut/blunted by klenow, there is a single G left. Therefore, in order to put back two Gs, the first oligonucleotide (Oligo 1a) should start with GG and the second oligonucleotide (Oligo 1b) should end with CC.

In the second pair of oligos, Oligos 2a should have CCC. After the CCC, TTTTT were put (for RNA Pol III transcription termination) and an EcoRI site (to subclone into the vector). In the oligo 2b, there should be GGG and the sequence for EcoRI site.

To generate a loop between the sense and antisense sequence, the HindIII sequence was added to connect them. Therefore the HindIII sequence should be in all four oligonucleotides. In other constructs, another loop generating sequence TTCAAGAGA was tested.

After this, the oligonucleotides sequenced were checked to make sure they have no EcoRI and HindIII site. If they do, other sites for sub-cloning had to be chosen.

The HindIII sequence that connects the two repeat motifs was itself an inverted repeat. As a result the final siRNA product was a perfect inverted repeat. This RNA was expected to fold back to generate a shRNA.

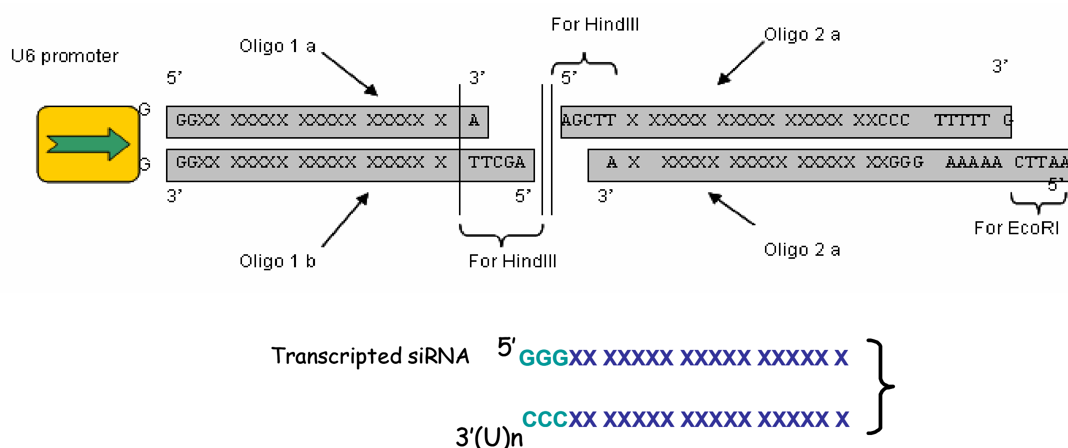


Figure 32: The basic scheme for the shRNA construction.
The proposed short hairpin RNA and the predicted transcribed siRNA are shown.

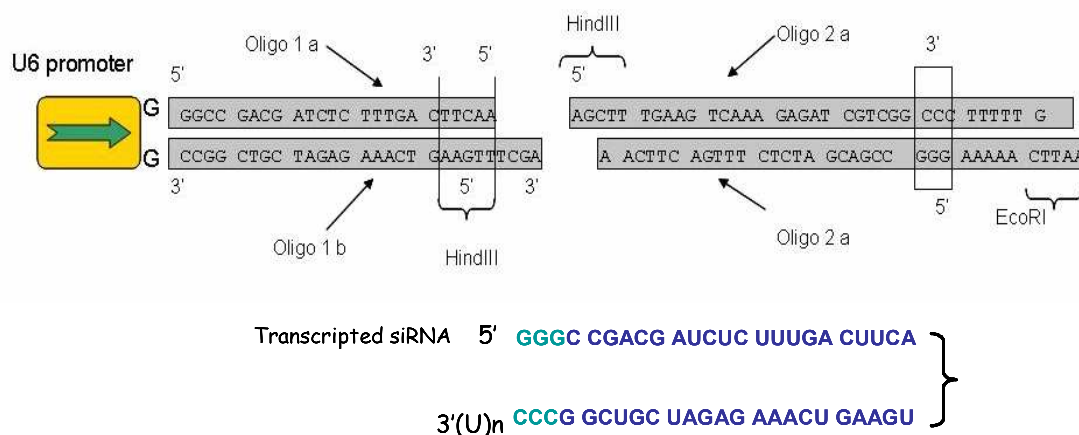


Figure 33 : Representation of the first shRNA construct.
The hairpin structure of the transcribed siRNA is shown.

The U6-shRNA plasmid co-transfected with the reporter gene is supposed to induce a very strong inhibition of the luciferase gene.

3. Preparation of reporter luciferase gene plasmid

In order to avoid virus culture and manipulation to test the efficiency of the shRNA constructs, we first designed a simplified model in which a single transcript, containing both the IE and the firefly luciferase reporter sequences, is produced. The production of an efficient shRNA leads to the destruction of this transcript and thus to a drop of the luciferase expression and activity. For construct validation, target plasmids which contain the luciferase reporter gene were prepared. Three different reporter luciferase constructs gave the possibility to check the specificity of different shRNAs. These constructs were called: pLM24 IE Luc, pLM24 Luc 3'UTR IE, pLM24 IE Luc 3'UTR IE.

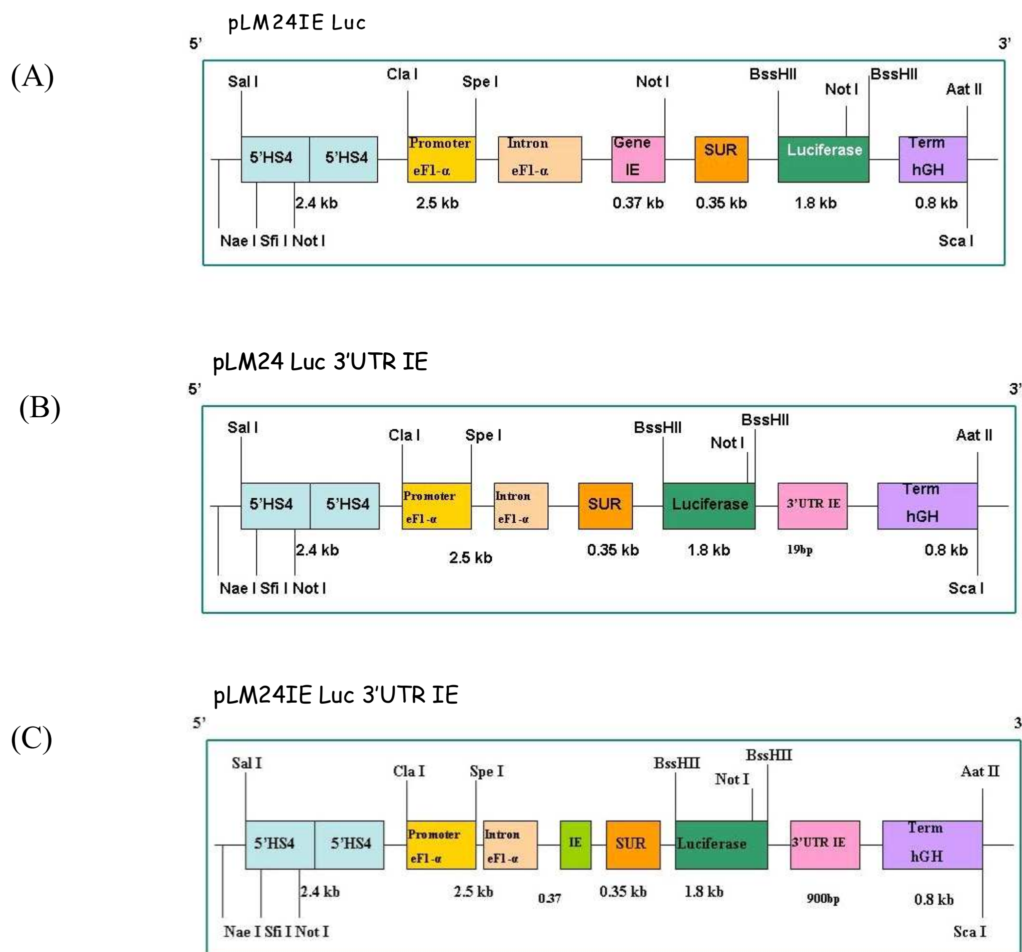


Figure 34: The target vectors with the reporter luciferase gene.
The luciferase gene was introduced after an IRES (internal ribosome entry site) which allows its efficient translation in all cases. The three plasmids are (A) pLM24 IE Luc prepared for testing the constructs designed to induce a degradation of IE mRNA. The target IE region contained 0.37 kb from the IE mRNA sequence (positions 5286 to 5679 in Genbank accession M34651 X12904) (Klupp et al., 2004). (B) pLM24 Luc 3'UTR IE. This plasmid contains a short part (positions 10266 to 10283 in sequence M34651) of IE 3'UTR corresponding to a shRNA construct (see later). (C) pLM24 IE Luc 3'UTR IE. This target designed from the plasmid pLM24 Luc contains the complete 3'UTR IE gene (positions 9855 to 10799 in sequence M34651).

4. Evaluation of the IE mRNA knockdown by transfection of pBS-U6-shRNA in CHO cells.

Transfection of the U6-shRNA construct in CHO cells reduced markedly (~90%) the expression of the luciferase gene whereas the empty U6 vector had no effect (Figure 35) The shRNA is therefore a potent interfering RNA, potentially capable of preventing mouse infection by PRV. This shRNA was used to evaluate the capacity of different vectors to knockdown the IE mRNA in cells and in transgenic mice. However, as it is known that U6 promoter used alone in transgenes often leads to silencing, we chose different strategies to try to overcome this difficulty. To tentatively favor the synthesis of the shRNA in transgenic animals, the vector U6-

shRNA was first introduced into the pM10 vector at three different sites. A second strategy was to use a RNA pol II promoter instead of the U6 promoter in order to produce either a shRNA (5T strategy) or a miRNA (miRNA strategy) (see paragraph 5 below)

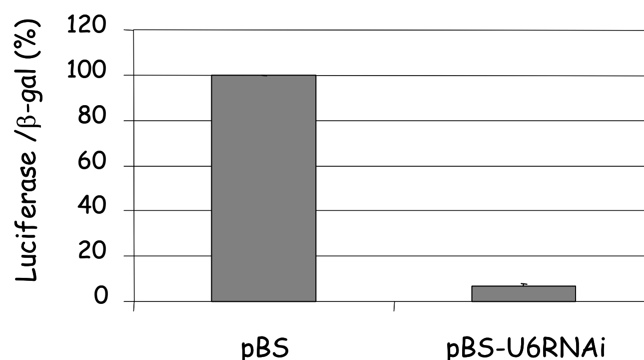


Figure 35 : Knockdown of the IE mRNA by the U6-shRNA construct in CHO.
The pBS-U6-shRNA construct was transfected into CHO cells with the reporter luciferase construct (plm24IE luc) and the β−galactosidase vector. Luciferase and β-galactosidase activities were measured 48h after transfection. The results are given as percentage of luciferase activity in cells transfected by plm24IE Luc and the empty pBS vector. All luciferase values were normalized to the β-galactosidase activities.

5. Construction and evaluation of vectors containing the U6-shRNA gene

The genes which are under the dependency of RNA Pol III promoters in transgenic animals are often silenced. It was thus postulated that an association of these vectors with others capable of working in a reliable manner in transgenic animals could make it possible their expression as transgenes ("rescue"). The U6-shRNA gene was therefore introduced in three different sites of the pM10 vector. In these conditions, the synthesis of the shRNA is expected to be driven by the U6 gene promoter and not by the eF1-α promoter.

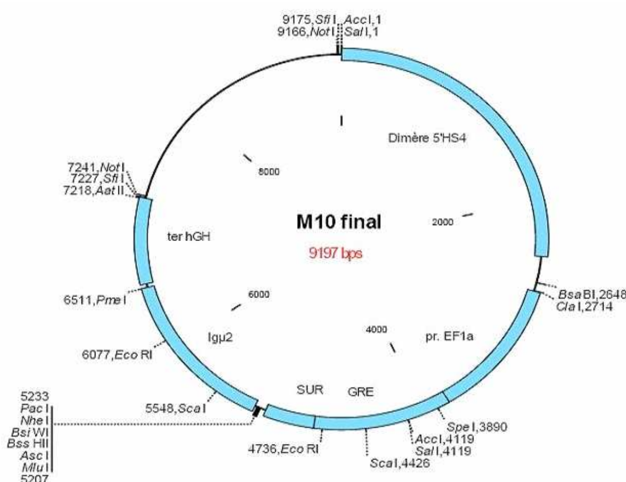


Figure 36 : Circular representation of the vector pM10.

The vector pM10 contains the insulator sequence 5'HS4 from the chicken β-globin (Chung et al., 1993). Two copies of 5'HS4 are located before the promoter eF1-α. The region 5'HS4 was used to increase the frequency as well as the expression level of transgenes integrated in a stable maner in the genome and to reduce the position effect (Taboit-Dameron et al., 1999).

The promoter eF1-α was located after the insulator and it contained a mouse mammary tumor virus enhancer. The pM10 vector was also deleted of UC containing region located just downstream of the cap site which prevents the mRNA translation in quiescent cells.

The intron contained in this vector is the second intron of the rabbit β-globin gene. It was selected because its splicing sites are close to the optimal consensus sequences and it is an AT rich region. This intron was already used successfully in other work to express transgenes in mice (Furth et al., 1994; Wagner et al., 1997).

