

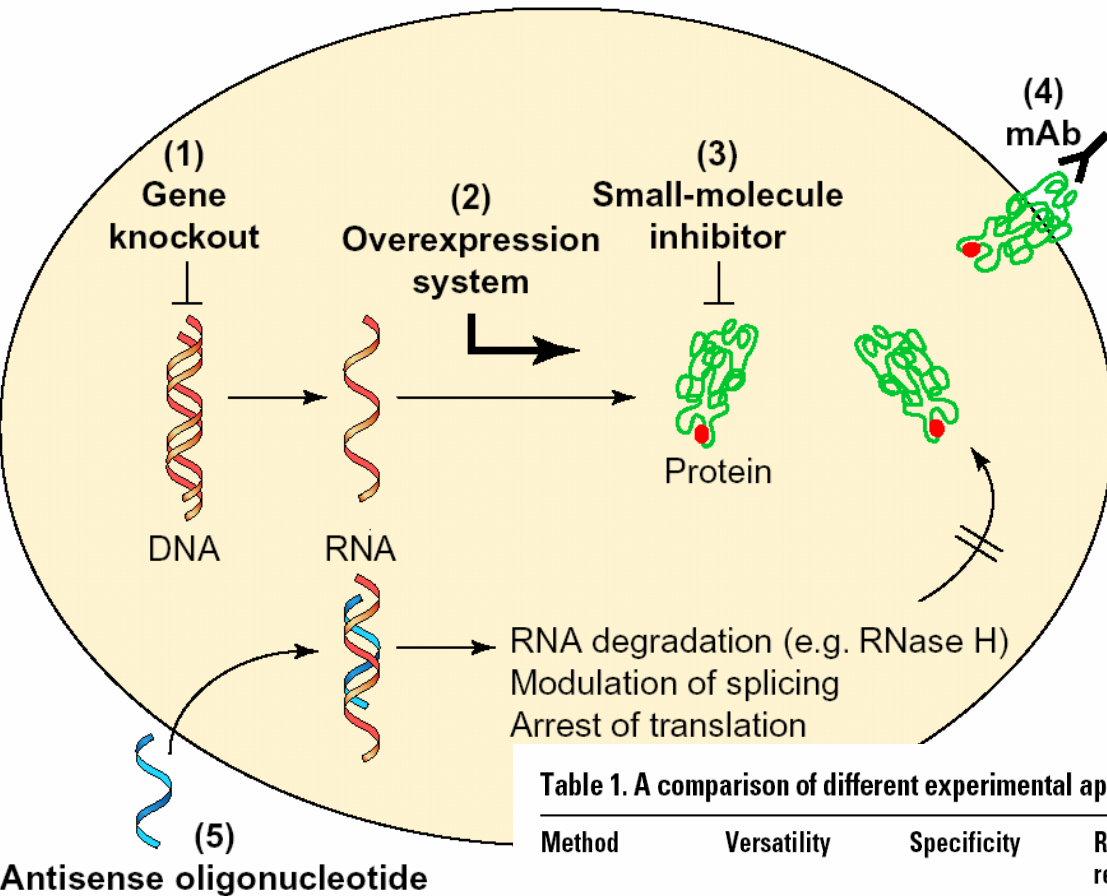
# Aplikovaná chemie a biochemie



## Přednáška č. 4

### Manipulace genové exprese

# Modulate expression of protein function:



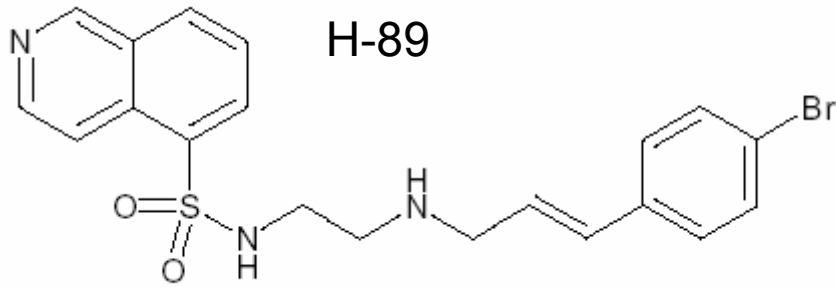
**Table 1. A comparison of different experimental approaches for modulating the function of cell signaling molecules**

Method	Versatility	Specificity	Required resources	Cost	Probability of success	Potential for drug development
Overexpression systems	Low to moderate	Moderate	Moderate	Low	Moderate	Low
Gene knockouts (mammalian)	High	High	High	High	Moderate	None
Small-molecule inhibitors	Low	Low	High	High	Low	Yes
Monoclonal antibodies	Low	High	Moderate	Moderate	Moderate	Yes
Antisense oligonucleotides	High	High	Low to moderate	Low to moderate	High	Yes

**Fig. 1.** Experimental approaches that are currently used to modulate the expression of a particular signaling molecule to determine its function: (1) gene knockout; (2) overexpression systems; (3) small-molecule inhibitors; (4) monoclonal antibodies; and (5) antisense technology. The advantages and disadvantages of each approach are discussed in the text. Antisense technology uses chemically synthesized oligonucleotides that target specific mRNA sequences through a Watson-Crick base pairing of adenine-thymine (A-T) and guanosine-cytosine (G-C) base pairs.

- chemická inhibice, aktivace
- změna genové exprese
- *selekce rezistentních klonů*
- *použití přirozených mutantů*
- *overexprese proteinu*
- *overexprese dominantně negativních mutantů*
  - *tranzientní vs. stabilní transfekce*
- *použití antisense oligonukleotidů*
- *RNA interference - tranzientní a stabilní*

# Chemická inhibice, aktivace:



**Forskolin**

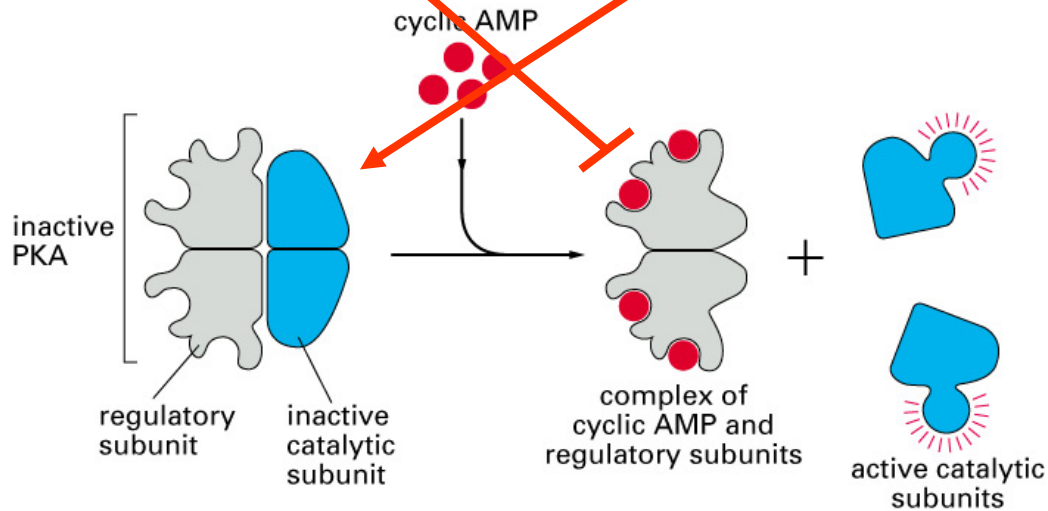
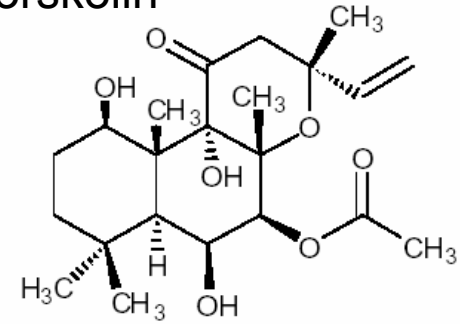


Figure 15-32. Molecular Biology of the Cell, 4th Edition.

