JAGIELLONIAN UNIVERSITY
IN KRAKOW

Regulation of gene expression in gene therapy

Lecture 7
28th November 2011

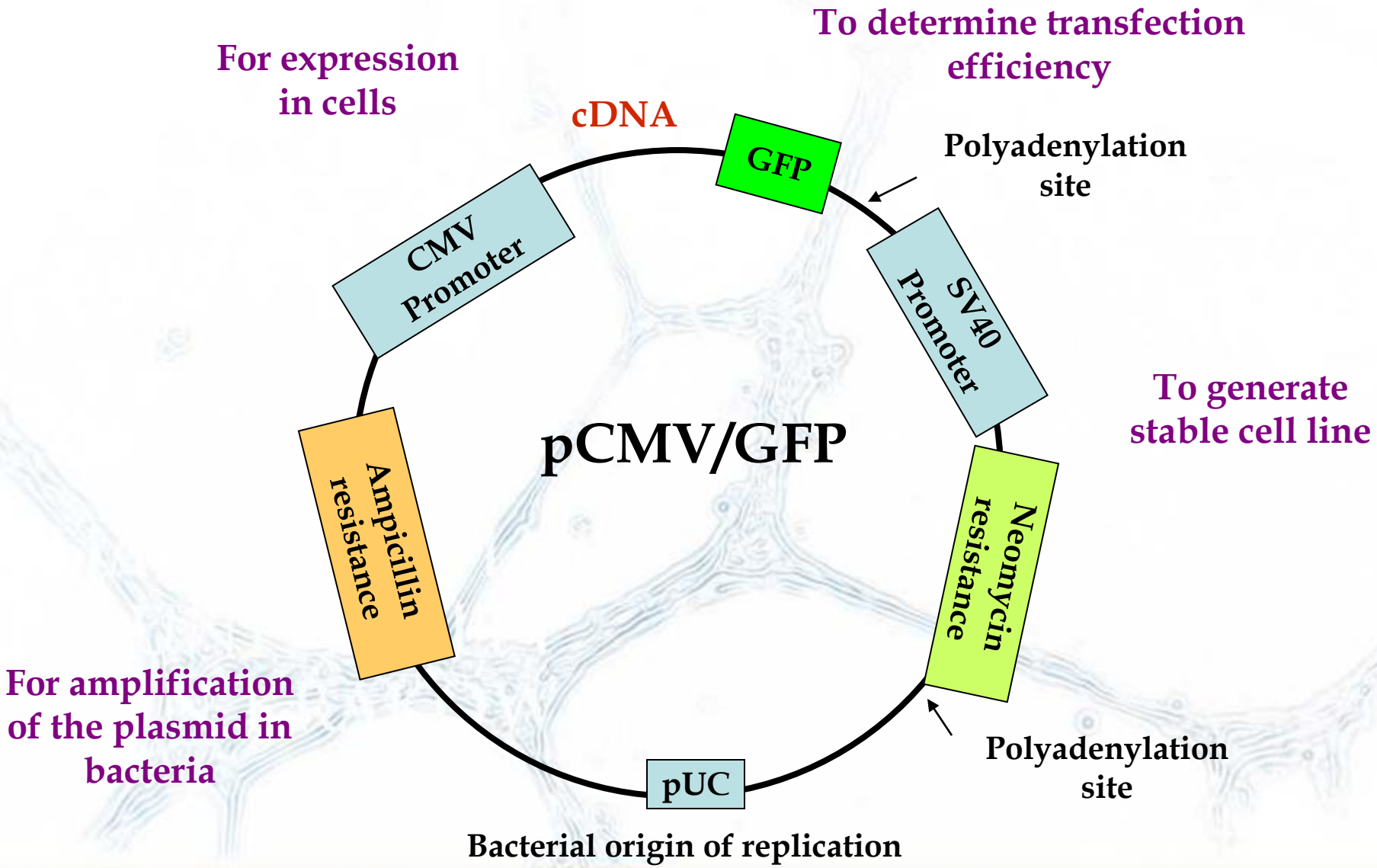
Successful gene therapy in the clinical setting

- should provide very tight regulation of gene expression

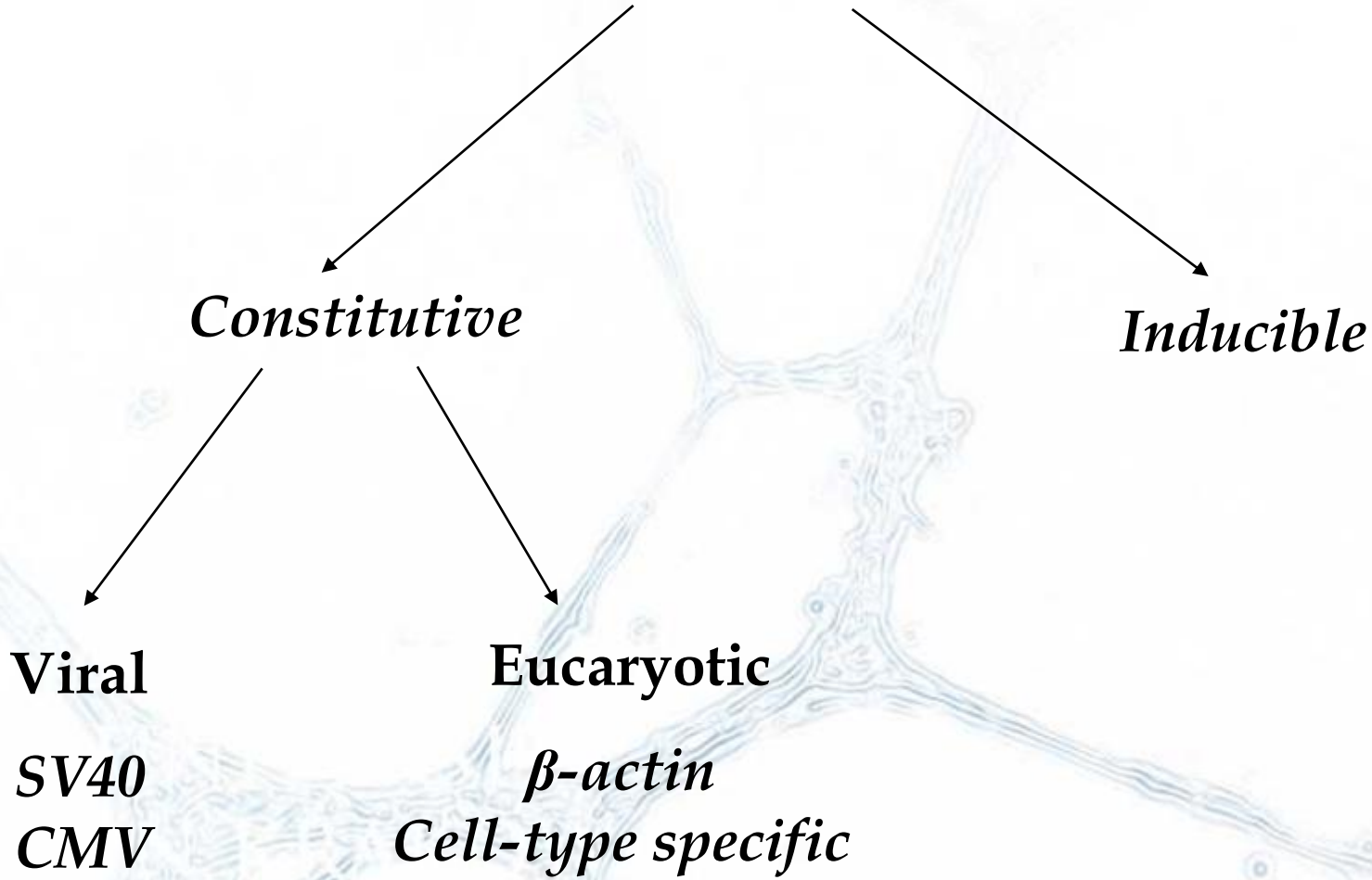
- the regulation should to be achievable using a compound that is **nontoxic**,
-Is able to **penetrate** into the desired target tissue or organ,
-It has a **half-life of a few hours** (as opposed to minutes or days) so that when withdrawn or added (depending on the regulatable system used) gene expression can be turned "on" or "off" quickly and effectively