The Igμ2 element contains an enhancer of expression bordered by two sequences of attachment to the nuclear matrix (MAR) coming from the locus of the heavy chains from immunoglobulins μ (Forrester et al., 1994). This enhancer was responsible for the local formation of euchromatin (Jenuwein et al., 1993). Its association with the two MAR sequences allows extending this effect of chromatin opening over a longer distance (at least 1 kb) (Jenuwein et al., 1997; Forrester et al., 1999). This enhancer has already been used successfully in vivo to express transgenes (Bodrug et al., 1994; Gross et al., 2000). The enhancer contained in the Igμ2 element is able to stimulate the expression with heterologous promoter in cell lines of non-lymphoid origin (murine fibroblasts, human cells HeLa) (Wasylyk & Wasylyk, 1986). The Igμ2 element was thus likely to contribute to locally maintain chromatin in an open structure favorable to the expression of transgenes even out of the lymphocytes B. In the vector pM10, the Igμ2 element was deliberately placed in the transcribed and not translated part of the genes in the 3'UTR of the cDNA. This choice was made to avoid disturbing the structure of the β-globin vector.

This vector also contains a fragment of the 5'UTR sequence of early gene of virus SV40 (86 bp according to the cap), associated to the totality of the R regulation region and the first 39 nucleotides of the U5 sequence of LTR sequence of the virus HTLV-1 (Human T-cell Leukemia lymphoma Virus) (Attal et al., 1996; Attal et al., 2000). The R and U5 regions located downstream the site of initiation of the transcription of the

HTLV-1 virus are necessary to obtain a maximum expression of viral genes (Ohtani et al., 1987). The association of these elements, placed downstream from heterologous promoters, is able to stimulate their level of expression in the absence of viral proteins of HTLV-1 (Takebe et al., 1988). This particular association is able to enhance the expression of various reporter genes and cDNA in several cellular types by increasing the level of transcription (Attal et al., 2000). We thus integrated this element downstream the promoter eFl-α in several vectors to stimulate gene and transgene expression.

The U6 promoter-shRNA construct was introduced in both orientations into three unique cloning sites (BssHII, BsaBI, and AatII) of the pM10 vector, giving the three following constructs Sh1, Sh2 and Sh3 (Figure 37). It appears later that the orientation of the U6 promoter-shRNA had no effect on its expression (data not shown). We thus performed all the following experiments with constructs in which the U6 promoter is in the same orientation as the eF1-α promoter.

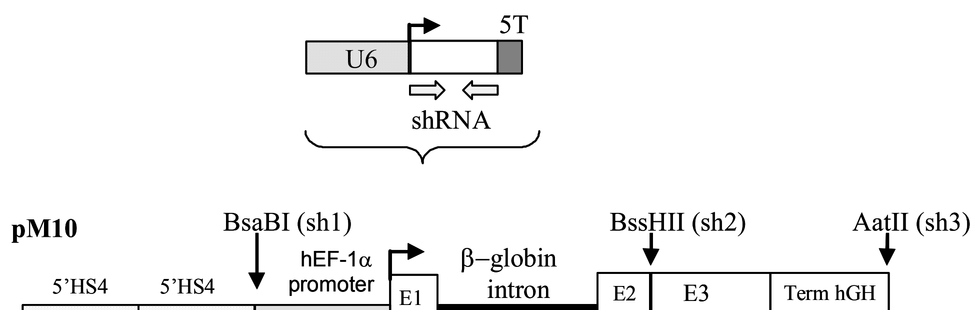


Figure 37: Introduction of the U6-shRNA gene in the three sites of pM10 vector, to give the Sh1, Sh2 and Sh3 constructs.

The three vectors pM10-U6-shRNA were tranfected into CHO cells with the IE-luciferase and the lacZ reporter gene. The results of the Figure 38 indicate that the three vectors were as efficient as the U6-shRNA alone. This suggests that the three vectors could express the shRNA gene at a high level in transgenic mice.

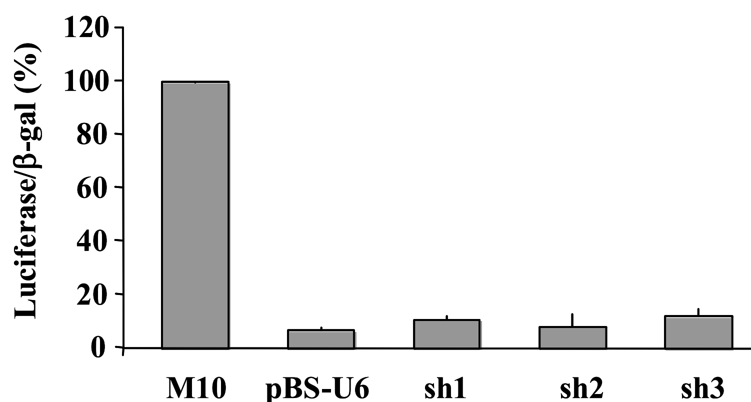


Figure 38 : Knockdown of the IE mRNA by the three vectors containing the pM10 and the U6-shRNA constructs.
The different constructs U6-shRNA, sh1, sh2 and sh3 constructs were transfected into CHO cells with the reporter luciferase construct (plm24IE-luc) and the β–galactosidase vector. Luciferase and β-galactosidase activities were measured 48h after transfection. The results are given as percentage of luciferase activity in cells transfected by plm24IE-Luc and the empty pM10 vector. All luciferase values were normalized to the β-galactosidase activities.

6. Construction and evaluation of the 5T construct

The idea behind this construct was to discard the use of the U6 promoter by synthesizing the shRNA only by the action of the RNA polymerase II promoter eF1-α. Inserting 5T at both sides of the target sequence was expected to improve and facilitate the shRNA excision from the mRNA. Indeed, the oligonucleotide T in the vector becomes an oligonucleotide U in the mRNA, a RNA structure which is particularly sensitive to RNAase and thus able to release efficiently the shRNA. For testing this hypothesis, the shRNA containing the sequence GCCGACGATCTCTTTGACTTCA was bordered by the oligo TTTTT. This insert was introduced into the BssHII of the multi-cloning site of pM10 vector.

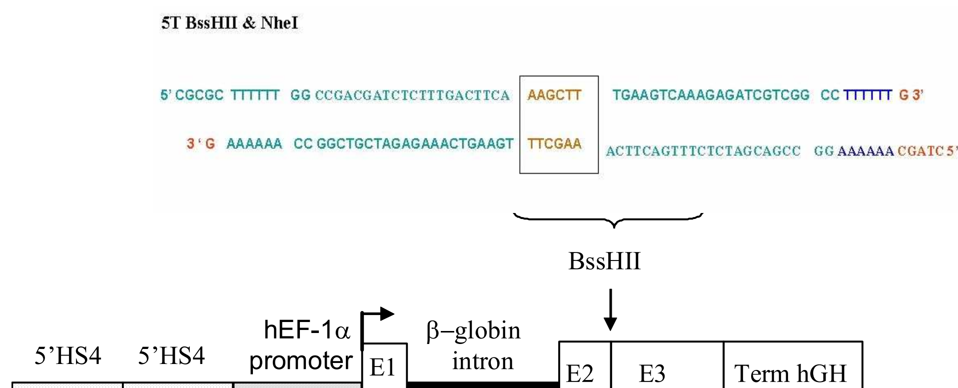


Figure 39 : Representation of the 5T construct.

As for the other constructs previously described, the 5T construct was transfected into CHO cells with the same two reporter genes. The IE mRNA was knocked down by the 5T construct but only to a limited extent (~45%) (Figure 40), indicating that a functional shRNA was released from the mRNA produced by this construct. However, the 5T construct could still be able to prevent or attenuate PRV infection in transgenic mice.

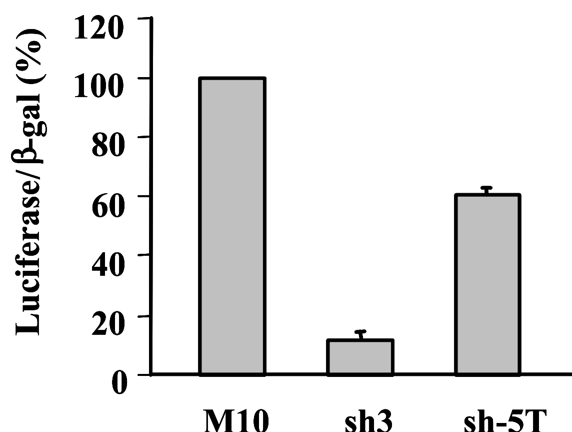


Figure 40 : Knockdown of IE mRNA by the 5T construct.
The sh3 and 5T constructs were transfected into CHO cells with the reporter luciferase construct (plm24IE-luc) and the β−galactosidase vector. Luciferase and β-galactosidase activities were measured 48h after transfection. The results are given as percentage of luciferase activity in cells transfected by plm24IE-Luc and the empty pM10 vector. All luciferase values were normalized to the β-galactosidase activities.

7. Construction and evaluation of the miRNA construct

The second approach to produce a siRNA without the use of U6 promoter was based on the use of a miRNA vector.

The naturally occurring Mir-30 RNA gene was used to prepare the proposed miRNA construct. Figure 41 illustrates the naturally occurring Mir-30 skeleton. As also shown in Figure 41, short hairpin RNA constructs were expressed as human microRNA-30 (miR30) primary transcripts (Boden et al., 2004). The hairpin stem consisted of 22 nt of dsRNA and a 19 nt loop from human miR30.

The shRNA sequence was introduced into the naturally occurring human mir-30 skeleton and replaced the original mir-30 sequence keeping the overall mir-30 structure as it is naturally occurring. At that time, it was not clear if the processing of miRNA by the cellular enzymes (Dicer and Drosha) occurred, because the siRNA sequence was just introduced into the skeleton as described in Figure 42.

Endogenous mir-30 primary transcript

mir-30 context Drosha cleavage site mir-30 sequence mir-30 loop

Open biosystems mir-30 hairpin design

Drosha Dicer

Antisense sequence of shRNA

Figure 41 : Mechanism of miRNA processing in cells.
The naturally occurring human Mir30, Drosha cleavage sites as well as Dicer cleavage sites are shown.

Hsa-mir-30a

Endonucléase Dicer

Endonucléase Drosha

30

Figure 42 : Representation of the miRNA construct.
The siRNA sequence was introduced into the miR30 gene. The expected siRNA is written in red.

The above miRNA construct was then introduced into pM10 vector as described in Figure 43.

BssHII

5'HS4 5'HS4 hEF-1α promoter E1 β–globin intron E2 E3 Term hGH

Figure 43 : Representation of the pM10-miRNA construction.
The miRNA construct was introduced into the BssHII site of the multicloning site of pM10.

As the other constructs, the pM10-miRNA construct was evaluated in CHO cells. Its capacity to knockdown the IE mRNA was significant but limited (~50%) (Figure 44).

This construction is an additional candidate to prevent PRV infection in transgenic mice.

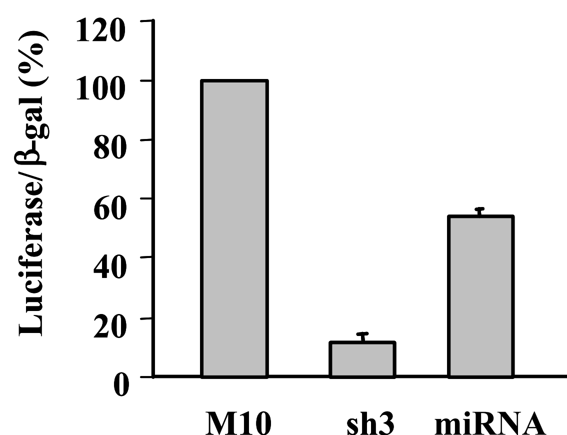


Figure 44 : Knockdown of IE mRNA by the miRNA construct.
The sh3 and miRNA constructs were transfected into CHO cells with the reporter luciferase construct (plm24IE-luc) and the β−galactosidase vector. Luciferase and β-galactosidase activities were measured 48h after transfection. The results are given as percentage of luciferase activity in cells transfected by plm24IE-Luc and the empty pM10 vector. All luciferase values were normalized to the β-galactosidase activities.

8. Measurement of siRNA concentration in transitionally transfected CHO cells

The different vectors depicted above have thus a various capacity (from 40% to 90%) to knockdown the IE mRNA in cells. This may reflect the concentration of the produced siRNA. In order to better understand the mechanism of siRNAs, it seemed important to measure their concentration in cells after transfection with the different vectors. SiRNA concentration may be roughly determined by Northern blotting. However, it is only a semi-quantitative method. A new method of RT PCR adapted to the quantification of small RNAs appeared more attractive because more capable of giving reliable measurements of the RNAs (Shi & Chiang, 2005). The rationale of this method is described in Figure 45.