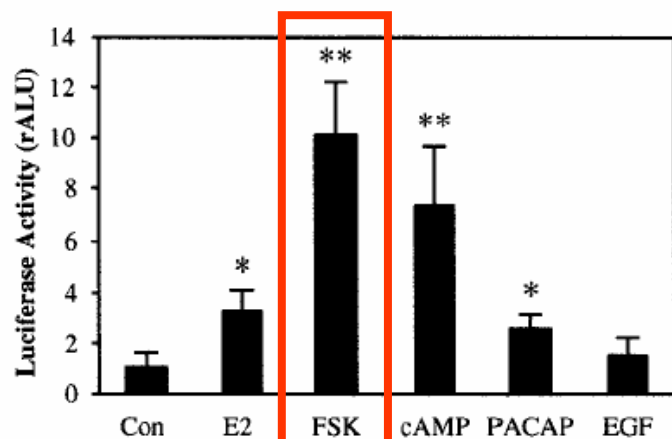
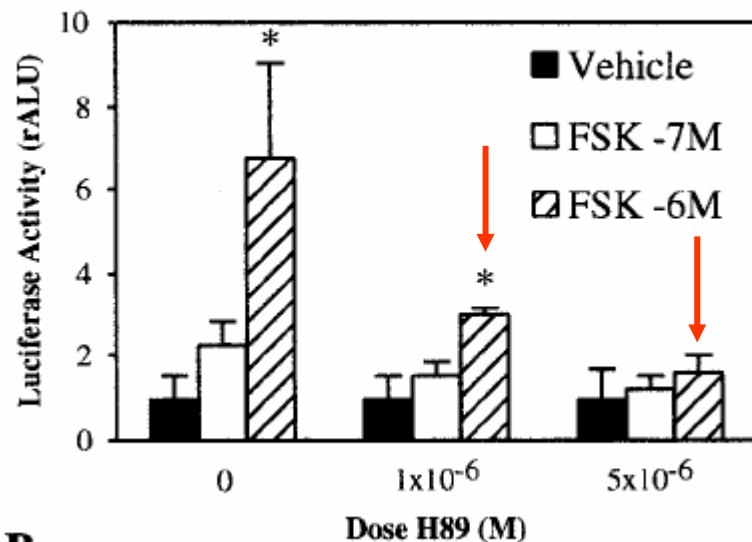


FIG. 1. Activation of an estrogen-responsive model promoter in  $\alpha$ T3 cells by cAMP. Cells were transfected with 2  $\mu$ g ERETkluc reporter plasmid as described and treated with vehicle (Con), 10 nM 17 $\beta$ -estradiol (E2), 1  $\mu$ M FSK, 2 mM 8-Br-cAMP (cAMP), 200 nM PACAP, or 100 ng/ml EGF for 24 h. Data shown represent the normalized mean  $\pm$  SEM from five to seven independent experiments performed in duplicate. Significant differences from control are denoted with asterisks (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ).

A.



B.



## Další kritické body pro použití inhibitorů:

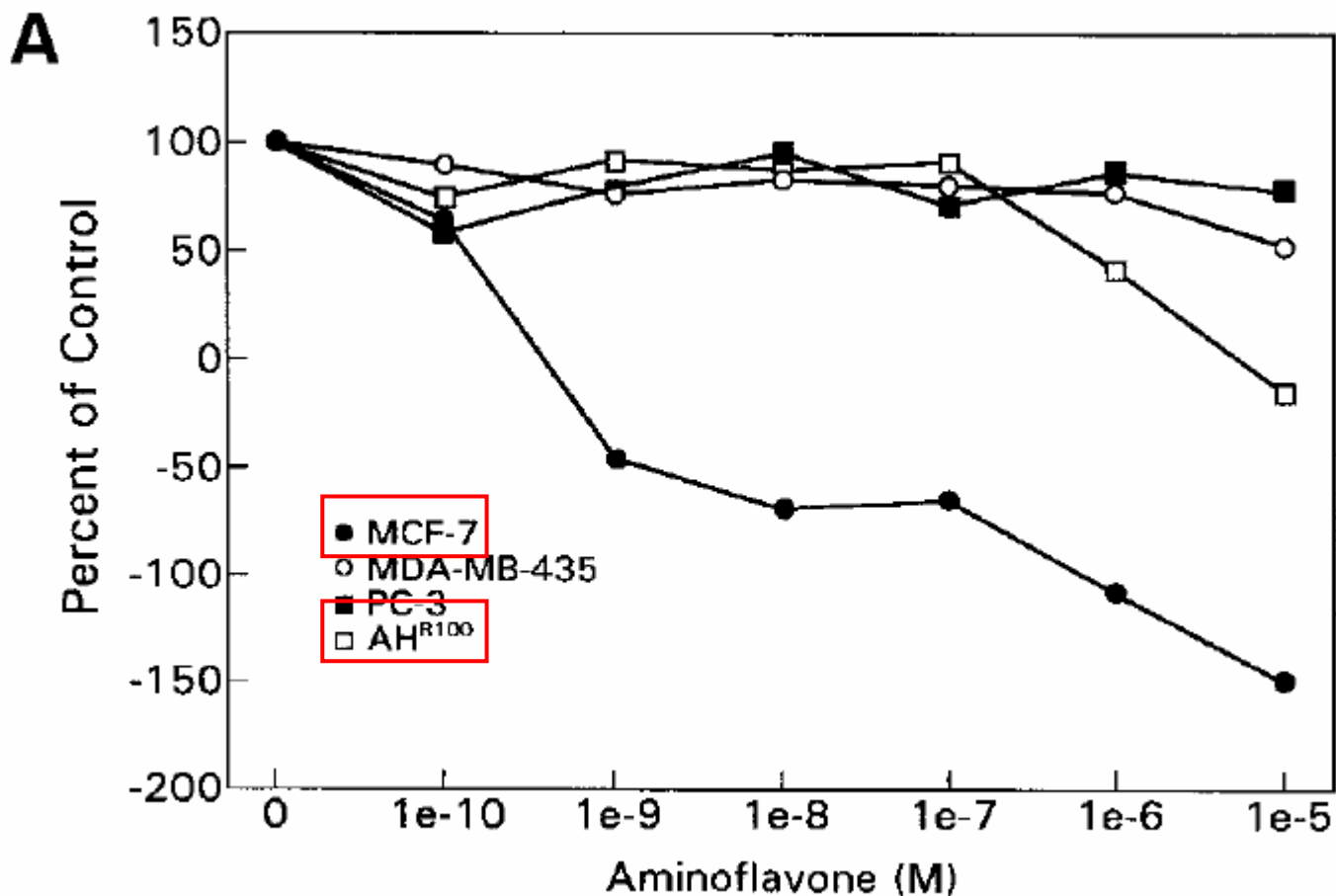
- rozpustnost;
- stabilita;
- biodostupnost;
- nežádoucí reakce s receptory;
- různá aktivita vůči izolovaným nebo rekombinantním proteinům a v buněčné kultuře nebo v in vivo podmínkách.