- the genetic switches should be **nonimmunogenic** in the host

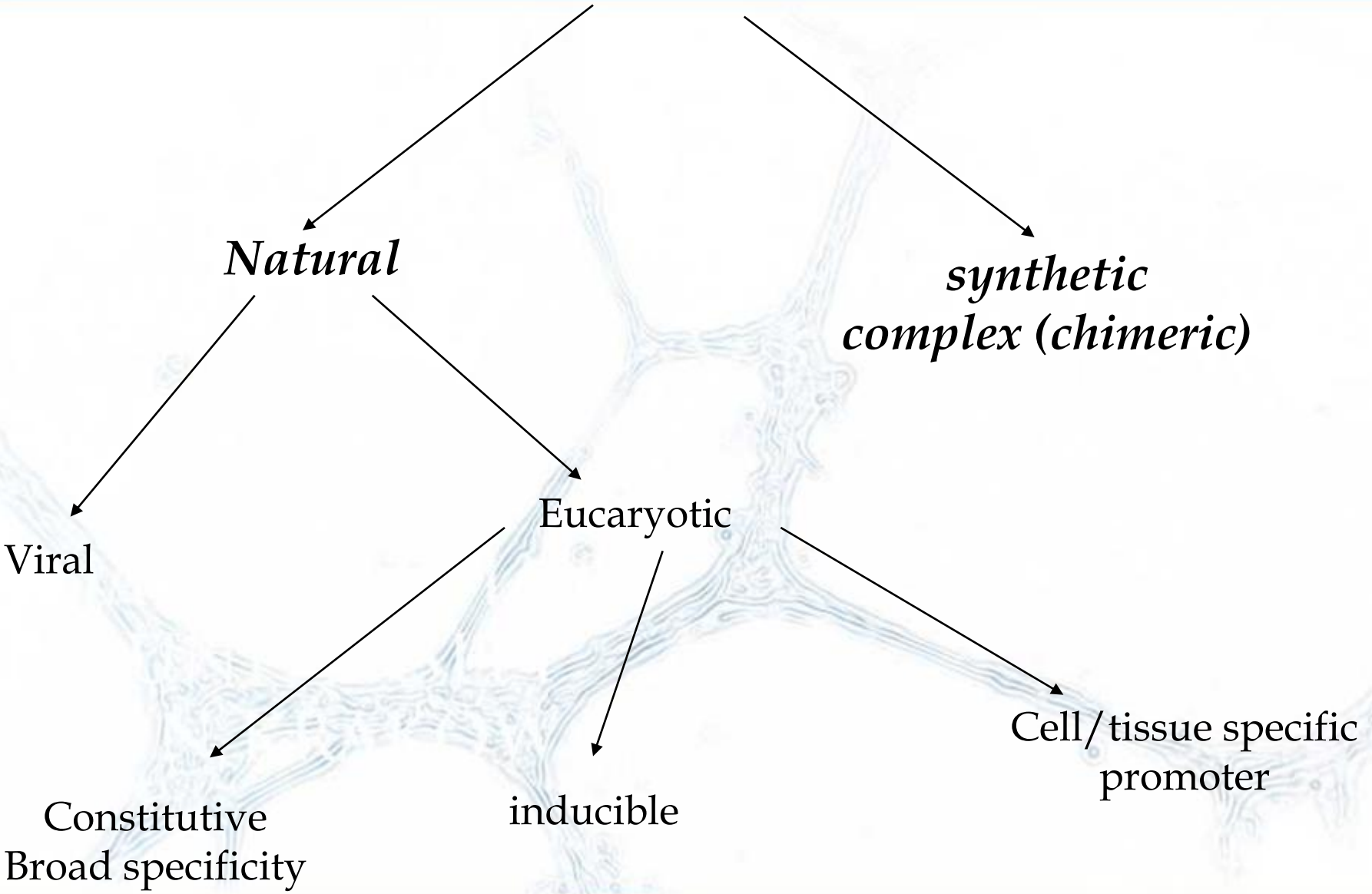
Mammalian expression plasmid



Promoters


Constitutive
Inducible
Viral
Eucaryotic
SV40
CMV
β-actin
Cell-type specific

Promoters

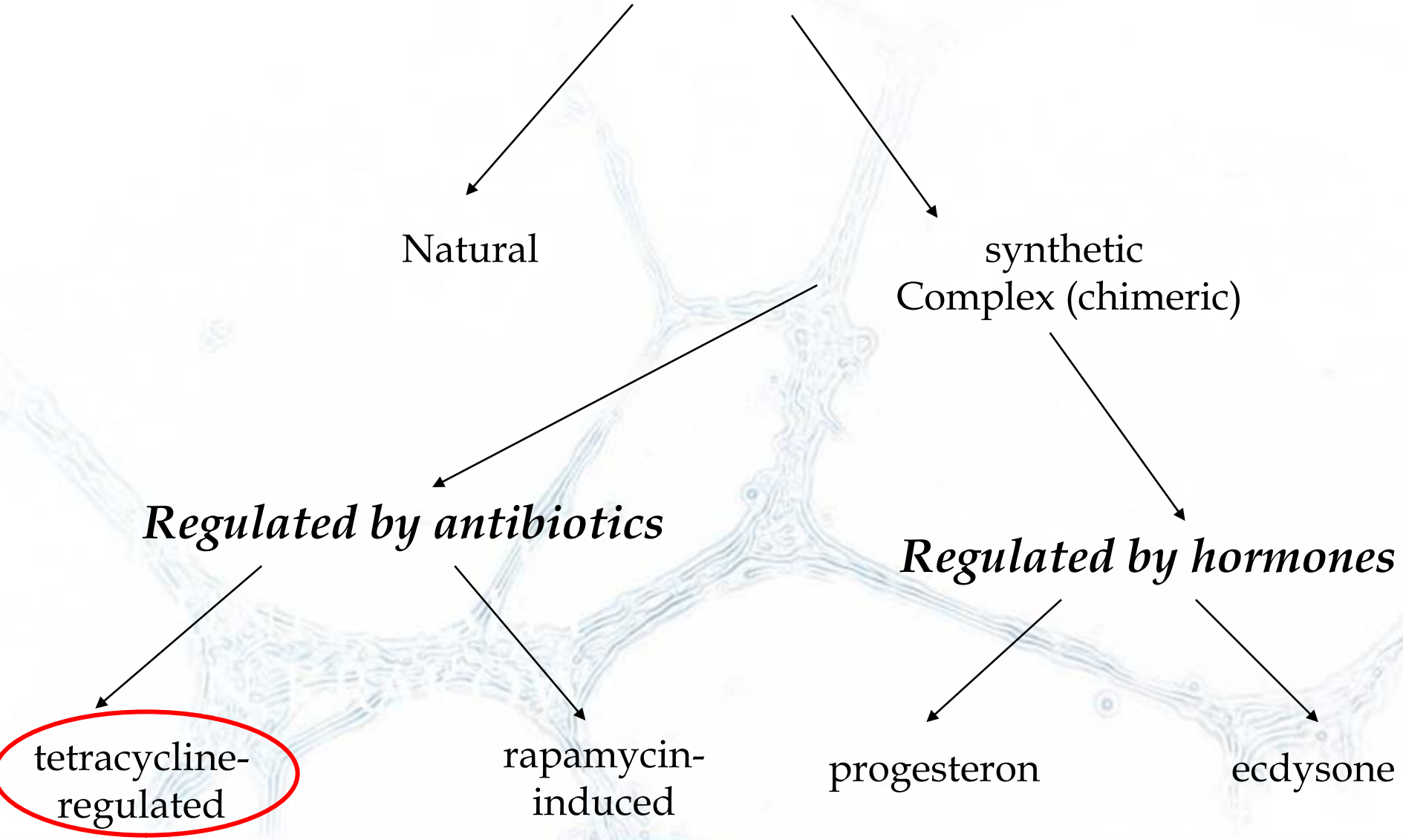

Natural
synthetic
complex (chimeric)
Viral
Eucaryotic
Constitutive
Broad specificity
inducible
Cell/tissue specific
promoter

Natural regulatable systems - based on naturally occurring inducible promoters

- weak induction of transgene expression,
- reliance on inducible agents that exert pleiotropic effects on mammalian cells

Chimeric regulatable systems - overcome these limitations, offer greater specificity than natural inducible promoters