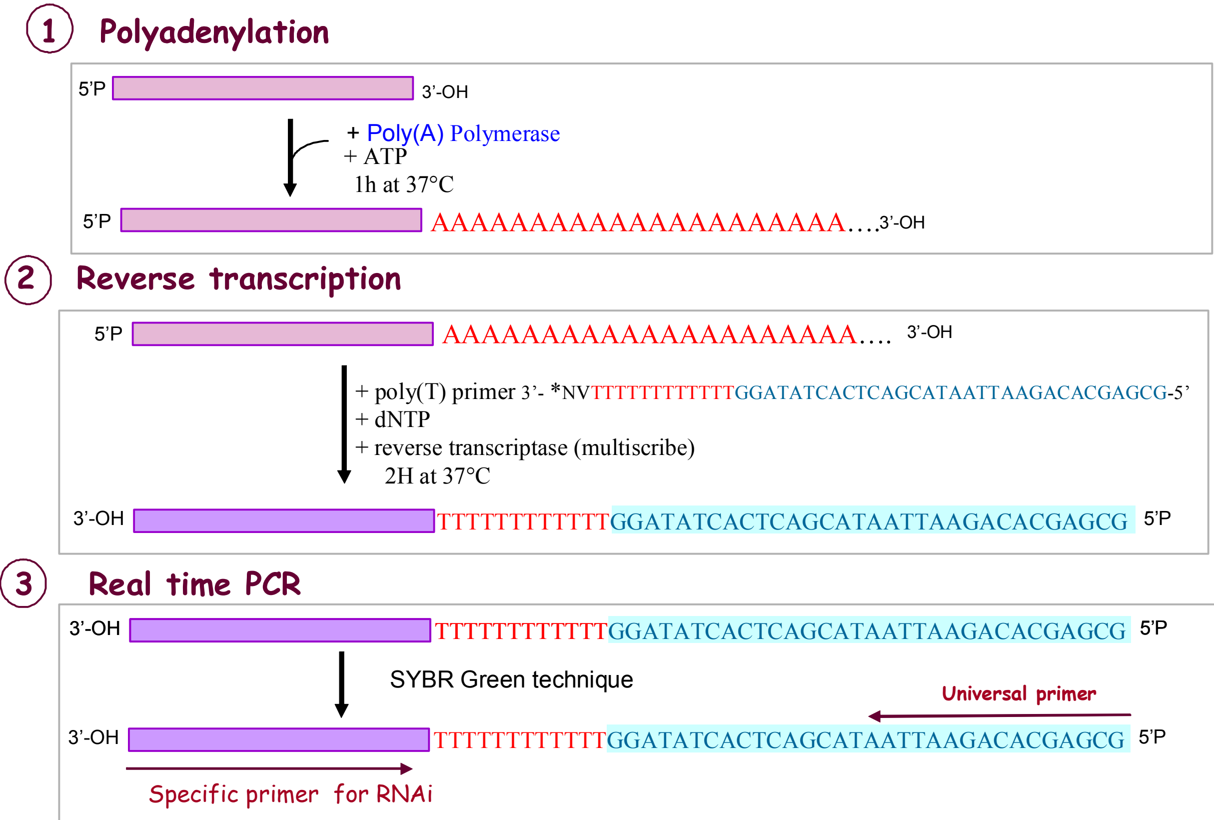


Figure 45 : siRNA quantification method using real time PCR.
Total RNA of transitionally transfected cells was first polyadenalated (1). A reverse transcription reaction was then carried on this polyadenylated RNA (2). Lastly, a real time PCR using one universal primer and a specific primer was performed (3).

To validate this technique with our siRNA, we ordered a synthetic siRNA for using as standard and to define the thresfold of detection of siRNA expression.

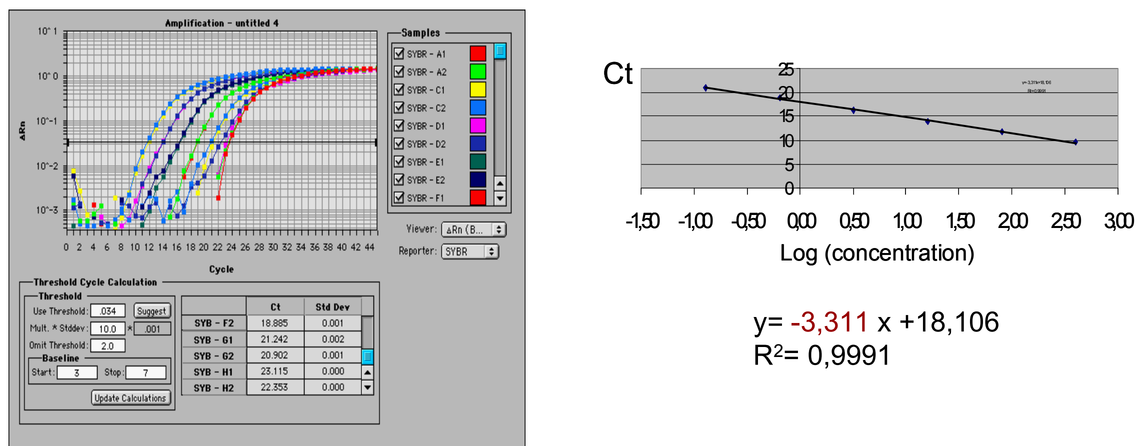
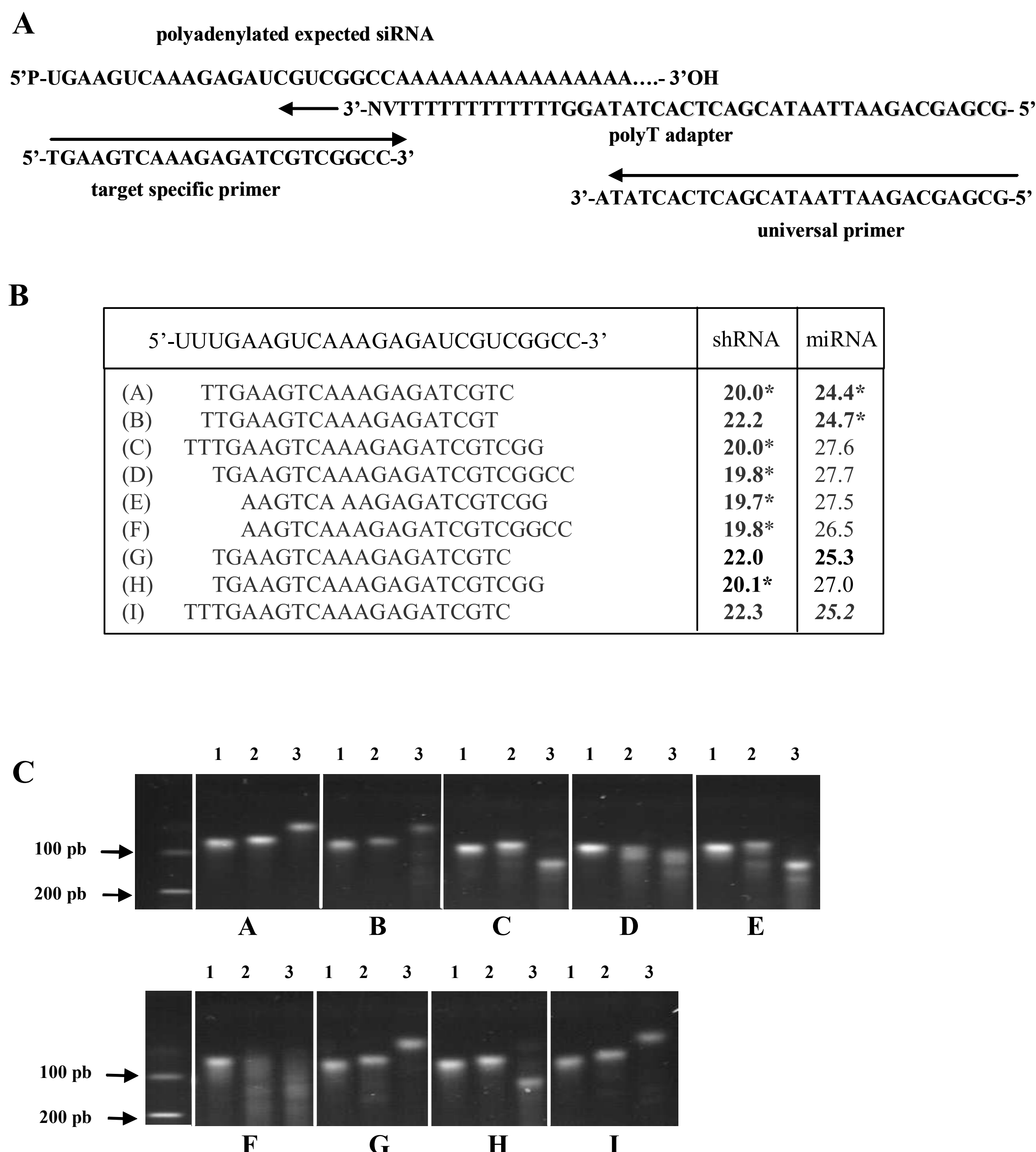


Figure 46 : Quantitative PCR validation using synthetic siRNA.
Standard curve was determined by using artificial RNAi (synthesized) added to the extracted RNAs of the CHO cells which has been prepared using the technique of Chomczynski (Chomczynski and Sacchi, 1987) without precipitation with lithium chloride. The RNA of CHO cells was used as a guide to make it possible to define the background related to endogenous RNAs.

As the efficiency of the final qPCR is strictly related to the relative location of the primer and the template, a series of primers complementary to various positions in the expected RNA was used (Figure 47B). All primers give a specific amplification product from the siRNA produced by the U6- shRNA construct (Figure 47C, lane 1), and give low Ct values indicating a high production of siRNA with the full correct sequence (Figure 47B, shRNA column). As also shown in Figure 47C the siRNA produced by the miRNA construct is always shorter than the siRNA produced by the U6-shRNA construct (compare lane 2 and lane 1 in A, B, C, G, H and I). A more detailed examination of the RT-qPCR products and the Ct values suggests that the siRNA produced by the miRNA construct is unlikely to contain the whole expected RNA target sequence. The two primers D and F fail to amplify any specific products from the siRNA produced by the miRNA constructs (see lane 2 in D and F in Figure 47C) and give high Ct values not significantly different from those obtained with DNA of non transfected cells. This allows us to exclude the presence of the two C at the 3' end of the siRNA produced from the miRNA construct. The three primers C, E and H, ending at the 3' end with the two G located just upstream the two C, give also high Ct values of the same order of magnitude with the siRNA produced from the miRNA construct (Figure 47B, miRNA column). However, they seem to give a specific amplification product from these siRNA (Figure 47C, lane 2 in C, E, and H). But it is to note that they also give a non specific amplification product of an unexpected long size with DNA of non transfected cells (Figure 47C, compare lane 3 in C, E and H versus in A, B, G and I). Even if it is not definitive evidence, it is our firm conviction that taken together these results show that the siRNA produced from the miRNA constructs also lack these two G in its 3'OH end. Both the Ct values and the amplification products obtained with primers A, B, G and I show that the following upstream C is present at the 3'OH end of the siRNA produced by the miRNA construct.



5'-UUUGAAGUCAAAGAGAUCGUCGGCC-3'		shRNA	miRNA
(A)	TTGAAGTCAAAGAGATCGTC	**20.0***	**24.4***
(B)	TTGAAGTCAAAGAGATCGT	**22.2**	**24.7***
(C)	TTTGAAGTCAAAGAGATCGTCGG	**20.0***	27.6
(D)	TGAAGTCAAAGAGATCGTCGGCC	**19.8***	27.7
(E)	AAGTCA AAGAGATCGTCGG	**19.7***	27.5
(F)	AAGTCAAAGAGATCGTCGGCC	**19.8***	26.5
(G)	TGAAGTCAAAGAGATCGTC	**22.0**	**25.3**
(H)	TGAAGTCAAAGAGATCGTCGG	**20.1***	27.0
(I)	TTTGAAGTCAAAGAGATCGTC	**22.3**	***25.2***

Figure 47 : quantification and sequence analysis of the siRNA produced in CHO cells transfected with different constructs.
(A) Oligonucleotides and specific RT-qPCR method used for the quantification of siRNA as described in Materials and Methods. (B) Nine primers (A-I) specific of the RNAi sequence were tested. The Ct values obtained for each primer are given. The stars point significant amplification. Other Ct values are not significantly different from non-transfected cells or non-transgenic mice background. (C) The qPCR products were separated on 2% agarose gel electrophoresis. Lane 1: cells transfected by a shRNA construct. Lane 2: cells transfected by miR30 construct. Lane 3: non-transfected CHO cells. The observed amplification in lane 3 was either a short dimer of primers (primers A, B, G, I) or a non-specific amplification (primers C, D, E, H).

RNAi expression in transient co-transfection of CHO cells

We can thus conclude that even if the siRNA produced from sh constructs (U6-shRNA and the three M10-U6-shRNA) or from the miRNA construct are both able to knock-down the target IE-luciferase gene, they do not have the same sequence. This

leads us to ask if the difference in the knock-down efficiency is due to the difference in sequence or to the difference in the amount of siRNA produced.

In order to answer this question, we first checked the amount of produced siRNA in CHO cells transfected with the different constructs. These amounts were high and roughly the same for the four shRNA constructs (Figure 498).

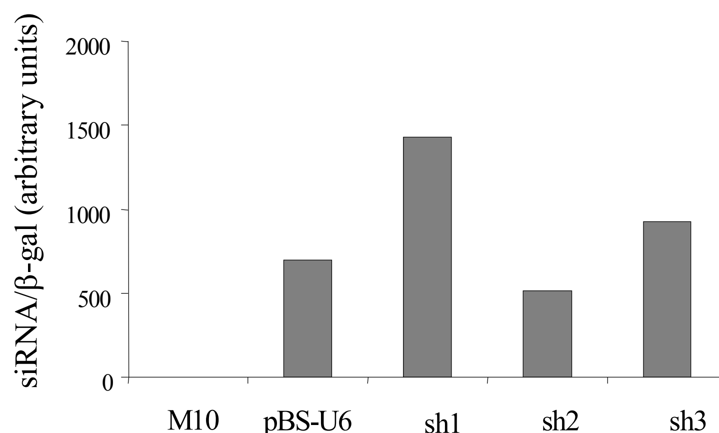


Figure 48 : Concentration of the siRNA in CHO cells transfected by the U6-shRNA.

The amounts of siRNA were deduced from a standard curve established with a synthetic siRNA corresponding to the IE targeted sequence. Values were then normalised to the level of β-galactosidase RNA determined in each sample. Comparison of siRNA produced by transfection of 1000ng of the pBS-U6, sh1, sh2 and sh3 constructs. Values are representative of two independent transfections.

The amount of siRNA obtained with sh constructs was in great contrast with the amount of produced siRNA from either the 5T construct or the miRNA construct (Figure 49). This low amount of siRNA is to compare with the low knock-down efficiency of these two constructs. This suggests that the difference in efficiency is mostly due to the difference in the amount of the produced siRNA, and not to the difference in sequence.