- selekce rezistentních klonů
- použití přirozených mutantů nebo linií KO myši:

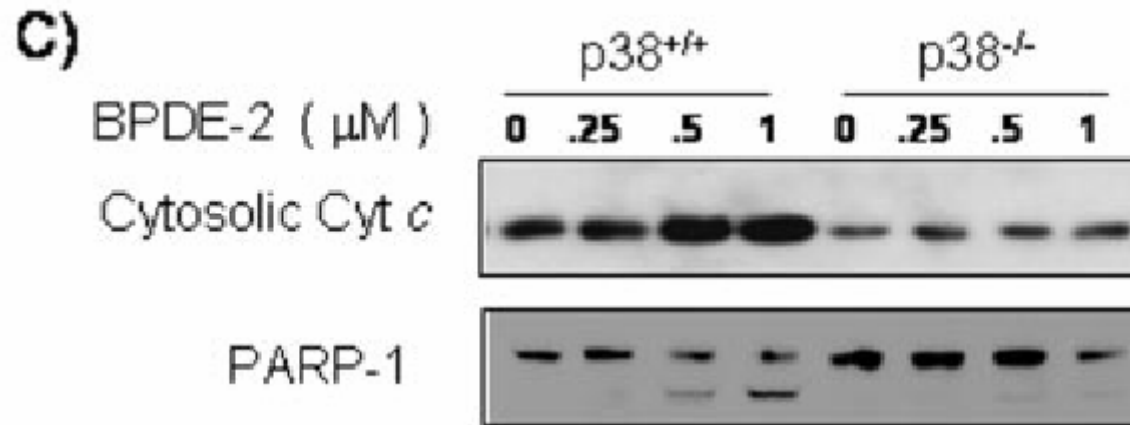
Buňky jsou dlouhodobě pěstovány v přítomnosti účinných koncentrací vysoce toxických látek - např. cytostatik - jsou vyselektovány přežívající buňky schopné růst v přítomnosti toxických látek - např. cytostatika, toxiny. Zpětně jsou pak studovány změny na úrovni exprese proteinů.



# Toxické účinky cytostatika na nádorové buňky



# Využití buněk izolovaných z knock-out myši: MEFs (mouse embryonic fibroblasts)

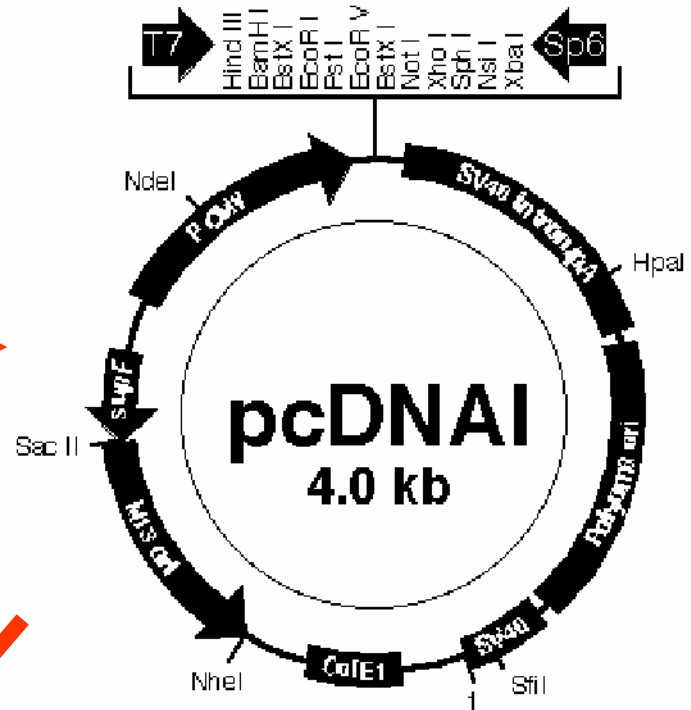
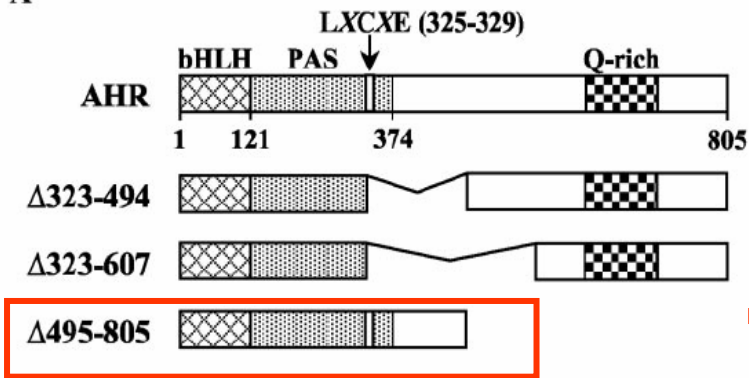


## Manipulace s funkcí proteinu prostřednictvím overexprese identického genu se změnou funkcí:

- **Loss –of-function mutations**
  - Null mutations – completely lacks function of gene
  - Infer function of wild-type gene
- **Hypomorphic mutations**
  - Partial loss-of-function
  - Infer function of genes expressed during different times in development
- **Conditional mutations**
  - Cause loss-of-function only under special circumstances
  - E.g., temperature sensitive mutations
  - Infer function of gene at different developmental stages
- **Dominant-negative mutations**
  - Mutant allele counteracts wild type allele in heterozygote
  - Also haploinsufficiency – mutant allele is dominant in heterozygote because two wild-type alleles required for development

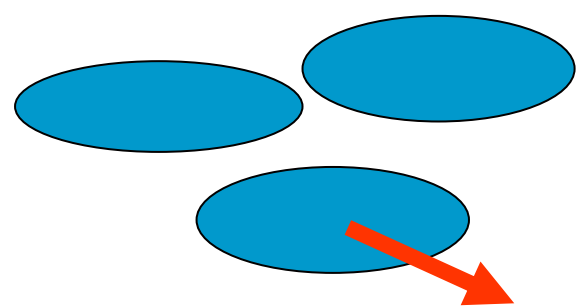
podmínkách in vitro můžeme vhodný model získat buď z existujícího organismu, nebo připravit gen kódující změnou funkci uměle.

A



**Transformace kompetentních bakterií (E.coli)**

- tepelný šok
- elektroporace



**Izolace plazmidu**

**Comments for pcDNA1:**  
4033 base pairs

- Col E1 origin: bases 1-587
- M13 origin: bases 588-1182
- SupF gene: bases 1183-1384
- CMV promoter: bases 1517-2170
- T7 promoter: bases 2171-2189
- T7 primer sequence: bases 2170-2189
- Polylinker: bases 2187-2306
- Sp6 primer sequence: bases 2307-2325
- splice and poly A: bases 2326-3024
- Polyoma origin: bases 3029-3870
- SV40 origin: bases 3871-4033

# Transfekce buněčných linií *in vitro*:

**Transfection** is the process of introducing naked DNA molecules into cells. Transfection can be categorized into 2 major types, stable and transient. Transient transfection is temporary and high level expression of foreign genes. Expression lasts for several days, but is lost as the DNA never integrates stably into the host cell DNA. In contrast, stable transfection occurs with a lower frequency (10 to 100-fold lower), but expression is maintained for the long term because the foreign DNA does integrate into the host DNA. In the case of stable transfection, cotransfection is often used to introduce a selectable marker (such as an antibiotic resistance gene). Since only one in 1000 cells might be stably transfected, it is necessary to select these cells from the total population. Cells that express the selectable marker also take up and express the other gene of interest.