Inducible promoters

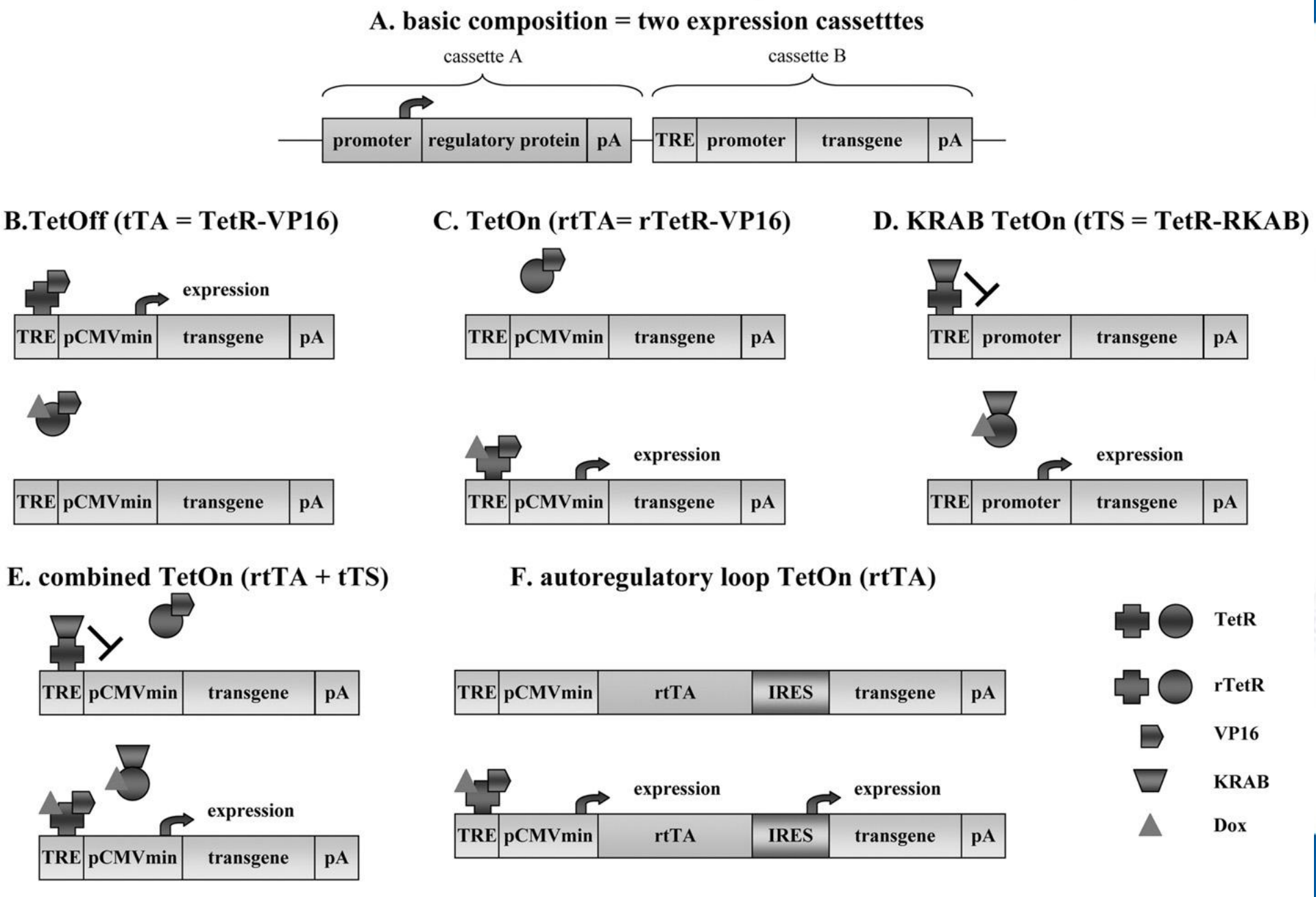


Tetracycline regulatable system

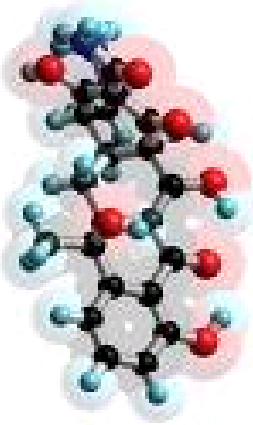
- tet off
- tet on

Tetracycline is used to **switch off** or **switch on** the expression of a gene

Five different Tet-regulatable systems



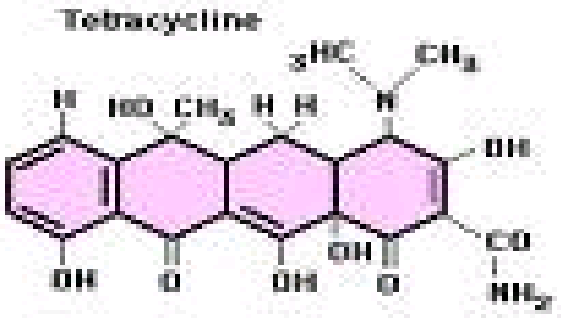
Tetracycline



- Has been used as an antibiotic for decades and it has been well characterized in a clinical setting (doxycycline, lymecycline and minocycline)

- Is nontoxic at doses required for gene activation in preclinical and clinical studies

- Is rapidly metabolized and cleared from the body, making it an ideal drug for the rapid increase in gene expression as well as rapid decrease in expression of the desired transgene



However,

because the protein was derived from bacteria, it may be immunogenic

Tetracycline-dependent regulatory system

- based on the *E.coli* Tn10-encoded **tetracycline resistant operon**

- **Tetracycline resistance operon** consists of two genes:

a) the resistance gene ***TetA*** – codes for a membrane protein that exports invaded tetracycline out of the bacterial cell

b) the regulator gene ***TetR*** *(repressor)* – codes for a dimeric DNA-binding protein

Tetracycline-dependent regulatory system

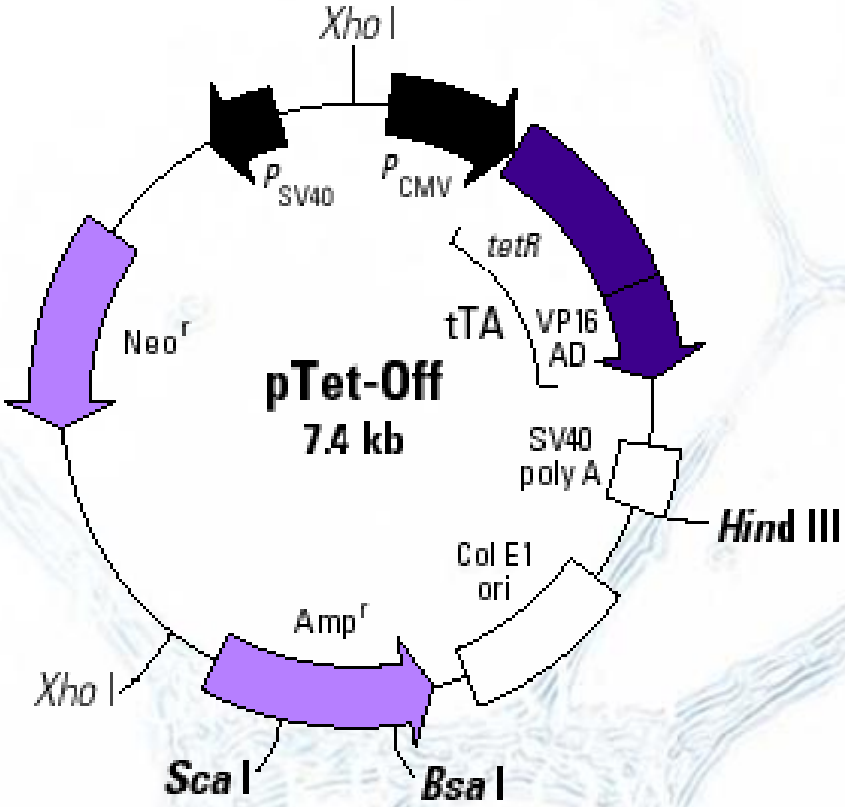
Bacteria:

In the absence of tetracycline, *TetR* protein inhibits its own expression as well as the expression of *TetA* by binding to operator sequence (*tetO*) of the *tet* operon. Tetracycline or other antibiotics (doxycycline) prevent this binding by binding to the TetR and inducing its allosteric change

Gene therapy:

TetR was converted to a transcriptional transactivator, called tTA, by fusing the VP16 transactivation domain of *Herpes simplex* virus to the C-terminus of TetR

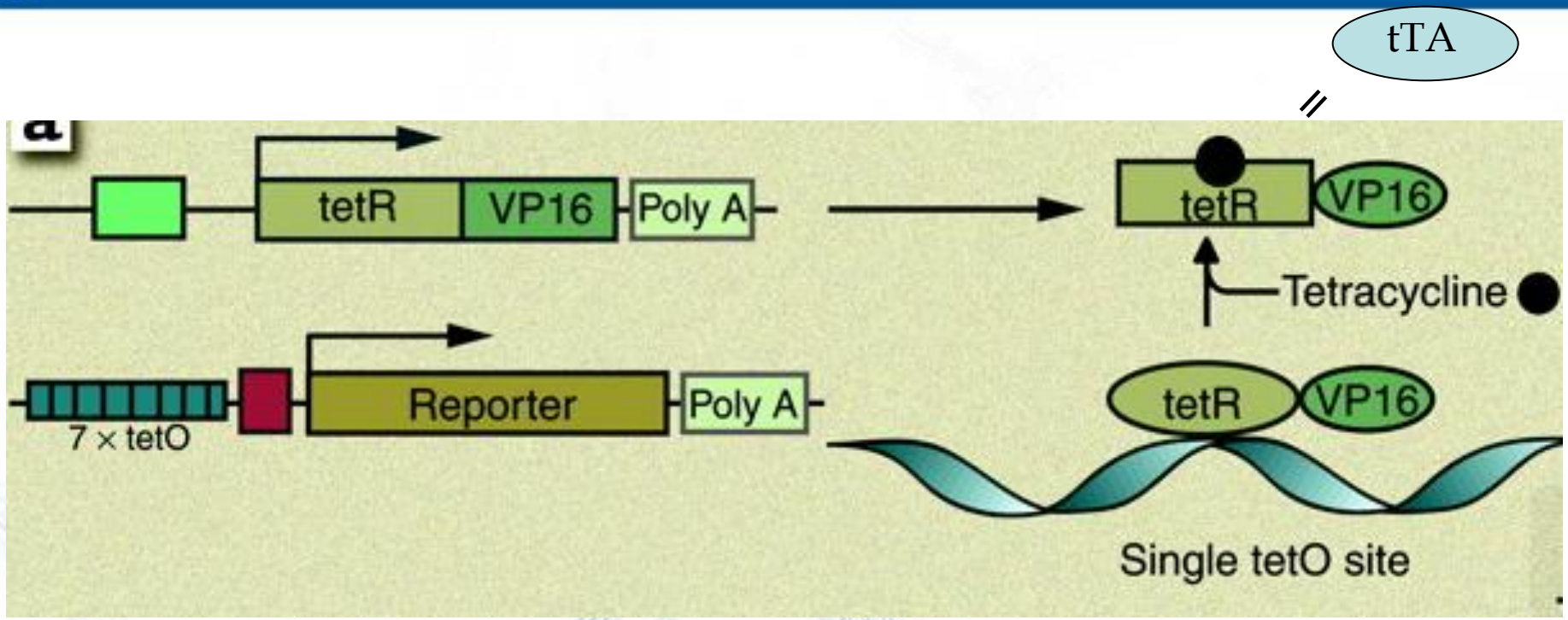
Tet-off system



- tet-responsive transcriptional activator (tTA) is expressed from the strong CMV promoter.