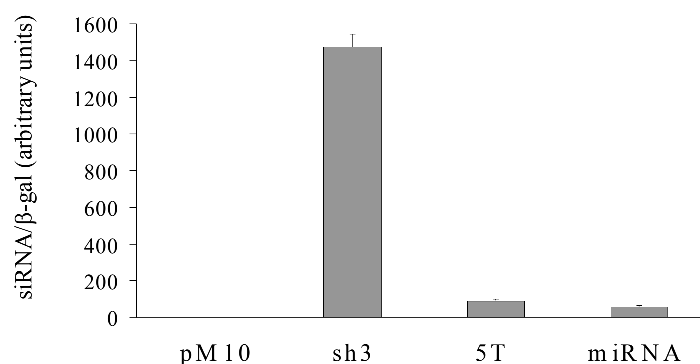


Figure 49 : Concentration of the siRNA in CHO cells transfected by the sh3, 5T, and miRNA constructs.

Comparison of siRNA produced by transfection of 1000ng of the sh3, 5T and miRNA constructs. The amounts of siRNA were deduced from the same standard curve than in the previous figure. Targeted sequence

In order to verify this hypothesis, CHO cells were transfected with various amounts of the pM10-SH3-U6-shRNA construct. As shown in Figure 50, the IE-luciferase knock-down is proportional to the concentration of the siRNA as long as this concentration is below a threshold level when the knock-down reaches 90%. This threshold level is reached when cells are transfected with 10 ng of the pM10-Sh3-RNA construct. If we compare Figure 49 (columns 5T and miRNA) and Figure 50 (column 1 ng), we observe that 1 µg of 5T or miRNA constructs are needed to roughly obtain the same production of siRNA than with 1 ng of pM10-Sh3-RNA construct (~50 siRNA/β-gal arbitrary units). In both cases this siRNA production leads to an IE-luciferase knock-down of about 50% (compare figure 40 “5T” column, figure 44 miRNA column and Figure 50 (column 1 ng)). We can thus conclude that the efficiency of the siRNA produced by the U6-shRNA and the miRNA constructs are similar in their ability to inhibit the IE-luciferase mRNA, despite the fact they do not have the same sequence. This implies that the difference in the knock-down efficiency of the two types of constructs is only due to the efficiency of siRNA production and thus to the strength of the promoter. We can thus conclude that the U6 promoter is 1000 fold more efficient than the eF1-α promoter for siRNA production (1 ng versus 1 µg).

It is to note that the same relationship between the level of siRNA produced and the inhibition of the luciferase activity has also been observed with the 5T construct and the miRNA construct (data not shown). It is thus a general property of the siRNA and not a specificity of the M10-SH3-RNA construct

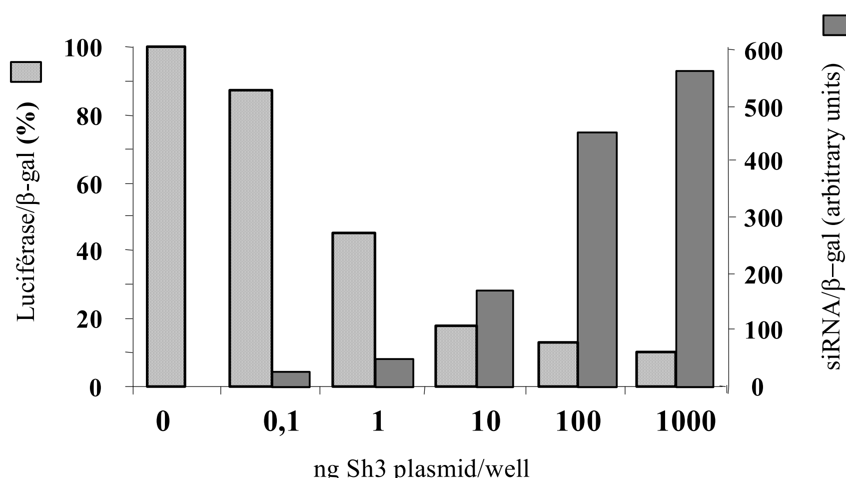


Figure 50 : Relation between the concentration of the siRNA in transfected CHO cells and the knockdown of the IE mRNA.
Various amounts (0.1 to 1000ng) of the plasmid sh3 were transfected in CHO cells with the reporter luciferase gene (plm25-IE-Luc) and the β–galactosidase vector. Luciferase values were normalised to the β–galactosidase activity. Luciferase inhibition (left scale, hatched bars) was given as the percentage of luciferase in cells transfected with the pM10 vector (no siRNA expression). The amounts of siRNA normalized to the β-galactosidase expression (right scale, grey bars) are given in arbitrary units. The figure is representative from three independent experiments.

9. Establishment of stable clones expressing specific IE siRNA

Before doing transgenic mice with the different constructs, we wanted to establish stably transfected cellular clones expressing shRNA to test their capacity to resist to a viral challenge (infection). We first tried with CHO cells in order to compare with results obtained by transient expression in the same cells, and then in PK15 cells which are commonly used for PRV infection studies. PK15 cells possess the receptor allowing PRV infection. It is not the case for CHO cells which can not be infected by the virus. Either with CHO cells or PK15 cells, it was very difficult to establish such clones as they were very unstable, with a lot of dead cells every day, and difficult to maintain and propagate. However, with a few clones we were able to perform a preliminary experiment. As shown in Figure 51, clones 4, 44 and 48 show an inhibition of IE-luciferase activity, whereas clones 7 and 8 have no effect. We measured the siRNA level expression in these clones (Figure 52). There is a good correlation between the level of the siRNA produced and the inhibition of the target IE luciferase mRNA. However, due to the instability of these clones, this promising result could not be reproduced and further investigated. As we were in the

impossibility to obtain reliable results with these clones, we decide to try directly with transgenic mice.

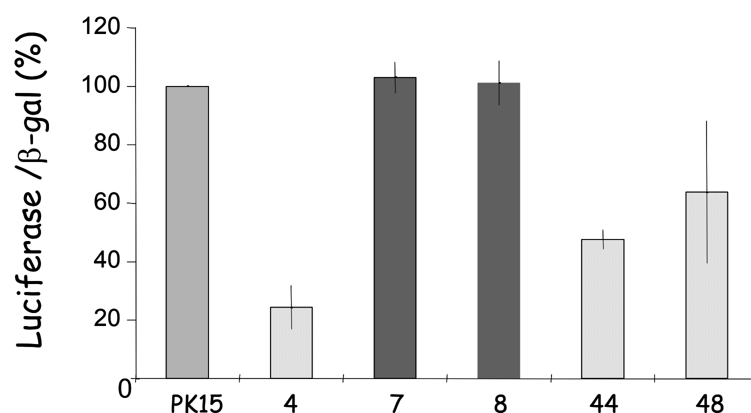


Figure 51 : Inhibition of IE-luciferase in stable clones prepared from PK15 cells.

Clones were transfected with the reporter luciferase gene (plm25-IE-Luc) and the β−galactosidase vector. Luciferase values were normalised to the β−galactosidase activity. Luciferase expression is given as the percentage of luciferase in PK15 cells transfected with the luciferase and β−galactosidase plasmids.

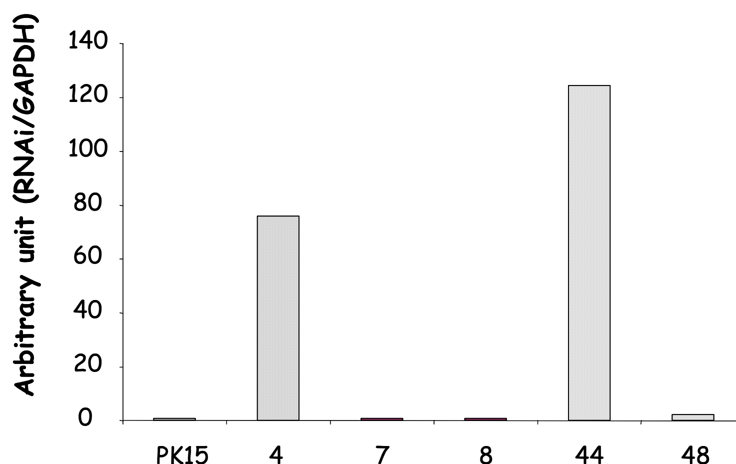


Figure 52 : Concentration of RNAi in PK15 cell stable clones harboring the integrated U6-shRNA construct.

10. Generation of transgenic mice harboring the pM10 U6-shRNA constructs and the 5T as well as the miRNA constructs

The U6-shRNA was not used as this type of vectors proved to be silent in transgenic mice. The different constructs used to generate transgenic mice are recapitulated in Figure 53.

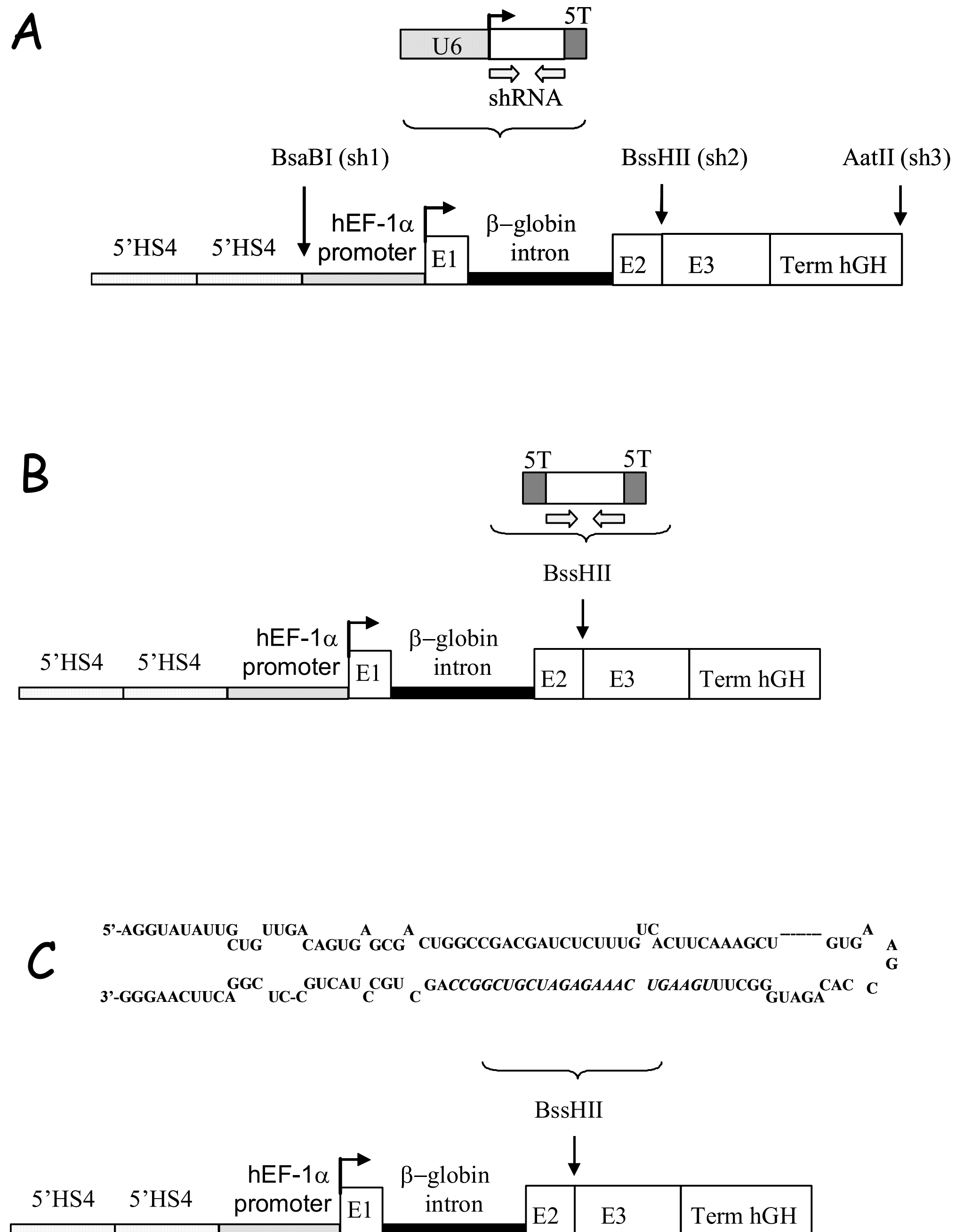


Figure 53 : Recapitulation of the constructs used to generate transgenic mice
The sh constructs (**A**), 5T construct (**B**) and miRNA (**C**) construct are represented.

The vector pM10 U6-sh1 gave 2 transgenic mice out of 76 newborns. With the pM10 U6-sh2 vector, no transgenic mice were obtained among the 26 newborns. The construct pM10 U6-sh3 generated 2 transgenic mice out of the 51 newborns. The transfer of the 5T construction allowed the generation of no transgenic mice out of 150 newborns. The proportion of transgenics was in all these cases particularly low. An examination of the transgene structure revealed that, in all the transgenic mouse lines, the integrated DNA did not contain the shRNA region. This strongly suggested that, for some reason, the shRNA is highly toxic and all the embryos or fetuses expressing the shRNA died. This conclusion is also supported by data reported previously in section 9 when stable clones of pig PK15 cells which are sensitive to the pig pseudorabies virus were obtained. The clones harboring the U6-shRNA construction were unstable. They could not be cultured easily and used to evaluate their resistance to the virus. An examination of the mouse genome revealed that it contains several sequences, some of them being transcribed, which show a significant homology with the sequence of the siRNA (pages 101 and 102). The instability of the PK15 clones and the absence of intact transgene in mice strongly suggest that the siRNA exerted potent off-targeting effects. The fact that the 5T construction gave no mice points the responsibility of the Sh sequence rather than those of the U6 promoter. This toxic effect did not alter cells expressing transiently the siRNA for the two days following transfection.