**Lipofection** is a procedure in which the DNA is complexed within lipid droplets. The droplets interact directly with the cell membrane and fuse. The DNA is liberated into the cytoplasm and some eventually reaches the nucleus. Lipofection is one of the most efficient methods of transfection. However, it is also relatively expensive, it can be toxic to cells, and it cannot be used with cells growing in serum. Lipofection, like other forms of transfection, works much more efficiently if the cells are rapidly growing. This is because the nuclear membrane is absent during cell division, allowing easier access to the host DNA.

**Calcium phosphate** is another popular method for transfection. In this procedure, the DNA is precipitated with calcium phosphate aggregates. The cells phagocytize the aggregates and the DNA is released into the cytoplasm and eventually reaches the nucleus. This is the oldest method and its main advantage is that it is cheap and easy to perform. However, calcium phosphate transfection is not as efficient as lipofection and the precipitates often cause cytotoxicity.

**Electroporation** is another method of transfection in which cells are exposed to an electric shock. This induces transient aqueous channels in the membrane for DNA to enter the cytoplasm. On the positive side, electroporation is rapid and simple, and it works on almost all types of cells. However, one needs special equipment such as an electroporator to shock the cells. The transfected cells also have high cytotoxicity after shocking.

There are several **viral vector systems** that have been developed for the study of gene expression in vitro or in vivo, including recombinant vaccinia viruses, retroviruses, and adenoviruses. Due to bio-safety regulations, a special lab facility must be available.

# Metody selekce

## Prokaryota

Antibiotic	Formula
<b>Ampicillin</b> Ampicillin is a semi-synthetic penicillin derived from the penicillin nucleus, 6-amino-penicillanic acid. It causes cell death by inhibiting cell wall biosynthesis. Resistance to ampicillin is mediated by $\beta$ -lactamase cleavage of the $\beta$ -lactam ring ( <i>bla</i> gene).	$C_{16}H_{18}N_3O_4 \text{ SNa}$
<b>Kanamycin Sulfate</b> Kanamycin is effective as a bacteriocidal agent by inhibiting ribosomal translocation and eliciting miscoding. Resistance is conferred by the Kan <sup>R</sup> -Tn5 gene product (aminoglycoside phosphotransferase), which modifies the antibiotic and prevents interaction with ribosomes. Liquid kanamycin (100X) contains 10 mg/ml kanamycin (base) utilizing kanamycin sulfate in 0.85% saline.	$C_{18}H_{36}N_4O_{11} \bullet H_2SO_4$
<b>Tetracycline</b> Tetracycline is a bacteriocidal agent that inhibits protein synthesis by preventing binding of aminoacyl-tRNA to ribosomes. Resistance is conferred by the Tet <sup>R</sup> -Tn10 gene product (an inner membrane protein that effluxes the antibiotic), which blocks cell wall permeability.	$C_{22}H_{24}N_2O_8 \bullet HCl$

## Eukaryota

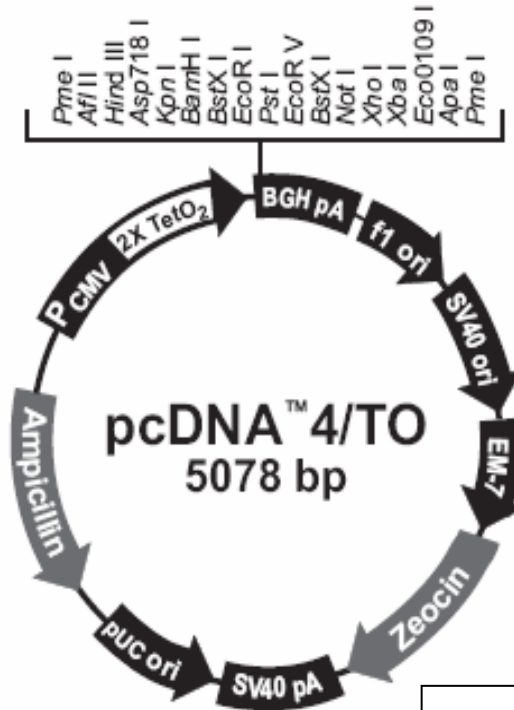
<b>Geneticin®</b>	HeLa	200-400	<ul style="list-style-type: none"> <li>• <b>pcDNA3.1™</b> vectors (Constitutive mammalian expression)</li> <li>• <b>pIND</b> vectors (Ecdysone-Inducible mammalian expression)</li> <li>• <b>pShooter™</b> vectors (Intracellular protein targeting)</li> <li>• <b>pDisplay™</b> vectors (Protein display)</li> <li>• <b>pVP22</b> vectors (Protein translocation)</li> <li>• <b>pBlue-TOPO®, pGlow-TOPO®</b> vectors (Assessing promoter activity)</li> </ul>
	NIH3T3	600-1,000	
	CHO	~ 400	
	293 HEK	600-800	
	Jurkat T cell	600-700	
<b>Hygromycin B</b>	HeLa	~ 550	<ul style="list-style-type: none"> <li>• <b>pcDNA5</b> vectors (Constitutive mammalian expression)</li> <li>• <b>pIND/Hygro</b> vector (Ecdysone-Inducible mammalian expression)</li> </ul>
	CHO	~ 250	
<b>Blasticidin S</b>	HeLa	1-3	<ul style="list-style-type: none"> <li>• <b>pcDNA6</b> vectors (Constitutive mammalian expression)</li> <li>• <b>BsdCassette™</b> vectors (Constructing customized Blasticidin-resistant vectors)</li> <li>• <b>pIB/V5-His-TOPO®</b> vectors (Stable insect expression)</li> <li>• <b>pMIB/V5-His</b> vectors (Secreted insect expression)</li> <li>• <b>pCoBlast</b> (Selection vector for DES®)</li> </ul>
	NIH3T3	5-10	
	CHO	5-10	
	COS-1	3-10	
	293 HEK	5-10	
	<i>S2 Drosophila</i>	~ 5	
	<i>S. cerevisiae</i>	~ 25	



## Overexpression/ectopic expression:

- exprese velmi vysokých hladin proteinu v buňce, která ho za normálních okolností neexprimuje, nebo jen v omezené množství;
- nevýhodou je častá nespecifita - aberantní lokalizace proteinu, aberantní interakce s dalšími proteiny, atd.