- tTA is a fusion of amino acids 1–207 of the tet repressor (TetR) and the negatively charged C-terminal activation domain (130 amino acids) of the VP16 protein of herpes simplex virus.

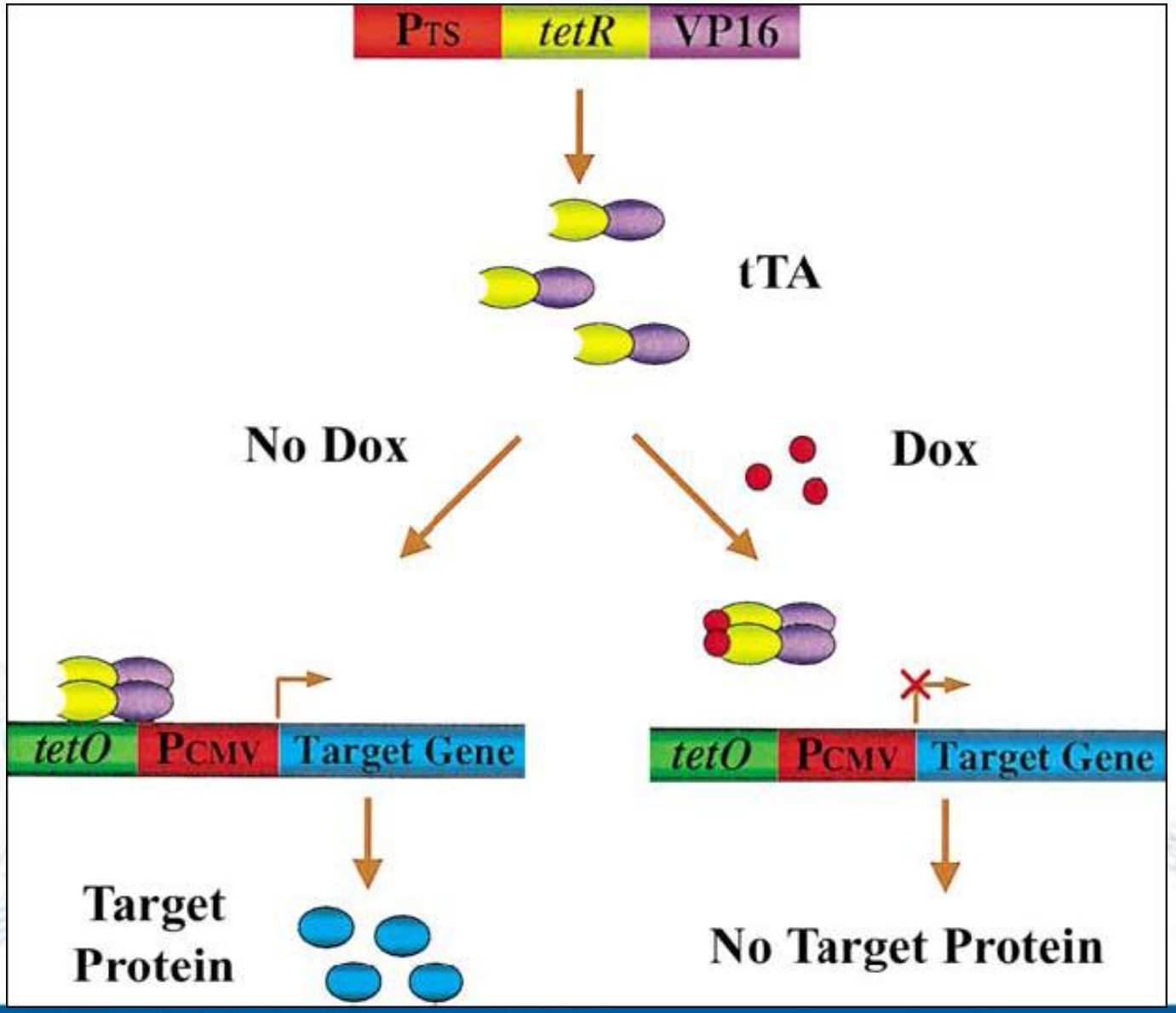
Tet-off system



In the absence of tetracycline, tTA binds with high affinity and specificity to a tetracycline-regulated promoter, a regulatory region comprising 7 repeats of tetO placed upstream of a minimal human CMV promoter.

tetR - tet represor – a prokaryotic dimeric DNA-binding protein that binds to specific operator sequences (tetO) of the tet-resistance operon
VP16 - virus transactivator

Tet-off system



PTS – tissue or cell type specific promoter

Tet-off system

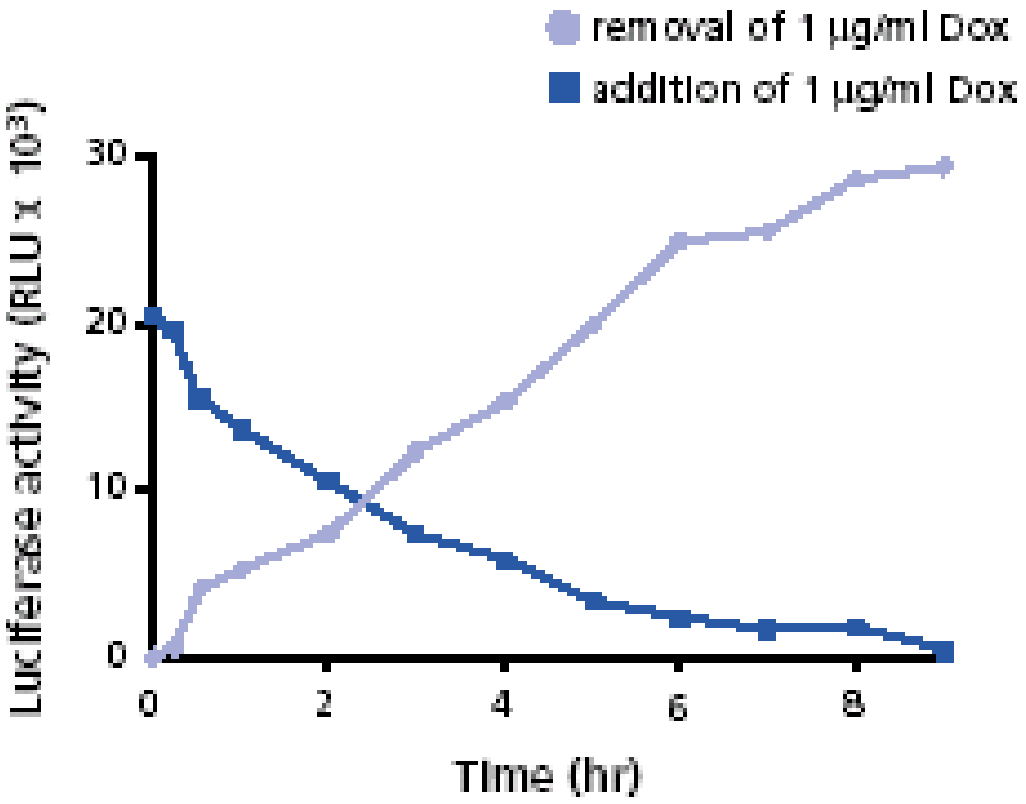
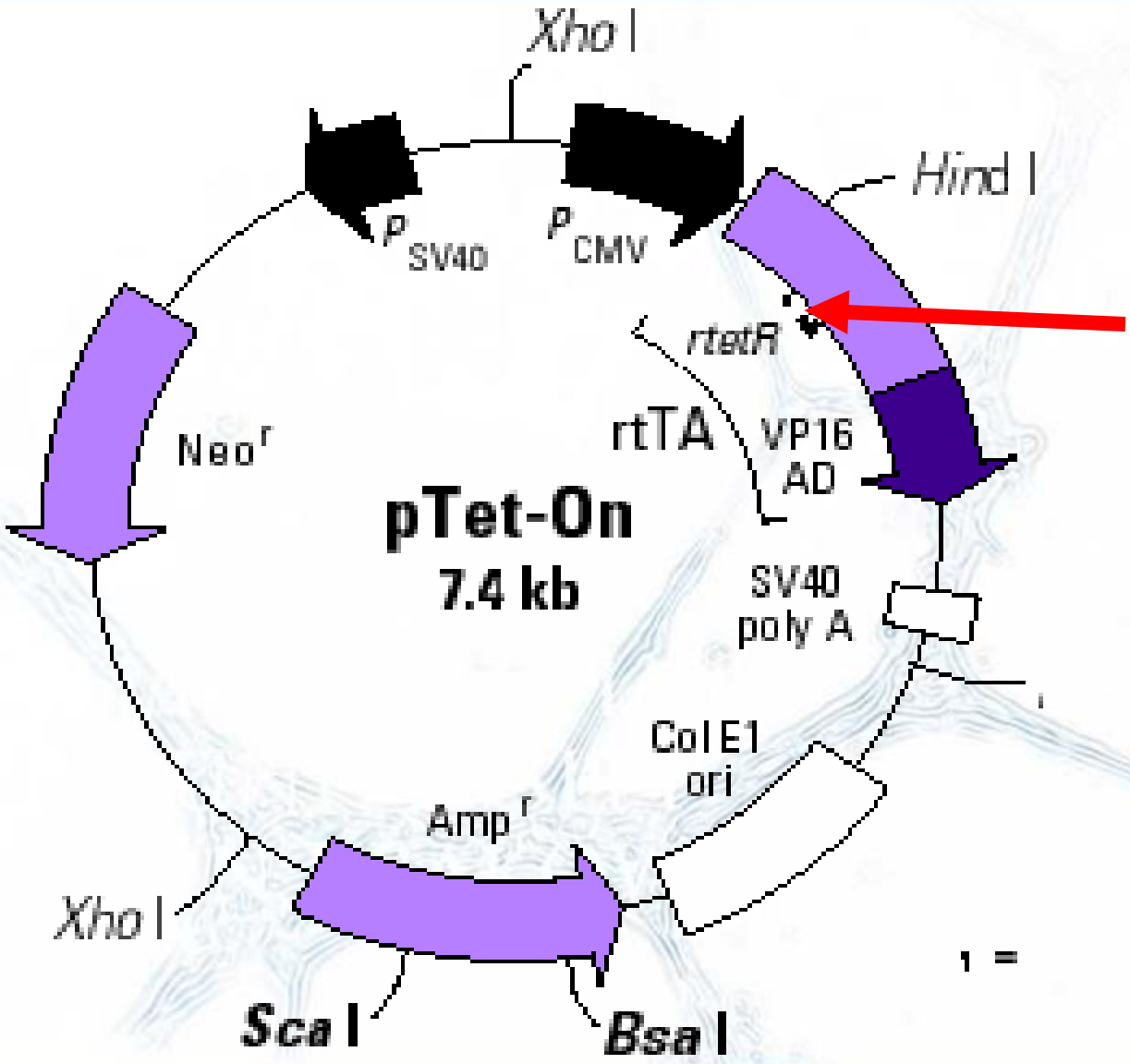