By contrast, seven transgenic mice harboring the miRNA construction were obtained from 70 newborns. The transgene was found unaltered using PCR covering the U6-shRNA region. Unfortunately two lines of transgenic mice could only be established as the other founders did not transmit the transgene. SiRNA level expression was measured in different tissues of these 2 lines. Total RNA was extracted from these mice to quantify the level of RNAi. It was found that in the two lines harboring the microRNA construct that has been tested, the amount of RNAi produced was low but detectable by using RT-PCR (Figure 54). The miRNA were perfectly detectable in different tissues of transgenic mice of the same lines, although the expression seems low compared to endogenous Let7c miRNA (Figure 54).

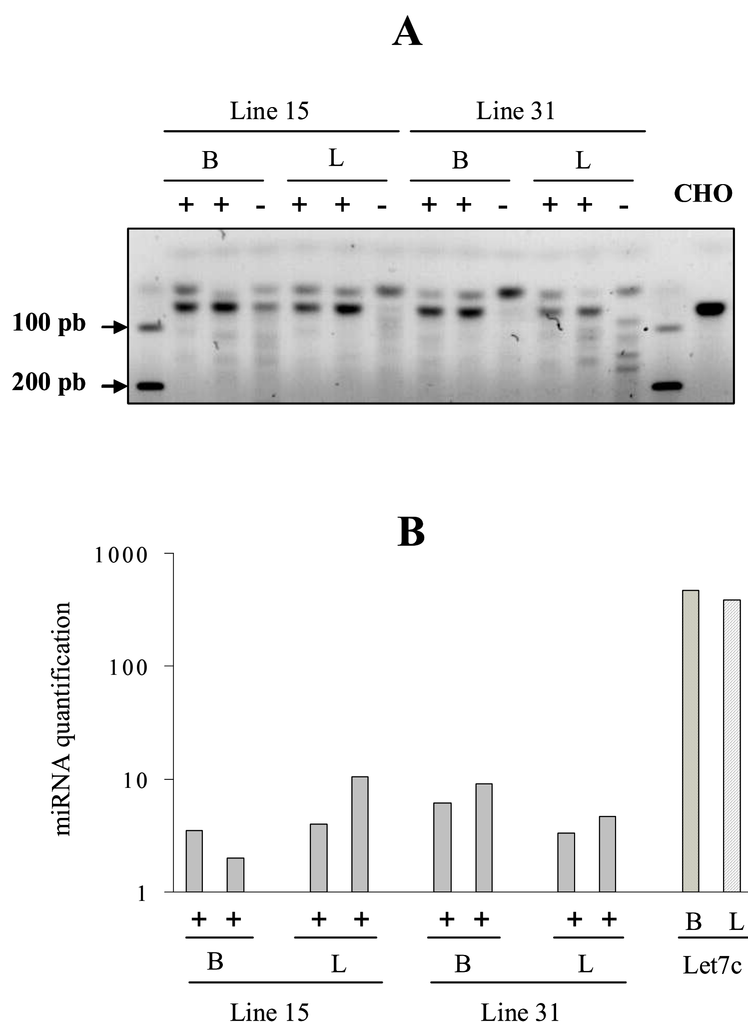


Figure 54 : Detection of siRNA produced by the miR30 construct in brain and liver of two transgenic mouse lines (line 15 and line 30).
The qPCR products obtained by amplification with primer J were separated on 2% agarose gel electrophoresis. Two transgenic mice (+) were assayed in each line in brain (B) and liver (L). With wild type animals (-), non-specific amplification products were observed. The specific signal amplified from brain of non-transgenic animal (lane 15) was negligible compared to the amount of the same fragment obtained from transgenic mice.

There are two possibilities to explain these results: either the difference in sequence between the siRNAs produced by the shRNA and the miRNA constructs is responsible for the lack of toxicity observed with miRNA construct, or the miRNA construct shows some toxicity but the low efficiency of the eF1-α promoter allows the survival of the mice. Although the lack of transmission of the transgene from 5 founders out of 7 suggests that the miRNA could have some deleterious effect at least in gonads, there is no evidence allowing us to choose between these two hypotheses.

11. Transcription gene silencing (TGS)

Transcriptional gene silencing (TGS) is induced by the same molecules that induce post-transcriptional gene silencing but it results in inactivation of the gene transcription rather than by mRNA translation inhibition or degradation. Practically, TGS can be distinguished from PTGS by the fact that TGS act on the promoter region of the genes. Such targeting can be accomplished by adding a transgene whose transcript will form a hairpin RNA that has the sequence of the gene promoter region (Mette et al., 2000; Jones et al., 2001). Such a gene construct cannot be evaluated in cells transfected by the reporter gene depicted above. A gene construct containing the IE gene promoter is required to evaluate a TGS effect. Rather than preparing specific vector containing the IE gene promoter to be used in cells, it was chosen to obtain transgenic mice harboring the miRNA gene construct shown in Figure 55.

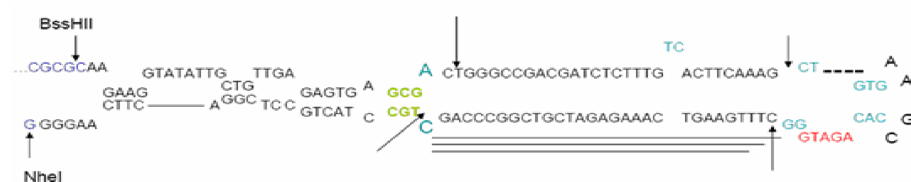


Figure 55 : miRNA construct harbouring the sequence of a shRNA similar to the promoter region close to the cap site of the IE gene.

In case of success, the siRNA generated by the construct should be able to protect mice from infection by PRV. No transgenic mice were obtained out of 58 newborns. This approach was abandoned. We had previously checked the mouse genome and transcriptome for homology with this new target, and we had found only partial homology (table 9).

Table 9 : Sequences in the mouse genome and transcriptome showing significant homologies with the TGS target sequence.

Number of conserved siRNA nucleotides	Part of the siRNA conserved	Position in mouse genome	Comments
17	7-23	Chr7: 102 695 883 - 102 695 899	In a genomic desert (no gene in 2Mb)
17	6-22	Chr14: 70 047 448 - 70 047 464	In the third exon of Loxl2 (lysyl oxidase-like 2) gene
17	9-25	Chr12: 51 665 743 - 51 665 759	In the first intron of Prkcm (Serine/threonine protein kinase D1)
16	2-17	Putative homeodomain transcription factor 1 Chr3, in 5' UTR (first exon)	
16	6-21	Proto-oncogene tyrosine-protein kinase Fyn Chr10, in 3' UTR (last exon)	

However, as in the case of the first Sh sequence, no transgenic mice were obtained despite no full homology with the mouse genome or transcriptome. This strongly suggests that off-target phenomenon can occur without full homology.

This result confirms the importance of the off-target phenomenon, and clearly suggests that it is the sequence itself which is the main parameter and not the mean of production of the siRNA (U6 or miRNA construct).

12. Infection of transgenic mice with PRV.

Although the level of miRNA expression was low in the two established miRNA mice lines (15 and 31), we wanted to know if it was sufficient to induce a partial resistance to the virus. In order to check this sensitivity to the virus, we infected 16 mice of each line and 16 of their non transgenic sibling with 4 or 6 DL50 of PRV and watched on the course of infection. This infection was performed by a nasal spray, following the protocol of Enquist (Brittle et al, 2004). After 10 days, the lethality results were the following:

Table 10 : Infection of transgenic animals by PRV virus.
Transgenic animals (L15 and L31) and non transgenic animals were subjected to infection by 4 DL50. Death occurs between day 2 and day 7. An infection by using 6 LD50 was also performed on transgenic (L31) and non transgenic animals.

4 DL50	J1	J2	J3	J4	J5	J6	J7	J8	J9	J10
L15	16	16	15	8	7	5	5	5	5	5
L31	15	15	15	10	4	3	1	1	1	1
T	15	15	15	10	5	5	1	1	1	1

6 DL50	J1	J2	J3	J4	J5	J6	J7	J8	J9	J10
L31	15	15	14	6	5	2	2	1	1	1
T	16	16	16	10	3	1	0	0	0	0

.

The 15 L31 mice and 15 negative sibling control mice submitted to 6 DL50 of PRV show roughly the same death pattern, and their survival rate is not significantly different (Table 10). In the infection experiment with 4 LD50, the same conclusion can be drawn for the L31 line: the L31 mice behave like the control mice with no apparent protection against the virus. This is in sharp contrast with the behaviour of

the L15 mice which present a higher survival rate than the control mice (Figure 56), despite a faster response to the virus infection leading to a slightly earlier death (Figure 57).

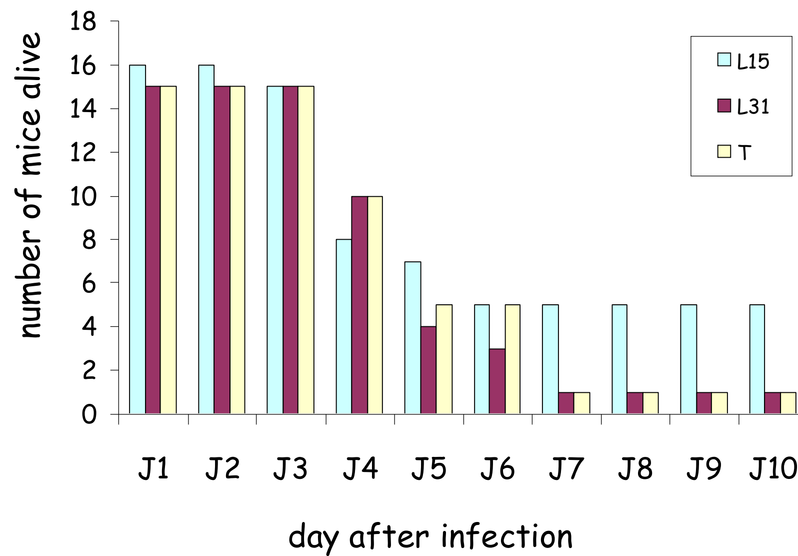


Figure 56 : transgenic mice infection by PRV (4 LD50).
The number of L15 (light blue), L31 (violet) and non transgenic (pale yellow) mice still alive is indicated for every day after infection.

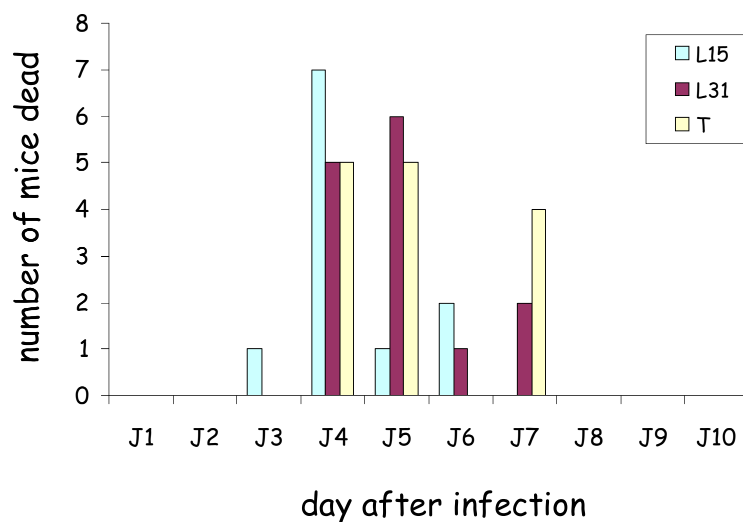


Figure 57 : Transgenic animal death pattern following infection by PRV.
For each day after infection, the number of animal found dead is indicated in L15 (light blue), L31 (violet) and non transgenic (pale yellow) mice

As the number of mice in the experiment is low, the $\chi 2$ test can not be used, even with the Yates correction. Because we cannot use statistic tests, we were thus obliged to use probability and to calculate the probability to obtain these results by chance if the mice in the two groups had the same probability to die (equiprobability hypothesis). There is two ways to do that.

In the first method, we postulate that the two groups (L15 and T) are equivalent, and thus we have 6 surviving mice on 31. The question is to determine the probability to have by chance the observed repartition of death in the two groups. We have 24 died mice, 6 living mice, 15 control mice (Ctrl-mice) and 16 transgenic mice (Tg-mice), and there are 7 possible repartitions of these animals:

1) 15 dead Ctrl-mice, 0 living Ctrl-mice, 10 dead Tg-mice and 6 living Tg-mice.
2) 14 dead Ctrl-mice, 1 living Ctrl-mice, 11 dead Tg-mice and 5 living Tg-mice.
3) 13 dead Ctrl-mice, 2 living Ctrl-mice, 12 dead Tg-mice and 4 living Tg-mice.
4) 12 dead Ctrl-mice, 3 living Ctrl-mice, 13 dead Tg-mice and 3 living Tg-mice.
5) 11 dead Ctrl-mice, 4 living Ctrl-mice, 14 dead Tg-mice and 2 living Tg-mice.
6) 10 dead Ctrl-mice, 5 living Ctrl-mice, 15 dead Tg-mice and 1 living Tg-mice.
7) 9 dead Ctrl-mice, 6 living Ctrl-mice, 16 dead Tg-mice and 0 living Tg-mice.