# Inducibilní exprese:



Comments for pcDNA™4/TO  
5078 nucleotides

CMV promoter: bases 232-958

TATA box: bases 804-810

Tetracycline operator (2X TetO<sub>2</sub>) sequences: bases 820-859

CMV forward priming site: bases 769-789

Multiple cloning site: bases 967-1077

BGH reverse priming site: bases 1089-1106

BGH polyadenylation sequence: bases 1095-1319

f1 origin: bases 1365-1793

SV40 promoter and origin: bases 1803-2143

EM-7 promoter: bases 2183-2249

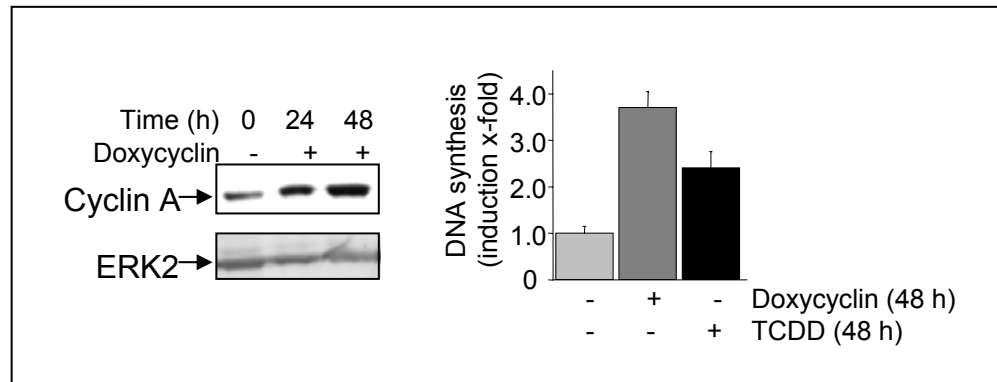
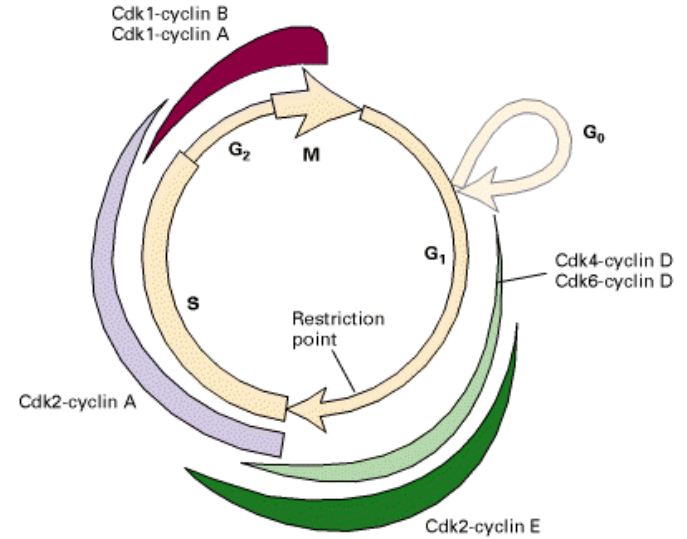
Zeocin™ resistance gene: bases 2250-2624

SV40 early polyadenylation sequence: bases 2754-2884

pUC origin: bases 3267-3937

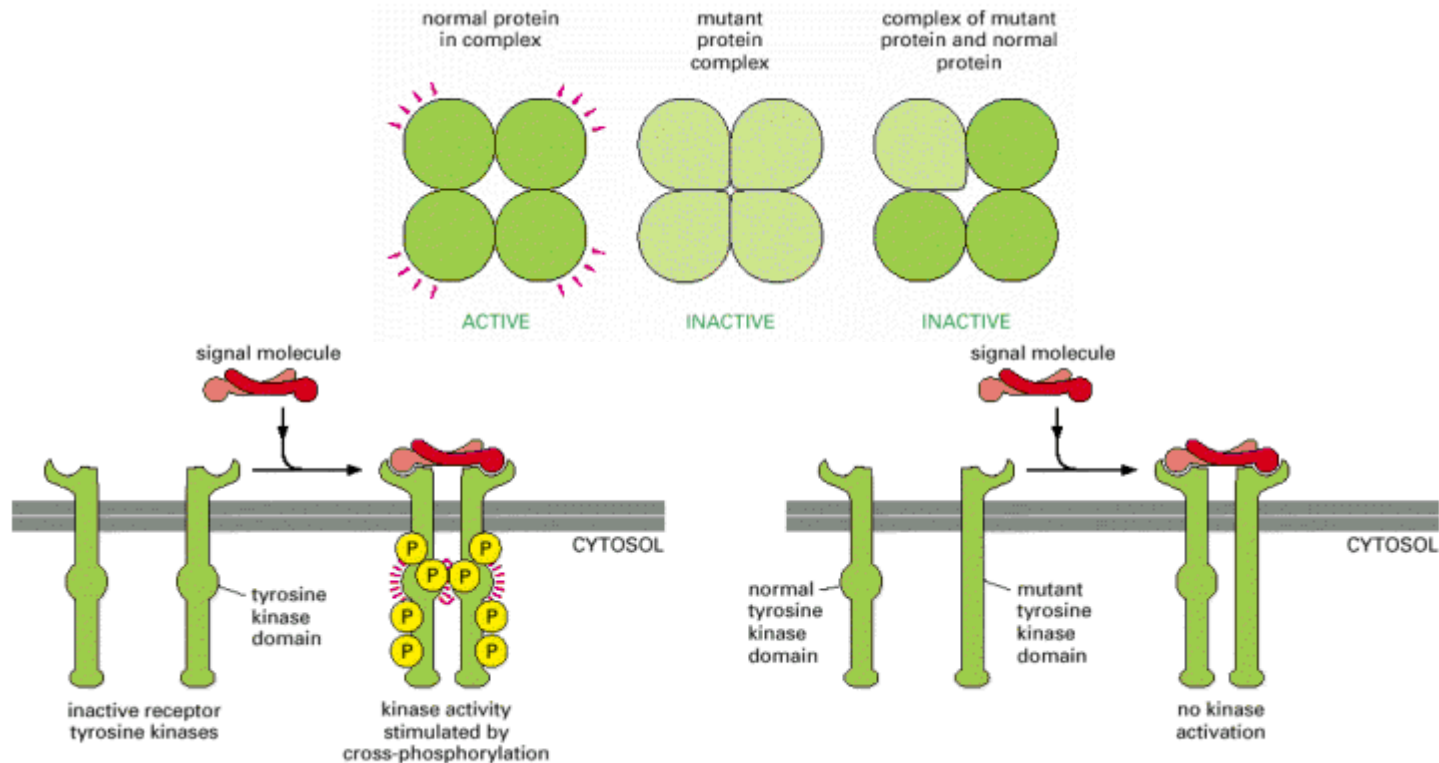
*bla* promoter: bases 4937-5041 (complementary strand)

Ampicillin (*bla*) resistance gene: bases 4082-4942 (complementary strand)



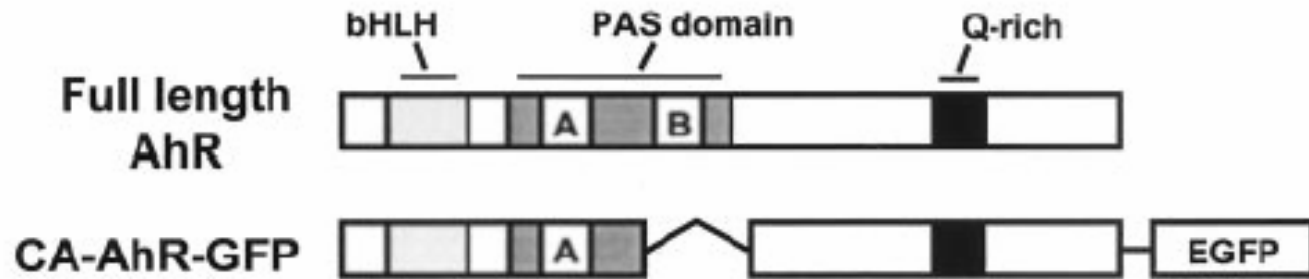
# Dominantně-negativní mutant - definice:

- mutantní protein, který potlačuje funkci wt proteinu v případě společné exprese;
- mechanismy - multimerizace, titrace (upstream or downstream targets), aktivní represe.