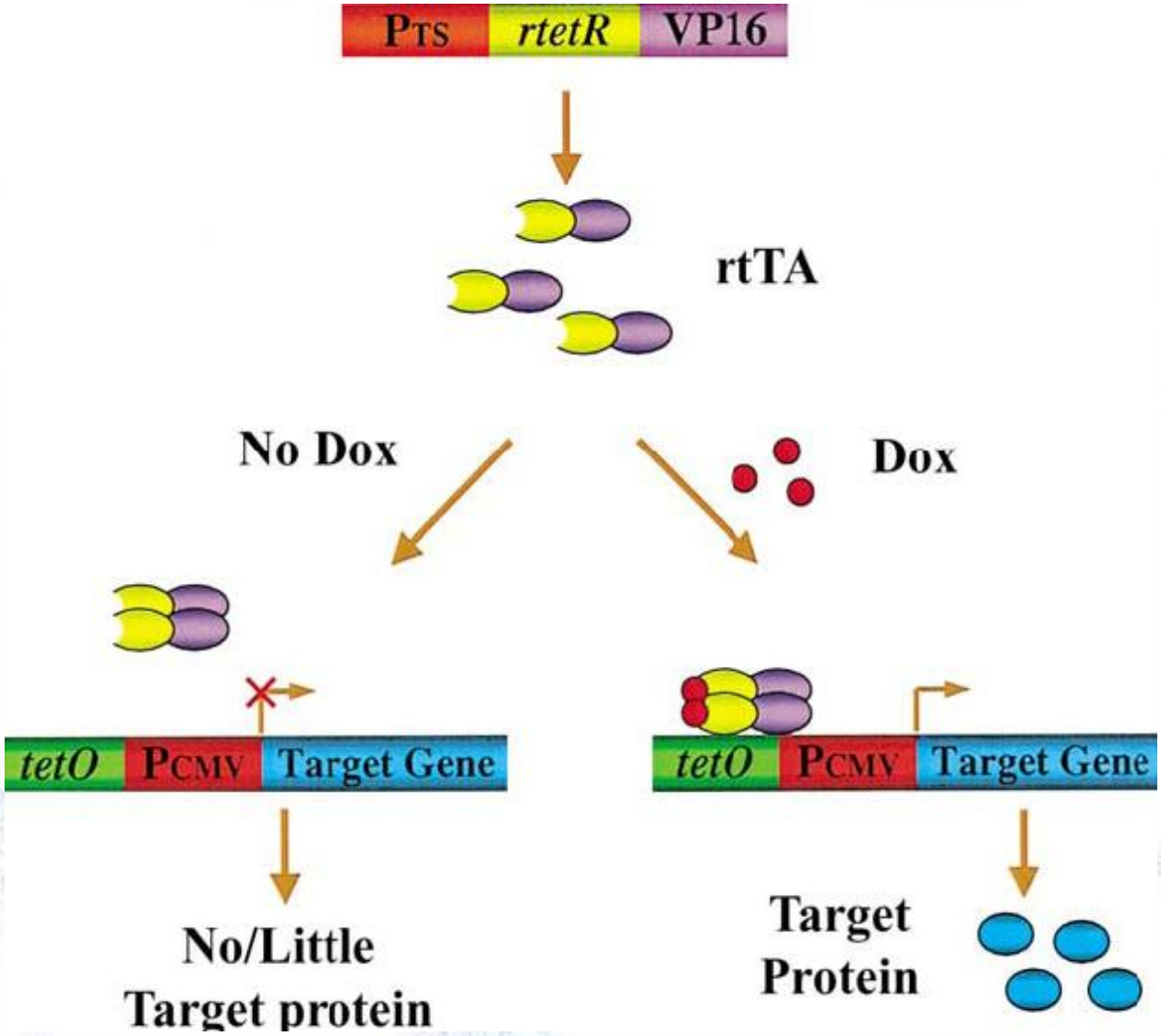
Figure 6. Change in luciferase activity is detectable within 1 hr of induction or suppression.

Tet-on plasmid



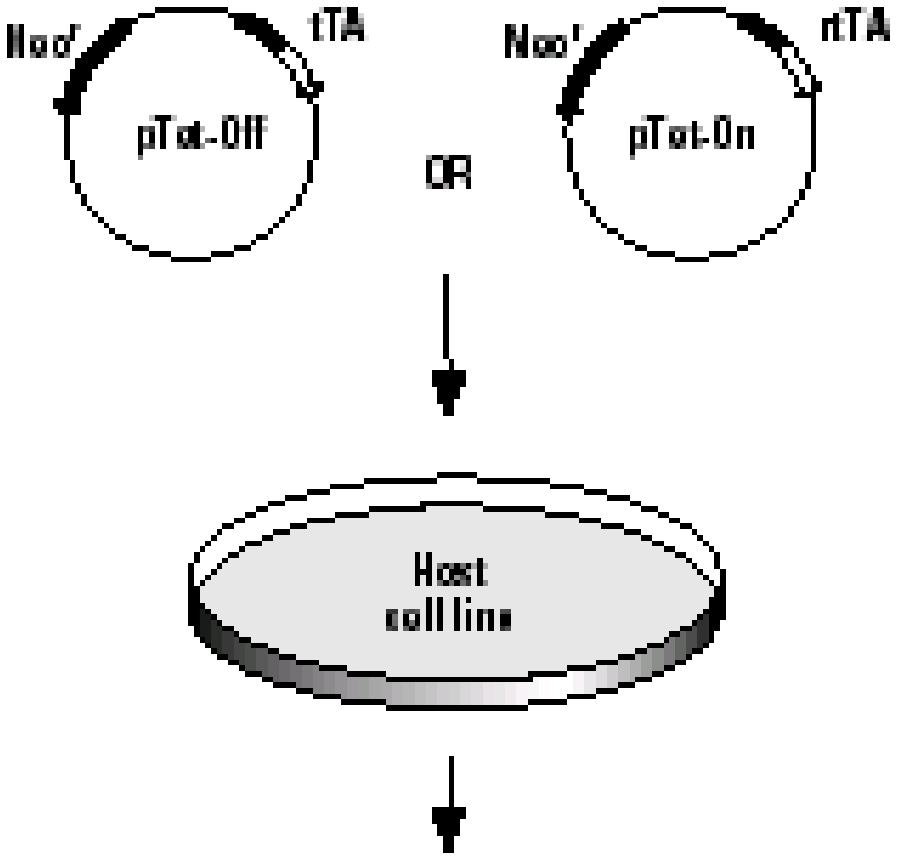
Mutations that convert TetR to rTetR (and tTA to rtTA)