In the case of the equiprobability hypothesis, if we call (A) the number of dead control mice, (B) the number of living control mice, (C) the number of dead transgenic mice and (D) the number of living transgenic mice, the probability to obtain each repartition is given by the formula:

$(25!\ 6!\ 15!\ 16! / 31!\ A!\ B!\ C!\ D!)$

As the observed repartition is repartition 2), the probability (P) to have 5 or more surviving transgenic mice is thus:

$(P) = P(1) + P(2) = (25!\ 6!\ 15!\ 16! / 31!\ 15!\ 0!\ 10!\ 6!) + (25!\ 6!\ 15!\ 16! / 31!\ 14!\ 1!\ 11!\ 5!)$

We thus have $(P) = 0{,}0108763 + 0{,}0889878 = 0{,}0998641 \approx 9{,}99\% > 5\%$

As 5% is the generally accepted threshold to consider a result as significant, we can not reject the equiprobability hypothesis in this case. It is the only rigorous calculation without supplemental hypothesis.

However, we have just taken into account the L15 and the control groups, and not the L31 group. If we decide to consider the L31 group as not different of the control group, we can do the same calculation for the L15 group and an "other" group (L31 + control). In this case, we have 39 died mice, 7 living mice, 30 "other" mice and 16 L15 transgenic mice, and the probability is:

(P) = P(1) + P(2) + P(3)

= (39! 7! 30! 16!/ 46! 7! 30! 9! 0!) + (39! 7! 30! 16!/ 46! 6! 29! 10! 1!)

+ (39! 7! 30! 16!/ 46! 5! 28! 11! 2!)

= 0,0002137 +0,0044884 + 0,0354991 = 0,0402012 ≈ 4,02% < 5%

In this case, we can reject the equiprobability hypothesis and are allowed to say that there is a significant resistance of the L15 mice to the virus. But it is not as rigorous than the first calculation because there is a supplemental and a posteriori hypothesis.

In the second method, we postulate that the control and L31 groups give us the true survival probability (1/15), and we calculate the probability to have 5 surviving mice and 11 died mice if the individual probability is 1/15. This probability is given by the formula:

$P(X=k) = C^k_n\, p^k (1-p)^{n-k} = (n!/ k!\,(n-k)!)\, p^k (1-p)^{n-k}$ with n the total number of mice, k the number of surviving mice and p the survival probability for a mice.

In our case, n = 16, k = 5, and p = 1/15, and the result is 0,002693 = 0,26% <<< 5%

So there is only 0,26% chance that the L15 mice have the same survival probability of 1/15 than the other mice. We are thus allowed again to say that the L15 mice are significantly more resistant to the infection by PRV. Here also, it is a less rigorous mean to see the facts because we add a supplemental hypothesis about the true survival probability.

To resume, we are close to, but outside, a significant result with the only rigorous probability calculation, and we have a very significant result with a few minor supplemental hypotheses. This is a promising result, but it is clear we have to repeat this experiment and perform other infections with more animals before being sure.

13. Search of new target for siRNA

Identification and study of new shRNAs targeting IE mRNA

Although the selected target sequence depicted above was highly effective in targeting the IE gene for its inhibition *in vitro*, it generated a lethal off-targeting effect, which led to low yield of transgenic animals. New target sequences were therefore necessary to reduce the off-target effect. Two regions of the IE mRNA were chosen: the 5'UTR and the 3'UTR.

Figure 58 illustrates part of the IE gene of pseudorabies virus with 5'P and 3'OH UTRs (untranslated region) where the different siRNA sequences were designed. Four new siRNA were chosen in the 5'P UTR, respectively called ASSR1, ASSR2, ASSR4 and ASSR5. Four siRNA were designed in the 3'OH UTR, respectively called SHOLD, N3, N4 and N5.

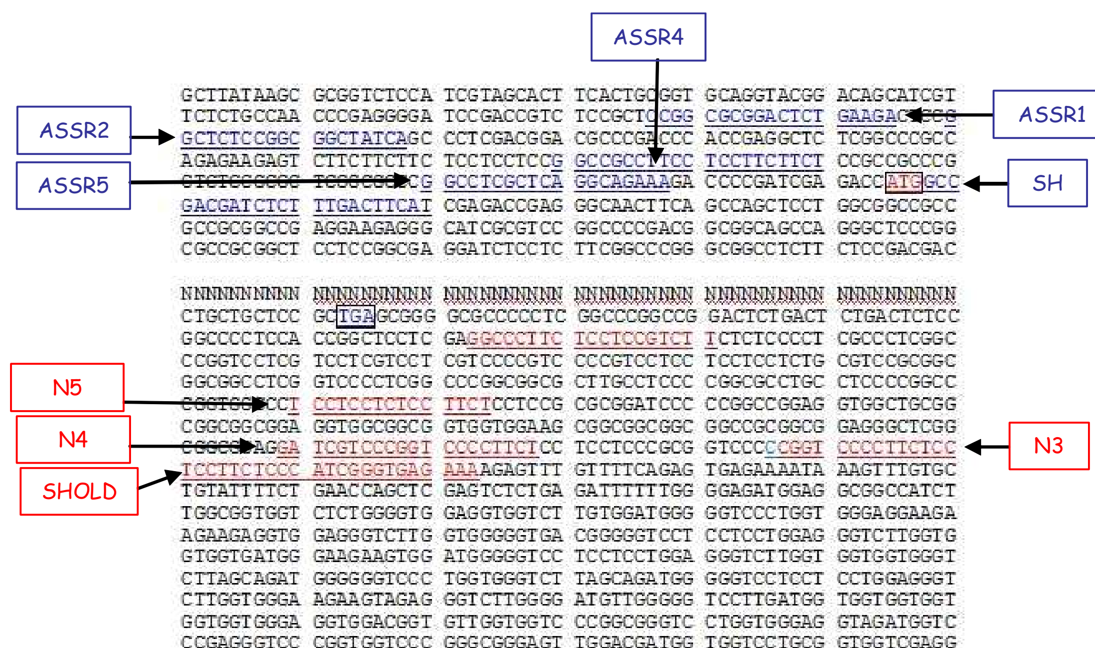


Figure 58 : The IE gene of pseudorabies virus with 5'P and 3'OH UTRs (untranslated region)
The selected target sequence was marked. ATG start codon is shown in red as well as the stop codon TGA in blue. Target sequences of the 5'UTR region and the target sequence after the start codon are marked with blue. The target sequences for the 3'UTR are marked with red.

The following siRNA was constructed with the same strategy than the previous Sh. The construct follows the rules described in Figure 32.

In *5'P* UTR:

ASSR1:	CCGGCGCGGACTCTGAAGA
ASSR2:	GGCTCTCCGGCGGCTATCA
ASSR4:	GGCCGCCTTCCTCCTTCTTCT
ASSR5:	GGCCTCGCTCAGGCAGAAA

In *3'OH* UTR:

N3	CCGGTCCCCTTCTCCTCCTTCT
N4	GATCGTCCCGGTCCCCTTCT
N5	GCCCTCCTCCTCTCCTTCT

The construct of the new shRNA of the 3'UTR called SHOLD was different of all the other constructs. Its hairpin was not formed with HindIII site but with the hairpin, preconised by Dharmacon site, "TTCAAGAGA" wich was composed of nine nucleotides instead of 6 nucleotides with HindIII site (see Figure 59)

SHOLD
shRNA targeted the '3TUR of the IE gene
Apa1
U6prom 5' CGCCG CTCCCATCGGGTGAGAAAA TTCAAGAGA TTTTCTCAC TC CCGATGGGAG TTTTTTG 3'
3' CCGGGCGGC GAGGGTAGCCCACTCTTTT AAGTTCTCT AAAAGAGTG AG GGCTACCCTC AAAAAACTTAA 5'
EcoR1
Transcripted siRNAUnt
5' G CTCCCATCGGGTGAGAAAA
3'(U) CGAGGGTAGCC CT CACTCTTTT

Figure 59 : New shRNA constructs targeting the 3'UTR of IE mRNA
The Dharmacon hairpin is written in brown (box).

New constructs targeting 5' and 3'UTR of the IE gene validation

Co-transfection of these constructs with pLM24 IE Luc 3'UTR IE reporter luciferase plasmid into CHO cells shows different level of reporter gene inhibition.

In the 5'UTR, only the shRNA called ASSR1 induced an inhibition of 60% (Figure 60), while the other shRNAs (ASSR2, 4, 5) did not significantly inhibit the reporter luciferase gene expression (not shown).

In the 3'UTR, only the SHOLD construct shows a significant inhibiting effect on the reporter gene. The SHOLD construct which has been designed to target the 3'UTR region of the IE gene were tested on CHO cells and the results obtained proved that this construct was highly effective in targeting the 3'UTR region of the IE sequence (Figure 60)

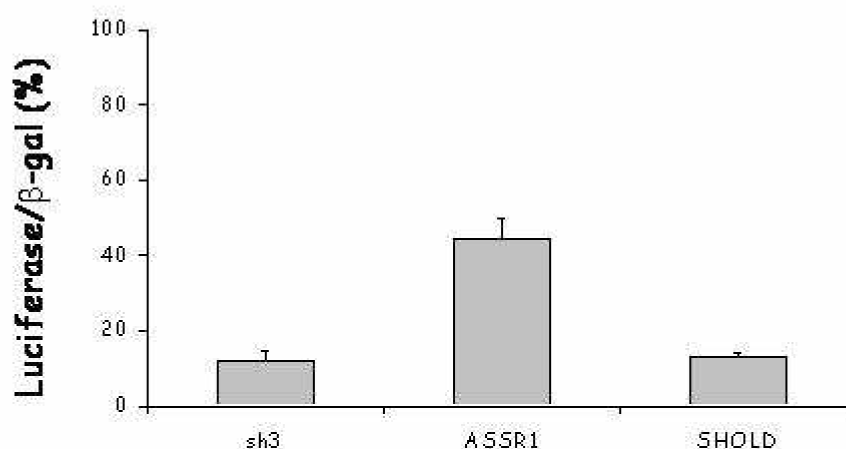


Figure 60: Knockdown of the IE mRNA by the sh3, ASSR1 and SHOLD constructs in CHO. The sh3, ASSR1 and SHOLD constructs were transfected into CHO cells with the reporter luciferase construct (plm24IE luc 3'UTR IE) and the β–galactosidase vector. The results are given as percentage of luciferase activity in cells transfected by plm24IE Luc 3'UTR IE and the empty pBS vector. All luciferase values were normalized to the β-galactosidase activities. The results represent the average of four independent experiments.

In order to compare more precisely the effect of the three different construct, we then performed dose –response experiments with various amounts of Sh3, Assr1 or Shold in CHO cells. In this case, the target luciferase gene used is the plm24IE luc 3'UTR IE which contains both the 5' part and the 3' UTR of the IE gene and can thus be degraded by the three types of si RNA. The results are shown in Figure 61.

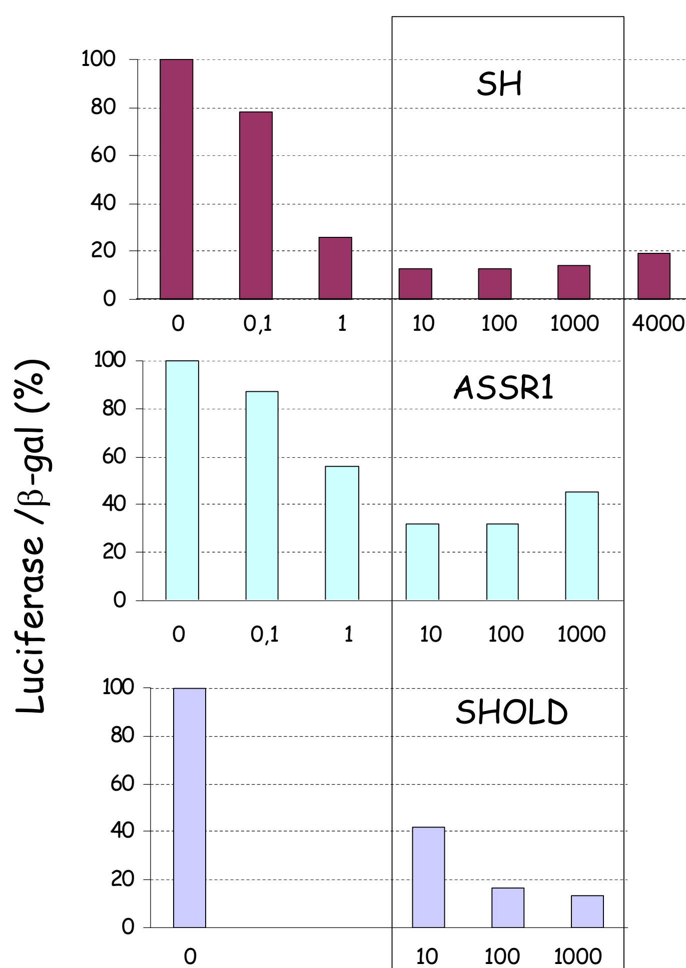


Figure 61 : Knockdown of the IE mRNA by the sh3, ASSR1 and SHOLD constructs in CHO cells transfected by different quantities of these constructs.
Various amounts (0,1 to 1000 ng) of the plasmids sh3, ASSR1 and SHOLD were transfected in CHO cells with the reporter luciferase gene (plm24-IELuc 3'UTR IE) and the β–galactosidase vector. Luciferase values were normalised to βgalactosidase activity. Luciferase inhibition was given as the percentage of luciferase in cells transfected with the pM10 vector (no siRNA expression).

We can conclude from the Figure 61 that construct ASSR1 is effective in targeting the 5'UTR region of IE gene. The best ratio to obtain a significant inhibition of the IE gene was obtained by co-transfection of this construct with the reporter luciferase gene into the CHO cells within 10 or 100 ng ASSR1 plasmid. The maximal inhibition of IE mRNA observed reach only 70%. It appears that if the ratio was higher, there was saturation of the system and the effect of this construct on inhibition were reversed. If we compare the inhibition observed by SH and by ASSR1, it was comparable except that the reversion of inhibition was observed more quickly with ASSR1 (1000 ng) than with SH (4000 ng).