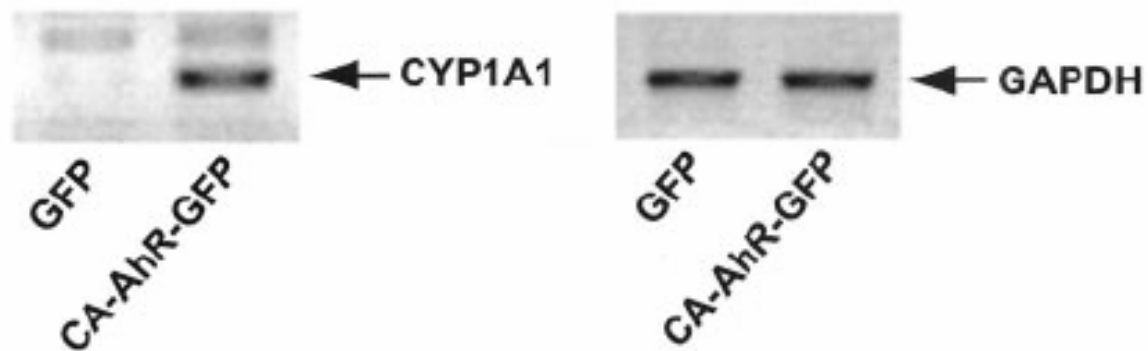


# Konstitutivně-aktivní mutant

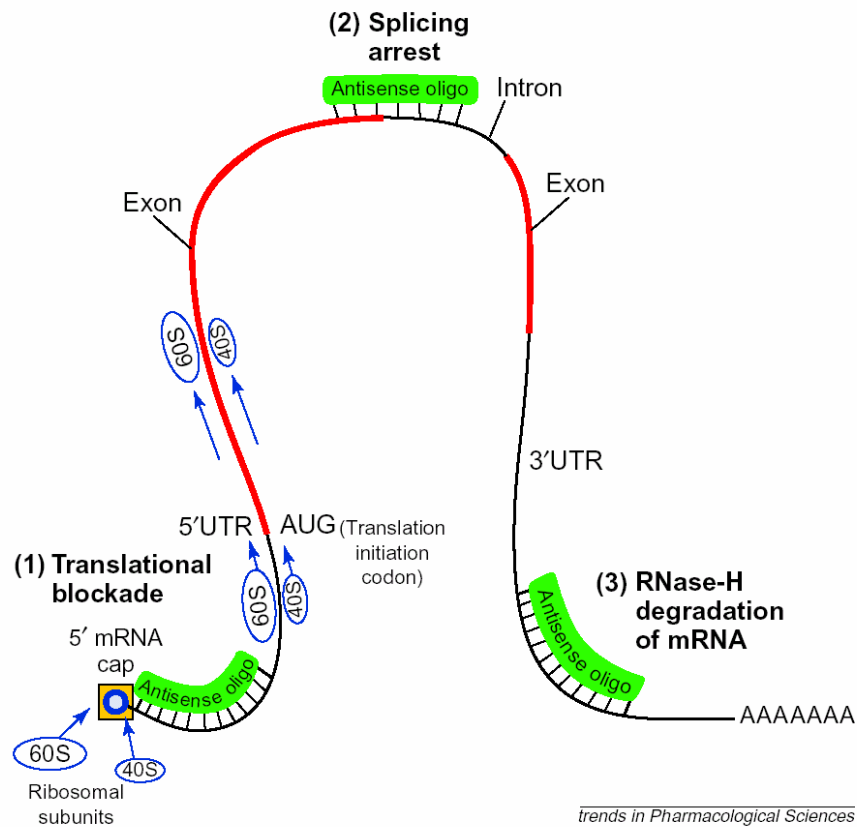
**A**



**B**



# Antisense technology:



*trends in Pharmacological Sciences*

**Fig. 1.** Experimentally demonstrated mechanisms by which antisense oligonucleotides disrupt protein synthesis include: (1) steric blockade of ribosomal subunit attachment to mRNA at the 5' cap site; (2) interference with proper mRNA splicing through antisense binding to splice donor or splice acceptor sites; and (3) RNase-H-mediated degradation of hybridized mRNA. The latter can occur anywhere in the mRNA where an antisense molecule binds with sufficient affinity, including the 5' and 3' UTRs, at the translation initiation codon, and in exons or introns.

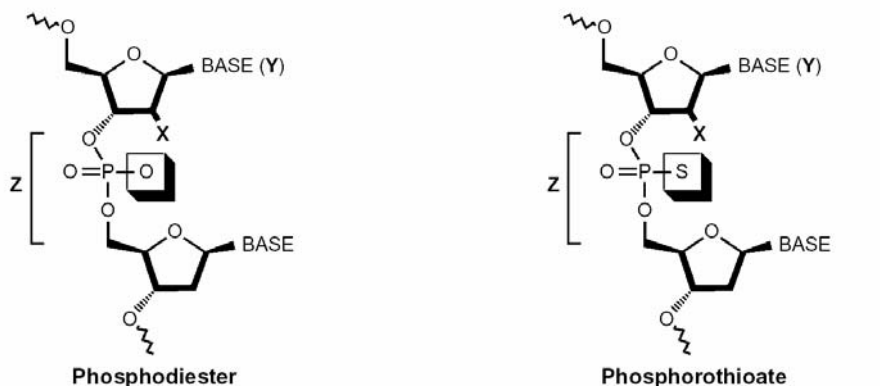
**Table 3. Antisense oligonucleotides currently in clinical trials or on the market**

Compound	Protein target	Indication	Sponsoring company	Development phase
Vitravene (ISIS2292) <sup>a</sup>	CMV IE2	CMV retinitis	Isis/Ciba vision	Approved
ISIS2302	ICAM-1	Crohn's disease, organ transplant, psoriasis	Isis	Phase II
ISIS3521	Protein kinase C $\alpha$	Cancer	Isis	Phase II
ISIS5132	RAF kinase	Cancer	Isis	Phase II
G3139	BCL2	Cancer	Genta	Phase II
INX3280	MYC	Restenosis	INEX	Phase II
GEM132	CMV UL36	CMV retinitis	Hybridon	Phase I
ISIS2503	Ha-RAS	Cancer	Isis	Phase II
ISIS13312	CMV IE2	CMV retinitis	Isis	Phase I
GEM92	HIV	AIDS	Hybridon	Phase I
GEM230	Protein kinase A	Cancer	Hybridon	Phase I

<sup>a</sup>Vitravene (Fomivirsin, ISIS2922) has been approved for the second-line treatment of cytomegalovirus (CMV) retinitis in patients with AIDS who are intolerant of or unresponsive to previous treatment(s) for the disease<sup>14</sup>. All drug compounds are phosphorothioate oligodeoxynucleotides except ISIS13312 (2'-methoxyethyl), GEM92 (2'-methoxy) and GEM230 (2'-methoxy), which contain 'second-generation' 2'-sugar modifications.

Abbreviations: ICAM-1, intercellular adhesion molecule 1; IE2, immediate early gene 2.