Tet-on system



Tet-off/Tet-on strategy in vitro (1)

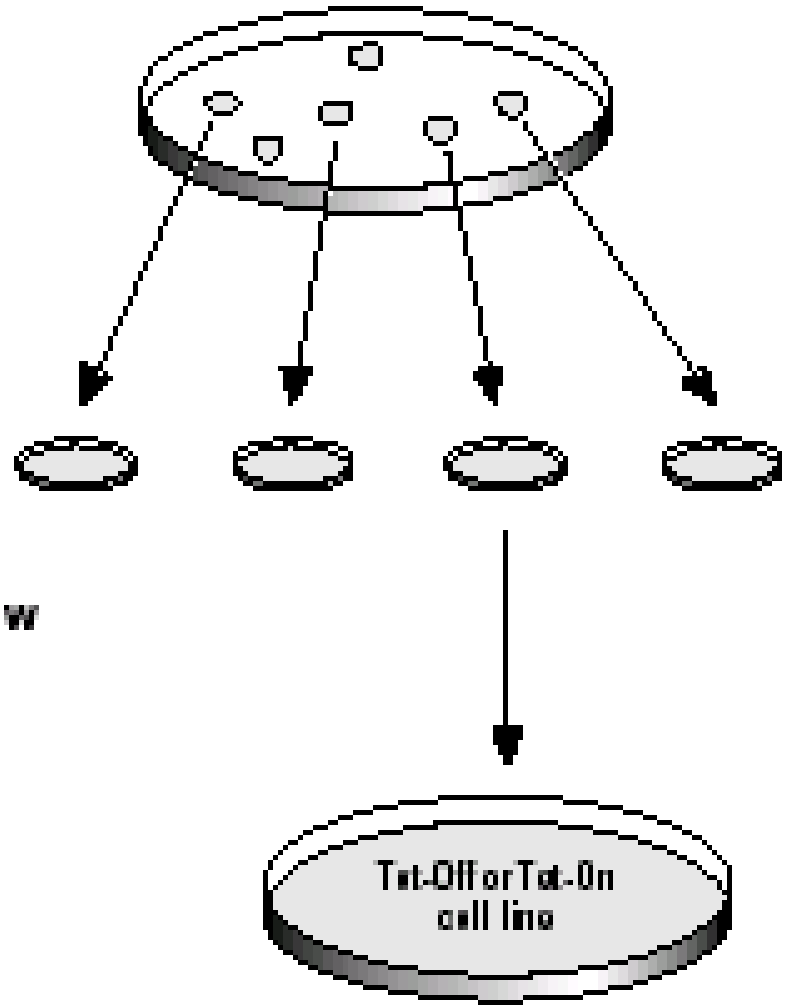
- Transfect host cell line with regulator plasmid (pTet-Off or pTet-On)



- Select in presence of G418

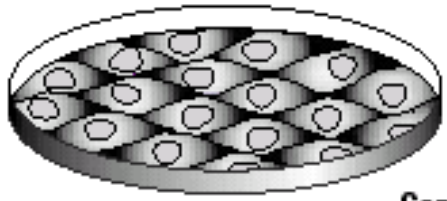
Tet-off/Tet-on strategy in vitro (2)

- Isolate at least 30 G418-resistant clones
- Screen by transient transfections with pTRE2hyg-Luc for clones with low background and high induction of luciferase in response to Tc or Dox
- Freeze stocks of Tet cell line



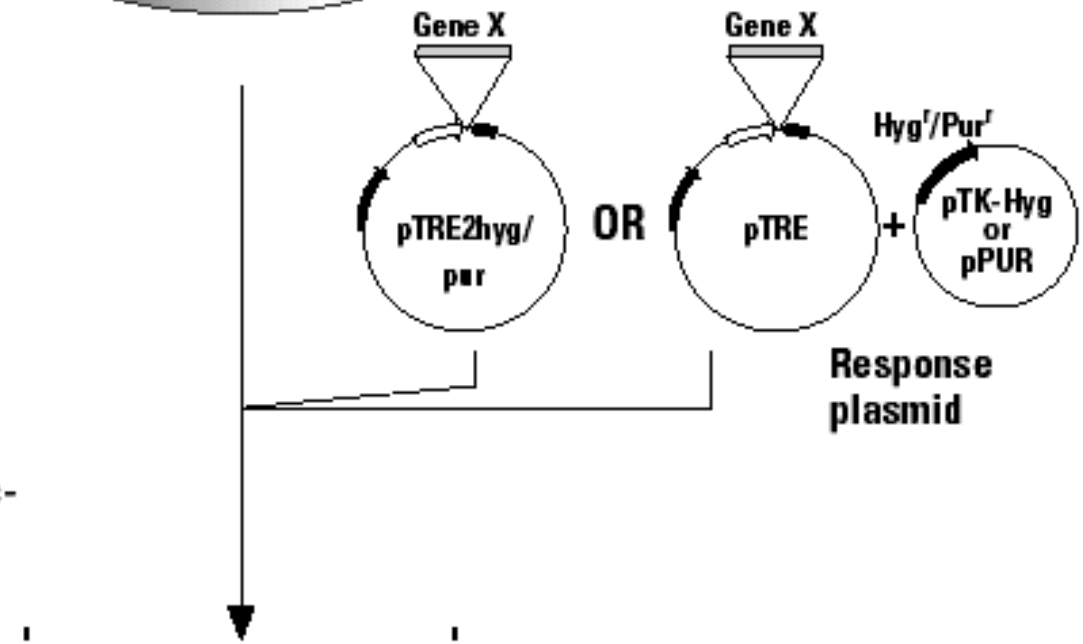
Tet-off/Tet-on strategy in vitro (3)

BD Tet-Off™ or Tet-On™ cell line (Premade cell lines are available from BD Biosciences Clontech)

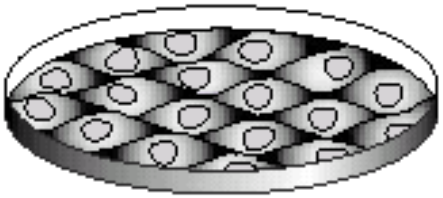


SECOND STABLE TRANSFECTION
(Section IX; ~ 2 months)

- Transfect with response plasmid; cotransfect with pTK-Hyg or pPUR, if necessary
- Select hyg- or puro-resistant clones
- Screen by a gene-specific assay for clones with low background and high Tc- or Dox-dependent induction of Gene X



Double-stable BD Tet-Off™ or Tet-On™ cell line



Modification of Tet-dependent systems

- TetR or rTetR cDNA is regulated by CMV promoter

- TetR or rTetR cDNA is regulated by cell-specific promoter

Table 1

New tissue-specific tTA (or rtTA) transgenic mice.

Tissue	Promoter	References
Brain	Neuron-specific enolase	[22]
	Calmodulin-dependent protein kinase II	[23]
	Glial fibrillary acidic protein	[24]
Lung	Clara cell 10 kDa	[15]
Class II positive cells	MHC class II	[25]
Mammary gland	Mouse mammary tumor virus	[26]
Pancreas	Proinsulin gene II	[27]
Epithelium	Cytokeratin	[28]
Skin	Keratin 14	[29]
Liver	LAP (C/EBPβ)	[30]