By contrast, with SHOLD construct, the best inhibition was observed with 1000 ng of SHOLD plasmid, and the inhibition decreased progressively when we transfected less amount of plasmid. The inhibition passed from 85% to 60% respectively with 1000 and 10 ng of SHOLD plasmid. To understand why we observed such results, we measured the level of RNAi produced for each construct (Figure 62).

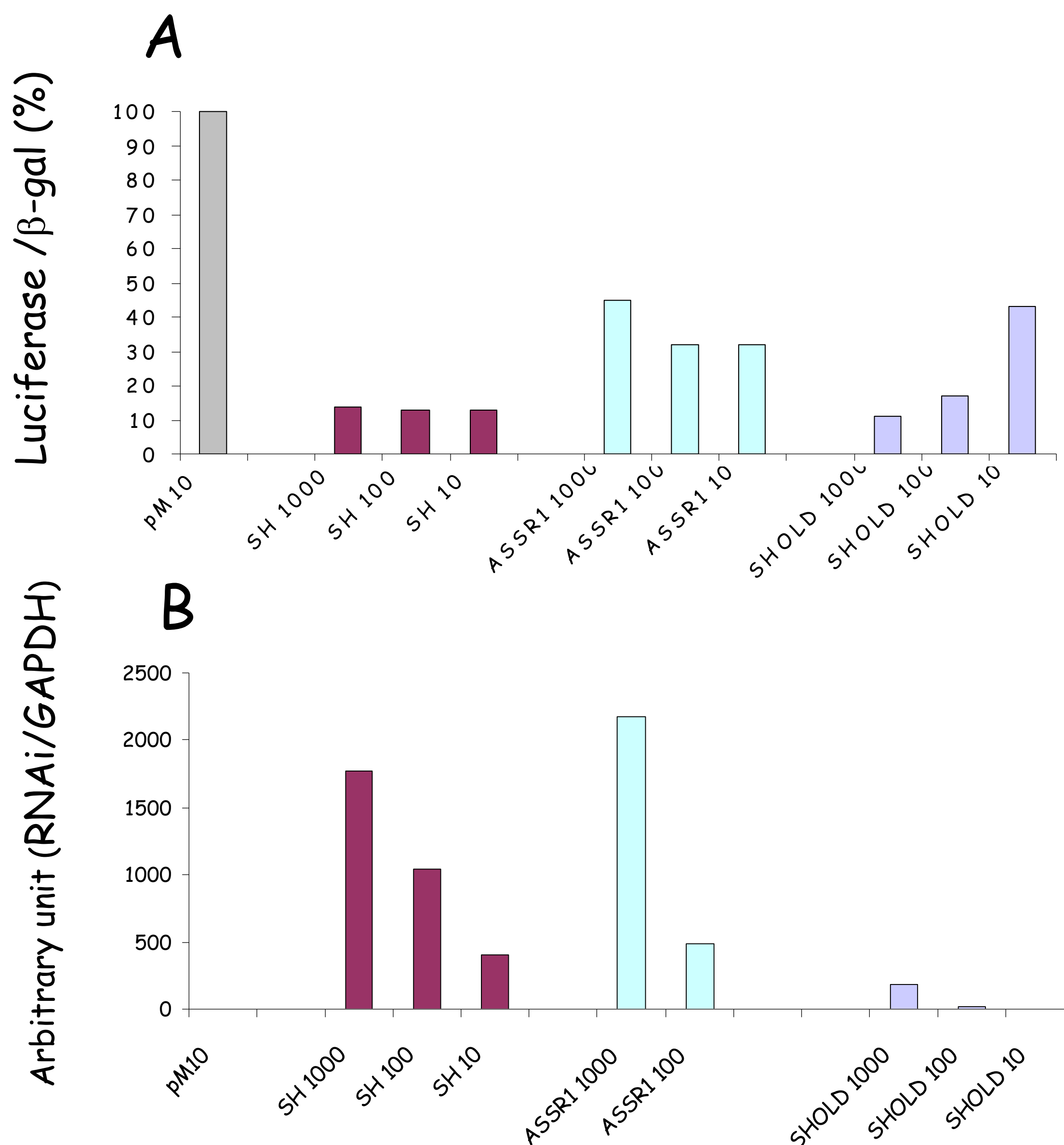


Figure 62 : Concentration of the siRNA in CHO cells transfected by the U6-shRNA, ASSR1, and SHOLD constructs.
Relation between the concentration of the siRNA in transfected CHO cells and the knockdown of the IE mRNA. Various amounts (10 to 1000ng) of the plasmids sh3, ASSR1 and SHOLD were transfected in CHO cells with the reporter luciferase gene (plm24-IELuc 3'UTR IE) and the β–galactosidase vector. (A) Luciferase values were normalised to β–galactosidase activity. Luciferase inhibition was given as the percentage of luciferase in cells transfected with the pM10 vector (no siRNA expression). (B) The amounts of siRNA normalized to the β-galactosidase expression are given in arbitrary units. The figure is representative from three independent experiments.

Despite the fact that we always found more siRNA when we transfect more plasmid, we observed a different behavior for each of the three constructs. In the case of the Sh construct, we observed a strong inhibition of the target mRNA with a moderate amount of produced siRNA when we tranfected with 10ng of plasmid. If we transfect with more plasmid (100 or 1000 ng) we obtained more siRNA but the same inhibition. With the AssR1 construct, the situation is different: we never obtained the same level of inhibition than with the Sh construct, even when we have more siRNA (compare the results obtained with 1000 ng of Sh3 or AssR1 in Figure 61). The intrinsic power of inhibition of the AssR1 siRNA is thus below the intrinsic power of inhibition of the Sh3 siRNA. This is in sharp contrast with what we observed with the Shold construct. When we transfect with 100 ng of Shold, a potent inhibition was observed with only a tiny amount of produced siRNA. With 1000 ng of transfected Shold, we obtained a similar inhibition than the one observed with the Sh3 construct despite an amount of siRNA produced far below the one produced by the Sh3 construct. The intrinsic power of inhibition of the Shold siRNA is thus higher than those of the Sh3 SiRNA. It would be thus of great interest to produce the Shold siRNA at a higher level.

From these results we conclude that the most important factor in determining the efficiency of an RNAi construct is the sequence itself and the concentration and the type of the produced RNAi in the cells.

RNAi-3'UTR (SHOLD) construct appears to be a promising construct to generate transgenic mice, as it produce a small amount of RNAi but the effect of which are very significant. Using this construct could result in reducing the off-target effect and toxicity we have obtained from the other constructs.

14. Effect of two different constructs on mRNA of IE gene expression inhibition (cumulative effect).

We then checked if there was a cumulative or a synergistic effect of the three different siRNA (SH, ASSR1 and SHOLD) we had found effective in their knock-down effect. For this purpose, we used one of the siRNA in conditions which are not optimal, and added different amounts of the other two siRNA. As shown in Figure 63, transfection

by different shRNA constructs plasmid gave a better IE mRNA inhibition results. In all cases, the transfection by two siRNA producing plasmids leads to a better inhibition. We found again than the Sh construct is a more potent inhibitor than the Assr1 construct (compare lanes 3 and 4).

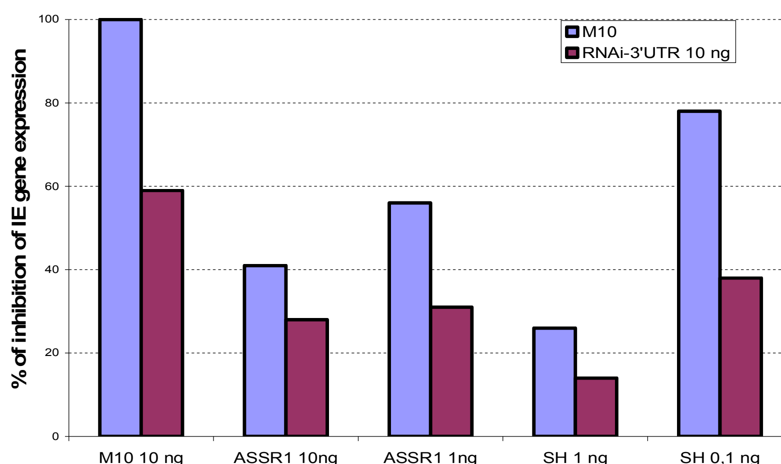


Figure 63 : Effect of co-transfection by two different shRNA constructs.
Lane 1 represents expression of the mRNA of IE gene following transfection of the M10 plasmid with the reporter gene into CHO cells (blue lane), while the violet lane represents the same expression when cells were cotransfected with 10 ng of RNAi-3'UTR plasmid. Lanes 2, 3, 4 and 5 show the same IE-luciferase expression when cells transfected with M10 and with (violet) or without (blue) 10 ng of RNAi-3'UTR plasmid , were cotransfected with 10 ng of ASSR1 construct plasmid, 1 ng of ASSR1 construct plasmid, 1 ng of SH construct plasmid and 0.1 ng of SH construct plasmid respectively.

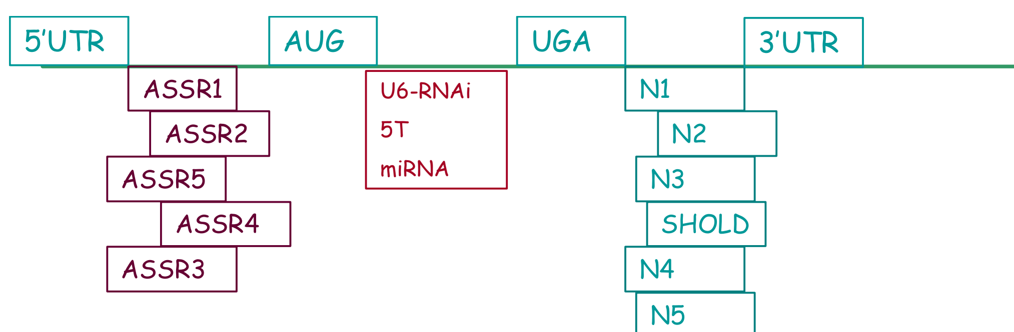


Figure 64 : Summary of the different target sequences in IE mRNA
The location of the sequences that has been chosen to prepare all the constructs are shown.

In summary, among the different shRNAs tested, only three of them showed a significant and exploitable capacity to knockdown IE mRNA. These sequences are SH, ASSR1 and SHOLD which inhibited luciferase gene expression of 95%, 60% and 85% respectively.

15. New miRNAs constructs

In order to construct new vectors to be used to produce transgenic mice, we first used the blast search to look for homology of ASSR1 and SHOLD target sequence in mouse genome. As shown in the following table, no full homology was found in mouse genome or transcriptome.

Table 11 : Sequences in the mouse genome showing significant homologies with the AssR1 and Shold target sequences.

Number of conserved siRNA nucleotides	Part of the siRNA conserved	Position in mouse genome	Comments
16	4-19	Chr5: 14 136 286 - 14 136 301	In first intron of Sema3e (Semaphorin-3E = Semaphorin-H) gene

Number of conserved siRNA nucleotides	Part of the siRNA conserved	Position in mouse genome	Comments
16	2-17	Chr1: 108 149 070 - 108 149 085	In first intron of Phlpp (PH domain leucine-rich repeat-containing protein phosphatase) gene
15	4-18	Chr2: 158 964 846 - 158 964 860	Between Dhx35 (DEAH box polypeptide 35) and Gm826 (gene model 826) genes
15	3-17	Chr8: 37 160 988 - 37 161 002	In first intron of D8Ertd82e (Tyrosine-protein kinase SgK223) gene
15	4-18	Chr18: 9 024 624 - 9 024 638	Between Wac (WW domain-containing adapter protein with coiled-coil) and Fzd8 (Frizzled-8) genes

Two new sequences were chosen to prepare miRNA based on MIR-30. The first sequence was chosen to target the 5'UTR region of IE gene of PRV virus, while the second sequence were chosen to target the 3'UTR of IE gene. These sequences correspond to the ASSR1 and SHOLD sequence respectively. Figure 65 shows their structure. These two miRNA constructs are under evaluation and if the results are good they will be used to generate transgenic mice.

CCGGCGCGGACTCTTGAAGA (miRNA)
CTCCCATCGGAAGTGAGAAAA (miRNA)

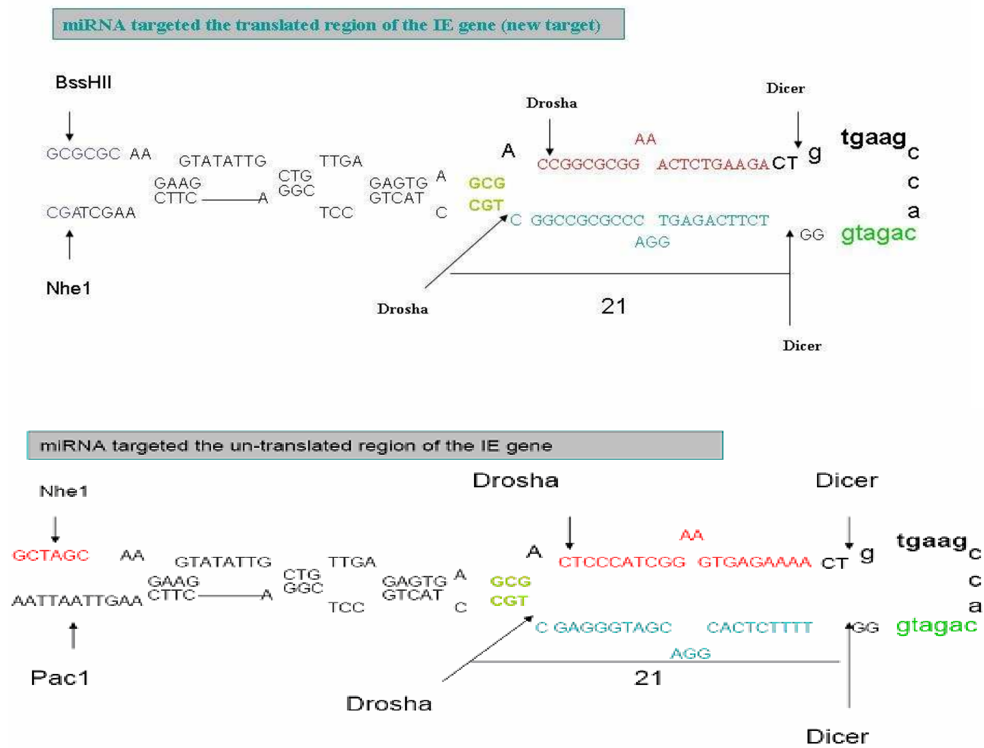


Figure 65 : new miRNA constructs.