# Design of a phosphodiester oligonucleotide:



## (a) 2' Sugar modifications (X)

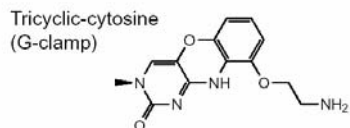
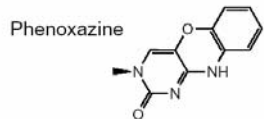
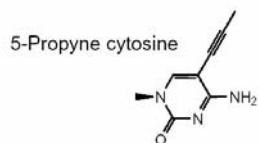
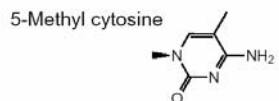
Fluoro  
—F

Methoxy  
—O—CH<sub>3</sub>

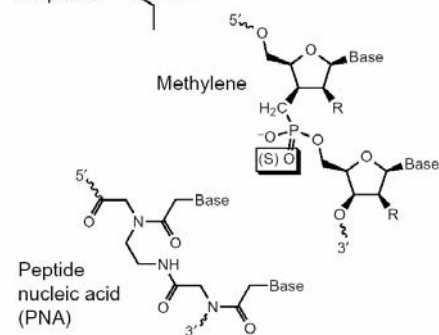
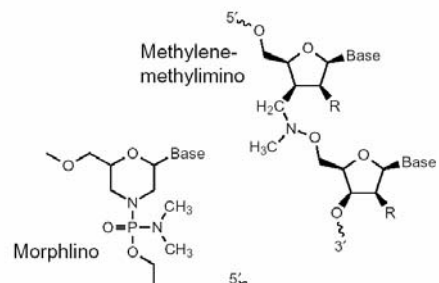
Propoxy  
—O—CH<sub>2</sub>CH<sub>2</sub>CH<sub>3</sub>

Methoxyethyl  
—O—CH<sub>2</sub>CH<sub>2</sub>—O—CH<sub>3</sub>

## (b) Heterocycle modifications (Y)



## (c) Backbone modifications (Z)



*trends in Pharmaceutical Sciences*

1) **Chemistry** - unmodified phosphodiester DNA is rapidly metabolized in both serum and cells - several chemical modifications can be incorporated into antisense molecules to boost their nuclease resistance. Two examples are phosphorothioate oligodeoxynucleotides and 2'-O-methyl oligonucleotides.

2) **Length** - most antisense molecules are 15-20 bases long, a length theoretically sufficient to pick out a unique sequence from others in the human genome and identify a target mRNA (Ref. 18). Antisense oligomers of this size have been successfully used to discriminate between two gene products that differ by a mutation of a single base. Longer oligonucleotide sequences (e.g. 30 nucleotides) are more expensive to synthesize and they might actually increase the risk of non-sequence specific mRNA cleavage because of growing probabilities that other mRNA hybridization sites will be included in long oligomers. Shorter oligomers, meanwhile, generally do not have sufficient affinity to result in adequate potency.

3) **Sequence selection** - empirical - not all areas of a mRNA molecule are equally amenable to antisense hybridization. The reasons for this are unclear but probably involve mRNA secondary structure, proteins bound to the mRNA or accessibility of hybridized mRNA to RNase H. 'gene-walk' approach involves synthesizing oligonucleotides that target regions scattered throughout the entire mRNA sequence and then evaluating these compounds in cell-culture assays

**Fig. 3.** Structures of chemical modifications that are employed in antisense technology. Phosphodiester oligodeoxynucleotides ('plain DNA') are not useful in antisense technology because of their inherent susceptibility to nuclease degradation. Chemical modifications involving substitution within the (a) sugar, (b) heterocycle (base) or (c) backbone substituents of DNA were designed primarily to reduce nuclease sensitivity, improve affinity for RNA hybridization, or both. Some of these modifications also provide pharmacokinetic and toxicological advantages. Phosphorothioate oligodeoxynucleotides are sometimes referred to as 'first-generation' antisense oligonucleotides whereas oligonucleotides containing other modifications are referred to as 'second-generation'. (See references for a description of the chemical and biological properties of the modifications indicated<sup>17,24,25</sup>.)

# RNA Interference

RNA molecules have been used for over two decades to reduce or interfere with expression of targeted genes in a variety of systems. Historically, these methods have been called **post transcription gene silencing (PTGS)** in plants, quelling in fungi and **RNA interference (RNAi)** in higher animals. Although originally thought to require use of long double-stranded (DS) RNA molecules, the active mediators are now known to be **short DS RNAs**. These short interfering RNAs (siRNAs) are naturally produced in vivo through nucleolytic processing of long DS RNAs. **Short DS RNAs can also be chemically synthesized and used to experimentally inhibit gene expression.**

## The Nobel Prize in Physiology or Medicine 2006

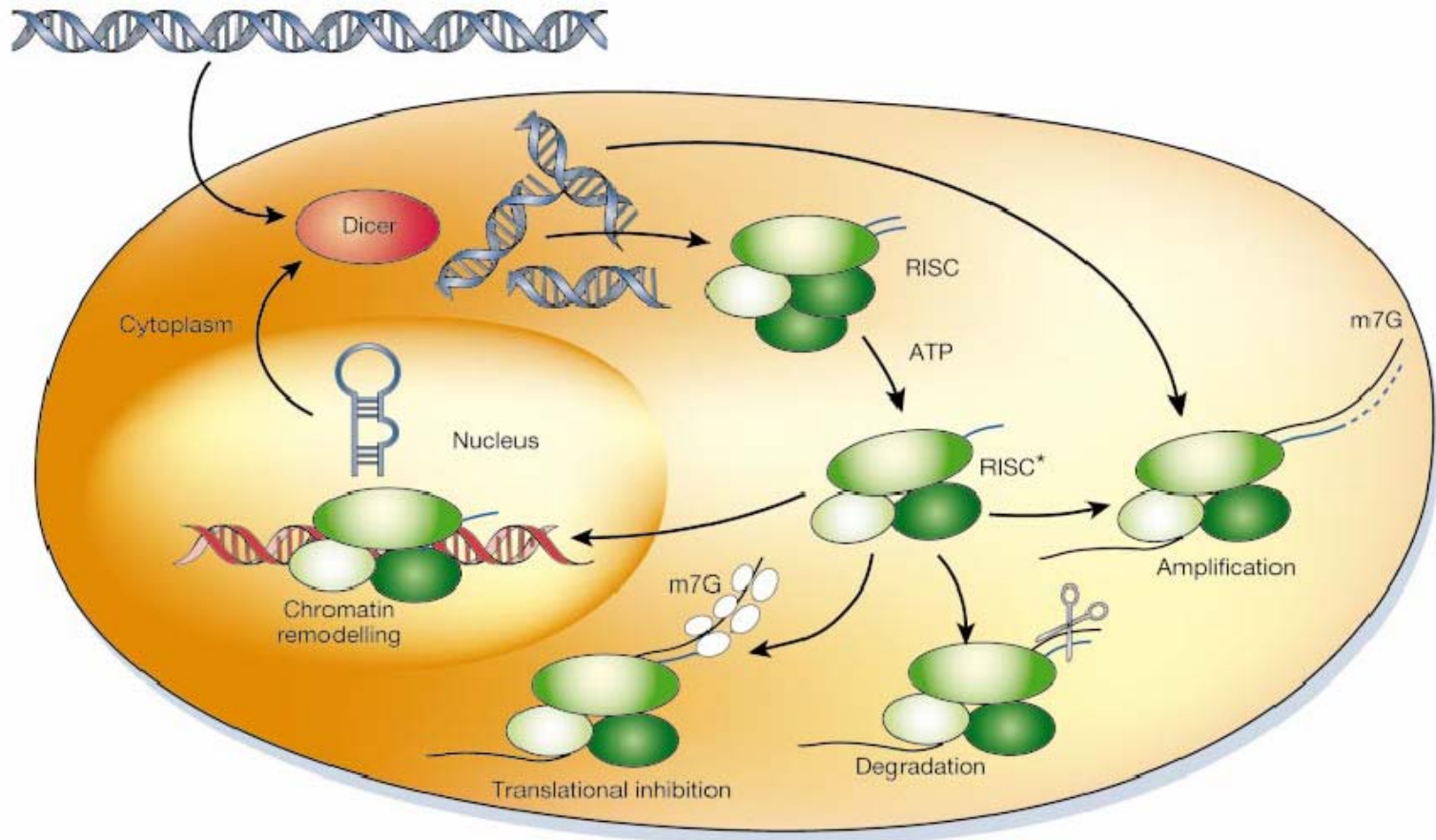
"for their discovery of RNA interference - gene silencing by double-stranded RNA"