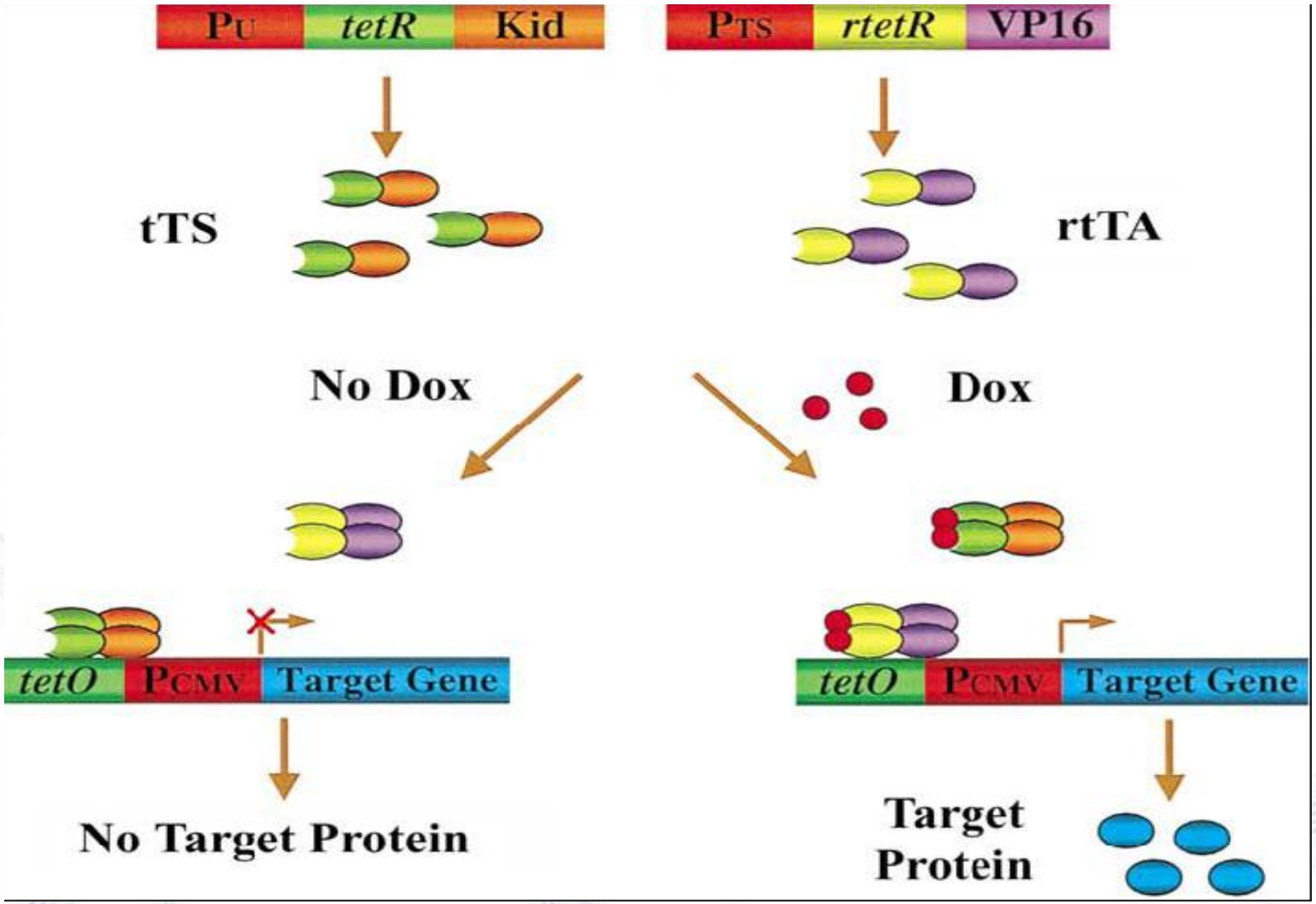
MHC, major histocompatibility complex; LAP (C/EBPβ), liver enriched activating protein (CCAAT/enhancer binding protein β).

New versions of tet-dependent gene expression

tTA (a fusion between TetR and VP-16)	Tet-off
rTA	Tet-on
tTS	Tet-on + tTS

tTS consists of TetR fused to the KRAB repressor domain
KRAB – Kruppel-Associated Box – transrepressing domain of human Kid-1 protein

Combined use of tetracycline-controlled transcriptional silencer (tTS) and rtTA system



Features of tet-induced expression system

1. **Tet-off** – widely used in animal models, but because of its unfavorable kinetics properties, its unlikely to be used in clinical setting

2. **Tet-on systems:**
   older versions – a significant basal activity; fully active only at high Dox doses
   novel versions: display a considerably lower basal activity in the OFF state

   However, tightness of the control may be partially lost at higher vector doses

3. **rtTA/tTS system** – significantly reduced the basal expression in vivo, provided that vector architecture was optimized

Features of tet-induced expression system

4. Tetracycline is **non-immunogenic** in mice, but i.m. injected into skeletal muscles of non-human primates elicited a cellular and humoral immune response

5. Dox, an analogue of Tet, is a well-documented antibiotic drug (used in the clinics for more than 30 years)

6. Dox is usually well-tolerated; can be taken orally. It can be also applied intravenously. The bioavailability of Dox is similar, not dependent on the way of administration

7. The tissue penetration is excellent and includes the brain. Concentrations are the highest in liver, kidney and digestive tract, as it is eliminated primarily via urine

Features of tet-induced expression system

8. Potential problems with Dox:

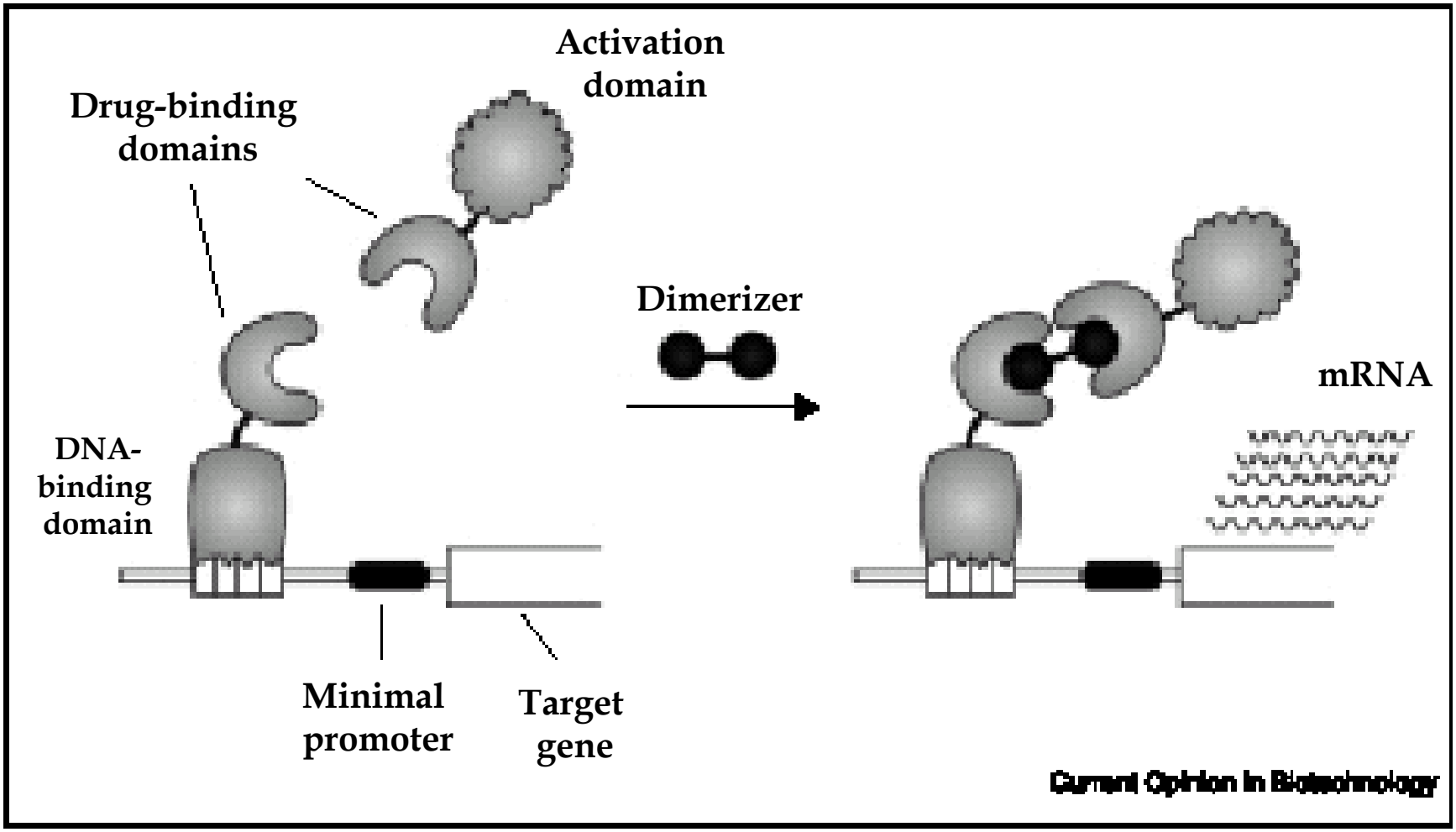
a) accumulates in bones; its slow release from bones may slow-down the silencing of a Tet-On system,