CHAPTER 7
DISCUSSION AND CONCLUSION

Some "small RNAs" do control plant and animal gene expression using different mechanisms. There are several classes of these small RNAs among which microRNAs (miRNAs), small interfering RNAs (siRNAs), and repeat-associated small interfering RNAs (rasiRNAs) and they are distinguished by their origins, not their functions (see for review (Zamore & Haley, 2005)). It is believed that the miRNAs alone regulate at least one-third of all human genes (Lewis et al., 2005). Eukaryotic genomes harbor several hundreds of genes encoding the RNA precursors of siRNAs. The small RNAs and their associated proteins act in distinct but related "RNA silencing" pathways that regulate transcription, chromatin structure, genome integrity, and, most commonly, mRNA stability and translation. Chronology of some of the major discoveries and events in RNA silencing in the last 15 years are illustrated in Figure 66.

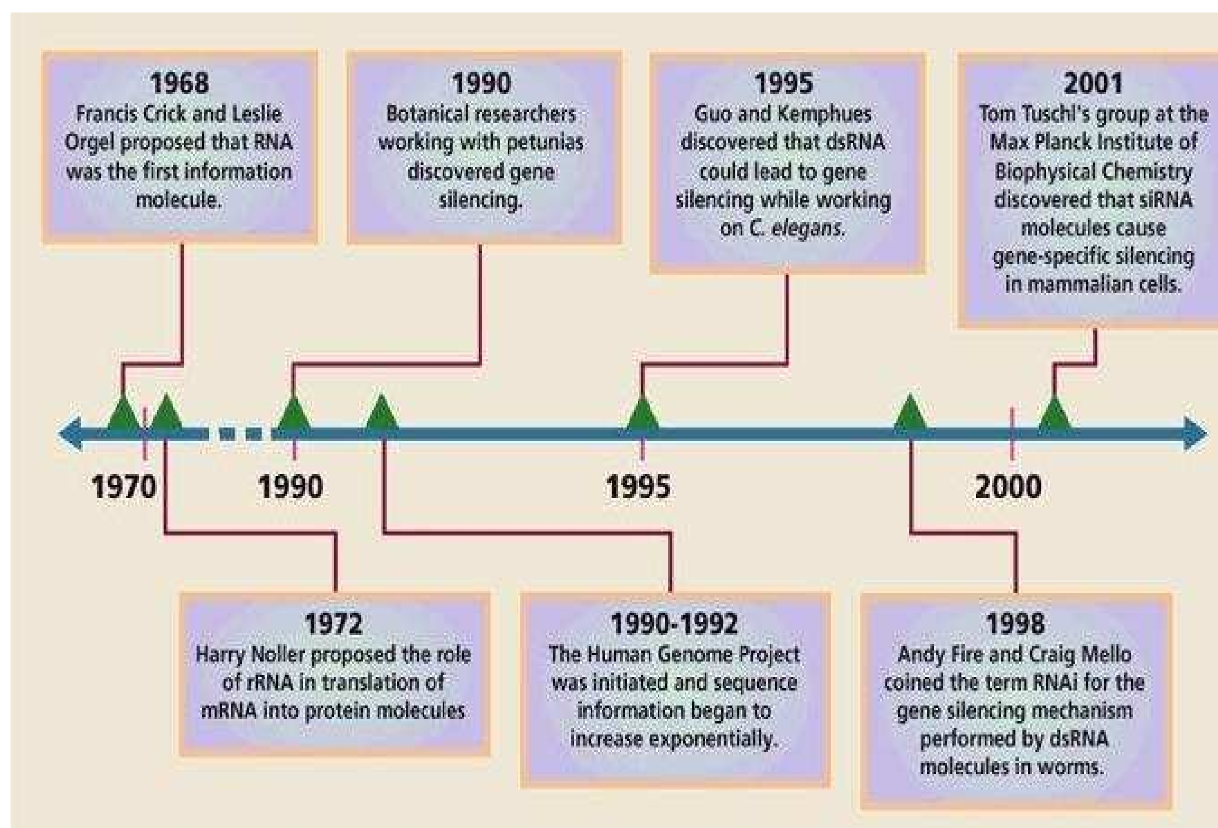


Figure 66 : a brief history of RNAi.
A few steps in the RNA knowledge in the last few years lead us towards more understanding of the RNAi mechanism of action.

The use of RNAi technology holds great promise as a novel nucleic acid–based therapeutic against a wide variety of diseases, including cancer, infectious diseases and genetic disorders (Dorsett and Tuschl 2004). Although for the last few years RNAi strategies against viruses and as an antiviral agent received much

attention it appeared later that for several reasons this approach was not as easy in practice as it thought to be (Joost Haasnoot et al., 2007). One of the reasons is that, in vertebrates, the long double strand RNA cannot be used as they trigger defense mechanisms particularly via interferon induction. Another limitation is that the RNAis are not auto-amplified in vertebrates as they are in plants and in some lower invertebrates. Moreover, all the experimenters noted that only a proportion of the RNAis was active for some still unknown reasons.

In all of these applications, the use of highly potent silencing constructs is expected to maximize the possibility of obtaining target knockdown and thereby is intrinsically important for the chance of success. Several attempts have been made to improve the potency of silencing constructs. Rules have defined to design si/shRNAs to select the appropriate strand. More recently, the structure of the targeted mRNA sequence has appeared an essential point to consider. Indeed, a given siRNA is fully active only when the targeted sequence is in open configuration (Katoh and Suzuki, 2007; Li et al., 2007; Sandy et al., 2005; Pei and Tuschl 2006). Several programs to determine the local secondary structure of RNA are available. These programs propose different structure and the fact that the larger sequence a siRNA is in open configuration cannot be always established with certainty (Li et al., 2007)

Another problem is this off-targeting which cannot be totally predictable as the genome sequences are not all known and as relatively short miRNA sequences, the seed regions made of 8 nucleotides, are sufficient to silence a mRNA (Haasnoot et al, 2007). The strand having the same sequence as the targeted mRNA may even be taken as a true siRNA inhibiting a unrelated mRNA (Clark et al., 2008).

A number of the projects implying the use of siRNA must be based on transgenesis. This is indeed in principle the best way to generate stable phenotypes by knocking down specific genes for basic research or applied projects. The toxicity problems generated by RNAi were noted in our work as well as several other recently published data (Grimm et al., 2006; Sioud 2006; Haasnoot et al., 2007).

An important point is to obtain shRNA gene expression at a sufficient level. The RNA polymerase III vectors are reliable tools to express siRNAs transiently or stably in cultured cells. Unfortunately, attempts to use the U6 or H1 vectors in transgenic mammals revealed that, unless inducible or protected by lentivial or adenoviral vectors, they were most of the time silenced and thus unable to direct in vivo the

synthesis of sufficient amount of siRNAs. The insertion of U6 or H1 vectors into lentiviral vectors proved to be an acceptable approach in a number of cases (Abbas-Terki et al. 2002; Morris and Rossi, 2006; Tiscornia et al. 2003). Lentiviral vectors are however not currently efficient to generate transgenic animals in all species. It was also proved that any selected siRNA sequence can be efficiently synthesized by vectors containing a sequence coding for a miRNA on condition to replace the natural miRNA sequence by the siRNA sequence. Several shRNAs and vectors were tested using in all cases as target gene, the IE gene, of the PRV as a model.

An initial shRNA was designed before rules were known for this step of the process. Interestingly, this shRNA expressed by a U6 vector induced a very strong gene knockdown on IE mRNA. An examination of this sequence indicated later that it fits well with the consensus sequence proposed by different groups to design a siRNA. Recent publications also made it possible to determine that the targeted sequence of the shRNA in the IE mRNA that we chose was in an open configuration allowing its efficient knockdown. Several vectors to express this shRNA could thus valuably be tested in transfected cells and later in transgenic mice.

The data obtained from our works exemplify the difficulty to implement siRNAs to knockdown genes in transgenic animals. First, our results in transiently transfected CHO cells showed that the U6–shRNA constructs were by far the most efficient to obtain a high level of siRNA and a strong inhibition of the IE mRNA target. Adding the U6-shRNA vector into a vector of pM10 validated the idea that the activity of the U6 gene promoter is fully compatible with the presence in its vicinity of a RNA polymerase II promoter (EF-1α) and expectedly protected from silencing in transgenic mice by the elements present in pM10. This hypothesis could not be directly verified *in vivo* with the shRNA gene used. Indeed, these constructs proved to be unable to generate any siRNA expressing transgenic mice suggesting that all the mouse embryos harbouring a functional shRNA gene expressed it at a high rate and could not survive. Transgenic mice were obtained with the miR30 construct only, which generated a siRNA having a sequence slightly different sequence of this obtained from the other constructs and probably less toxic than that produced by U6-shRNA constructs. From these observations we conclude that although this sequence which has been used to prepare (SH1, SH2, and SH3) was the most effective sequence to knockdown the IE gene, and it met the best criteria of siRNA sequence,

unfortunately this sequence was toxic. We believe that this off-target effect is essentially due to the siRNA sequence itself not to the use of the Pol III promoter (U6), because no mice were obtained with the 5T construct which have the same sequence but not the Pol III promoter.

One interest of the present approach is to better define the exact sequence of the produced siRNA by using the described RT-qPCR method which allowed us to predict the siRNA products having the lowest off-target effect.

The 5T vector appeared promising even if the level of siRNA produced was low. The advantage of this approach is that multiple regulated promoters and conventional vectors can then be used to generate transgenic animals. However, some of these promoters may be too weak to express sufficient amount of siRNA. It must be possible to introduce several copies of the DNA fragment coding for the shRNA of interest in tandem in the same vector. The amount of siRNA produced should be increased as a function of the number of copies introduced in the vector. This possibility is currently under study in our laboratory.

The miRNA vectors are attractive as they are dependent on regulated promoters using RNA polymerase II. The amount of siRNA produced by these vectors may however not be high. This was observed in our works and in others (Li et al. 2007). It seems that the mechanisms which process the pre-miRNAs to generate functional siRNAs are more complex than those transforming short shRNAs into siRNAs (Cullen, 2004; Tomari and Zamore, 2005). The sequence of the exogenous shRNAs introduced into miRNA vectors appears to interfere with the maturation processes leading to the generation of the corresponding siRNAs. Moreover, it was observed that the exportin-5 which transfers the pre-miRNA from the nucleus to the cytoplasm may be limiting. This may reduce the efficiency of exogenous miRNA and alter the normal action of the endogenous miRNA.

Recent publications reported unexpected results. Transgenes coding to long dsRNA under RNA polymerase II vector proved to direct the generation of efficient siRNA without inducing interferon. The difficulty to use the long dsRNA approach may have been over estimated (Strat et al., 2006).

One of the most and unexpected observation of our works is that the concentration of a siRNA has not to be high to exert a satisfactory knockdown effect. Overexpressing a siRNA may be a way to compensate the limited intrinsic knockdown capacity of this siRNA. However, it may be difficult in some cases to overproduce a siRNA,

especially if the expression is to occur specifically in a single cell type of the animals and if no strong specific promoters are available. The overexpression of a siRNA may also trigger deleterious off-targeting side effects and general toxic effects. It seems therefore that the design of appropriate shRNAs capable of acting at a low concentration is a key for the success of knockdown in transgenic animals.

The major conclusions that were drawn from this part of the work are the followings.
* The siRNAs produced from shRNAs need not to be present at a high concentration in cells to exert a potent and specific knockdown effect.
* The off-targeting of a siRNA may occur despite a BLAST examination of the targeted genome to identify homologous sequences.
* The off-targeting is amplified when the shRNAs are produced in high amounts.
* It is essential to spend a long enough time to design shRNAs capable to inhibit the targeted mRNAs even at a low concentration to reduce off-targeting and unspecific cytotoxic effects.
* The U6-shRNA constructs are probably highly efficient when introduced into (and possibly in the vicinity) of RNA PolII promoter vector known to contain elements favoring a reliable transgene expression.
* The miRNA vectors can be used to express shRNAs but the conditions to obtain a high production of siRNAs are not clear.
* The conventional vectors expressing cDNAs and thus dependent on RNA PolII can produce significant amount of siRNAs when the shRNA sequence is introduced in the transcribed part of the constructs. The level of siRNAs is relatively low but it can probably be significantly improved by different ways, including by adding several copies of the shRNA sequence in tandem in the constructs. This can potentially make it possible the use of relatively simple vectors capable of modulating shRNA synthesis and action in given cell types as well as at given times in animals.
* The approach of using gene coding for long dsRNA under the direction of RNA polymerase II promoters should be reevaluated even if multiple siRNA are generated by the dsRNA and if this may enhance off targeting.

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