Andrew Fire



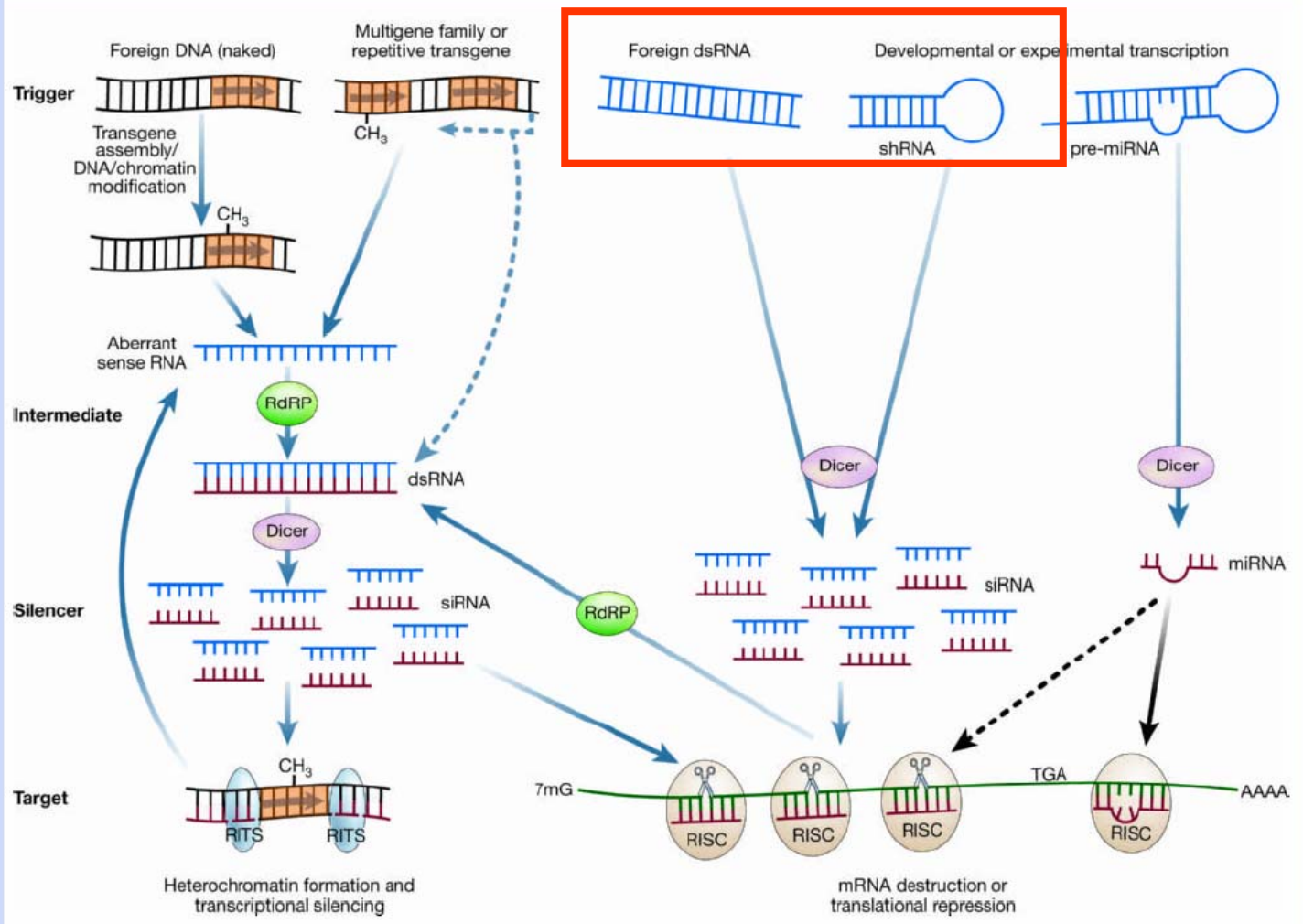
Craig Mello



**Figure 5** A model for the mechanism of RNAi. Silencing triggers in the form of double-stranded RNA may be presented in the cell as synthetic RNAs, replicating viruses or may be transcribed from nuclear genes. These are recognized and processed into small interfering RNAs by Dicer. The duplex siRNAs are passed to RISC (RNA-induced silencing complex), and the complex becomes activated by unwinding of the duplex. Activated RISC complexes can regulate gene expression at many levels. Almost

certainly, such complexes act by promoting RNA degradation and translational inhibition. However, similar complexes probably also target chromatin remodelling. Amplification of the silencing signal in plants may be accomplished by siRNAs priming RNA-directed RNA polymerase (RdRP)-dependent synthesis of new dsRNA. This could be accomplished by RISC-mediated delivery of an RdRP or by incorporation of the siRNA into a distinct, RdRP-containing complex.





**Figure 2** Model depicting distinct roles for dsRNA in a network of interacting silencing pathways. In some cases dsRNA functions as the initial stimulus (or trigger), for example when foreign dsRNA is introduced experimentally. In other cases dsRNA acts as an intermediate, for example when 'aberrant' mRNAs are copied by cellular RdRP. Transcription can produce dsRNA by readthrough from adjacent transcripts, as may occur for repetitive gene families or high-copy arrays (blue dashed arrows). Alternatively, transcription may be triggered experimentally or developmentally, for example in the expression of short hairpin (shRNA) genes and endogenous hairpin (miRNA) genes. The small RNA products of the Dicer-mediated dsRNA processing reaction guide distinct protein complexes to their targets. These silencing complexes include the RNA-induced silencing complex (RISC), which is implicated in mRNA destruction and translational repression, and the RNA-induced transcriptional silencing complex (RITS), which is implicated in chromatin silencing. Sequence mismatches between a miRNA and its target mRNA lead to translational repression (black solid arrow), whereas near perfect complementarity results in mRNA destruction (black dashed arrow). Feedback cycles permit an amplification and longterm maintenance of silencing. CH<sub>3</sub>, modified DNA or chromatin; 7mG, 7-methylguanine; AAAAA, poly-adenosine tail; TGA, translation termination codon.

## Kritické body pro aplikaci siRNA:

- vhodně navržená sekvence ([www.dharmacon.com](http://www.dharmacon.com); [www.ambion.com](http://www.ambion.com));
- stabilita siRNA;
- vhodný způsob transfekce;
- optimální stav buněčné kultury;
- především optimálně nastavený systém kontrol a vyloučení tzv. nespecifické interferonové reakce.



# RNA Interference pomocí shRNA