b) risk of raising resistance to antibiotics

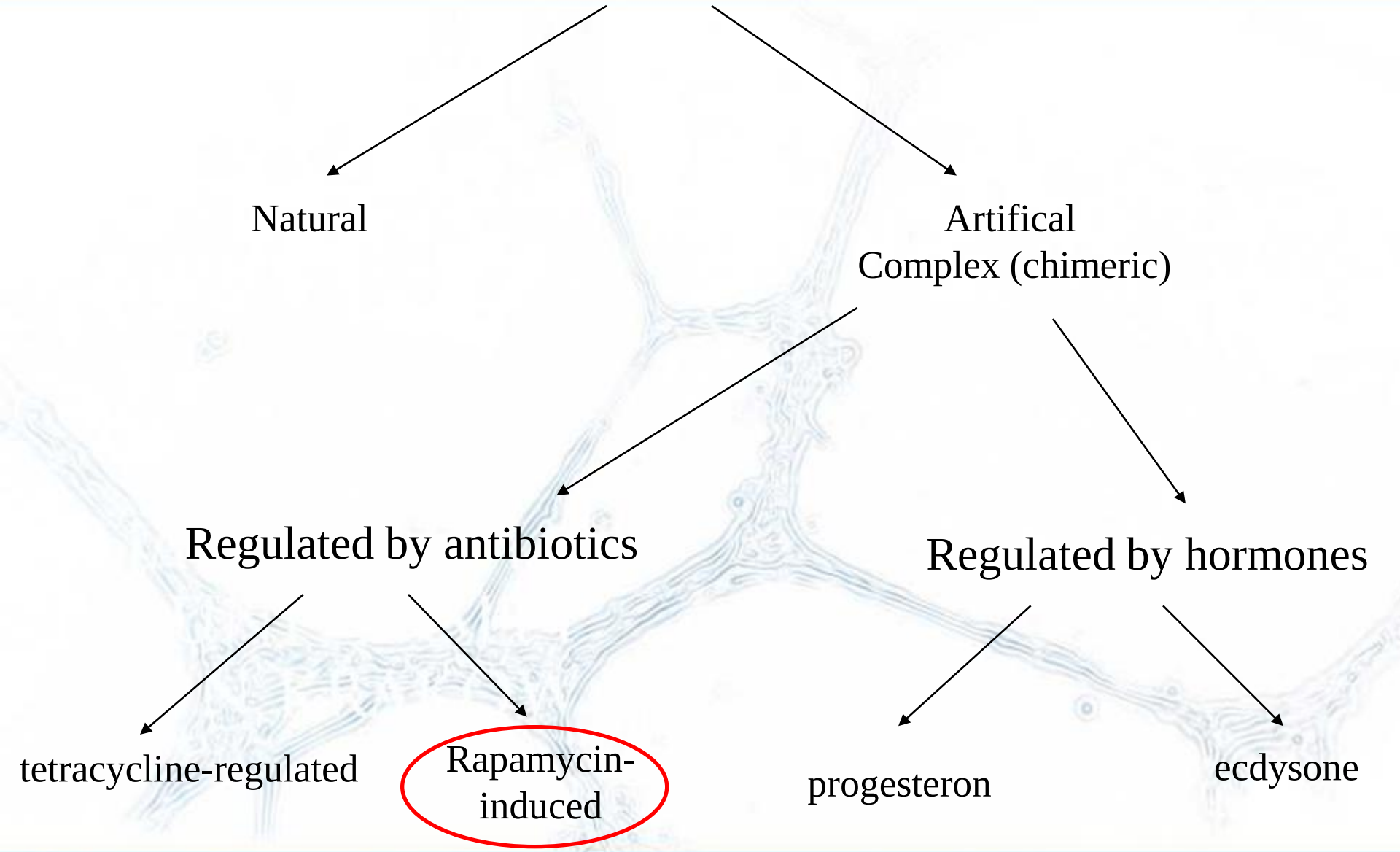
c) can stain developing teeth (even when taken by the mother during pregnancy)

How to decrease the leakiness of the promoters?

Dimerizer-induced gene expression



Inducible promoters

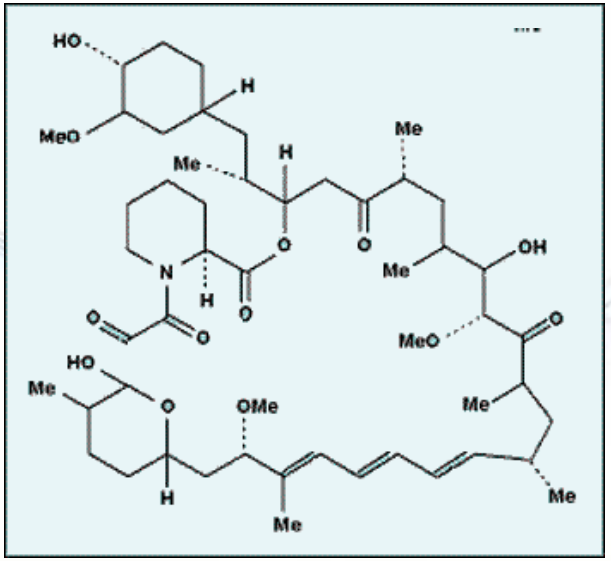


Rapamycin

Rapamune® (sirolimus) is an immunosuppressive agent

Sirolimus is a macrocyclic lactone produced by *Streptomyces hygroscopicus*. The chemical name of sirolimus (also known as rapamycin) is (3*S*,6*R*,7*E*,9*R*,10*R*,12*R*,14*S*,15*E*,17*E*,19*E*,21*S*,23*S*,26*R*,27*R*,34a*S*)-9,10,12,13,14,21,22,23,24,25,26,27,32,33,34,34a-hexadecahydro-9,27-dihydroxy-3-[(1*R*)-2-[(1*S*,3*R*,4*R*)-4-hydroxy-3-methoxycyclohexyl]-1-methylethyl]-10,21-dimethoxy-6,8,12,14,20,26-hexamethyl-23,27-epoxy-3*H*-pyrido[2,1-*c*][1,4] oxaazacyclohentriacontine-1,5,11,28,29 (4*H*,6*H*,31*H*)-pentone. Its molecular formula is **C51H79NO13 and its molecular weight is 914.2.**

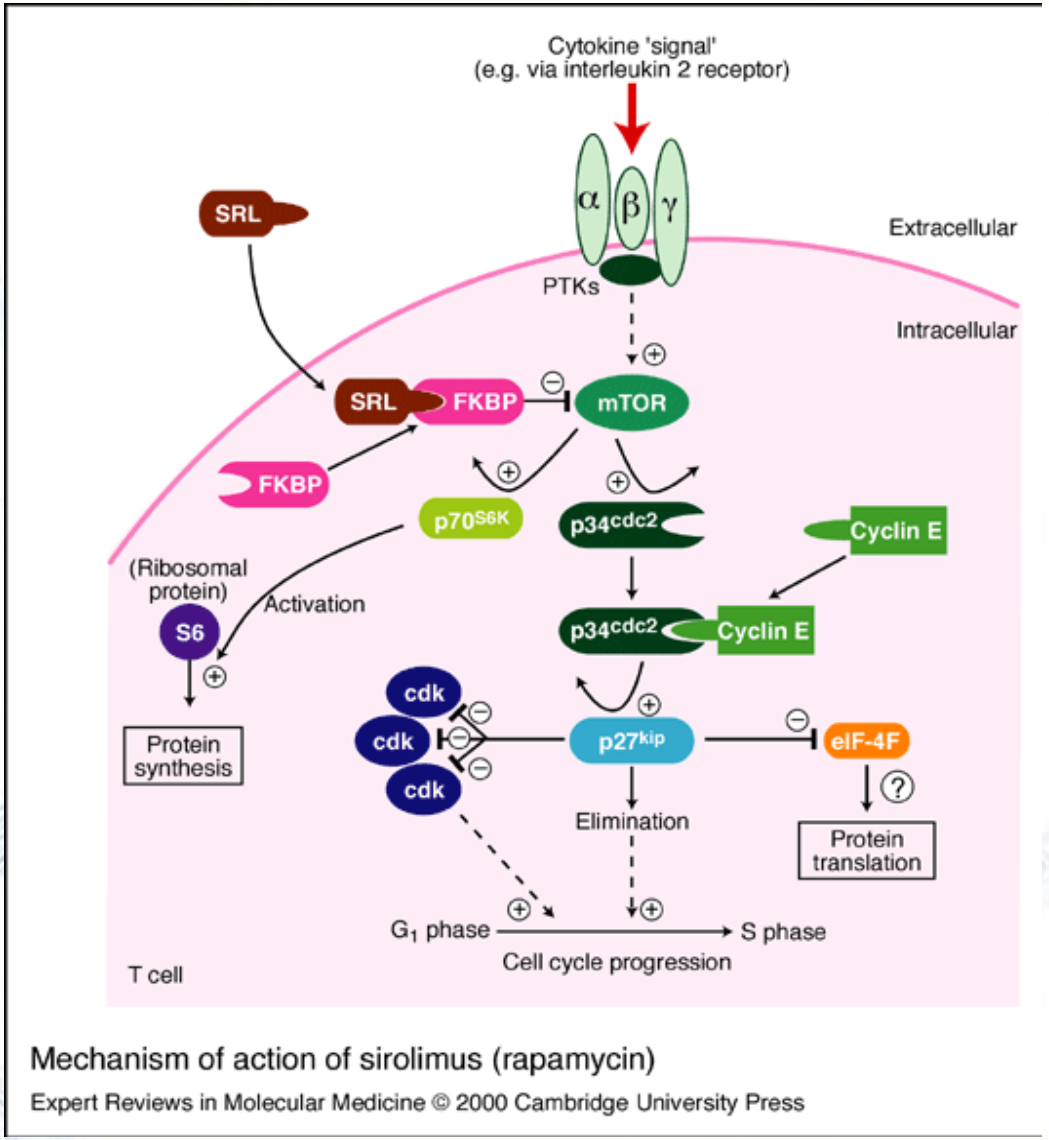
Rapamycin = sirolimus (SRL)



Rapamycin-induced gene expression

Sirolimus (SRL) binds to FK506-binding protein (FKBP) and then such complex binds to the mammalian target of rapamycin (mTOR)

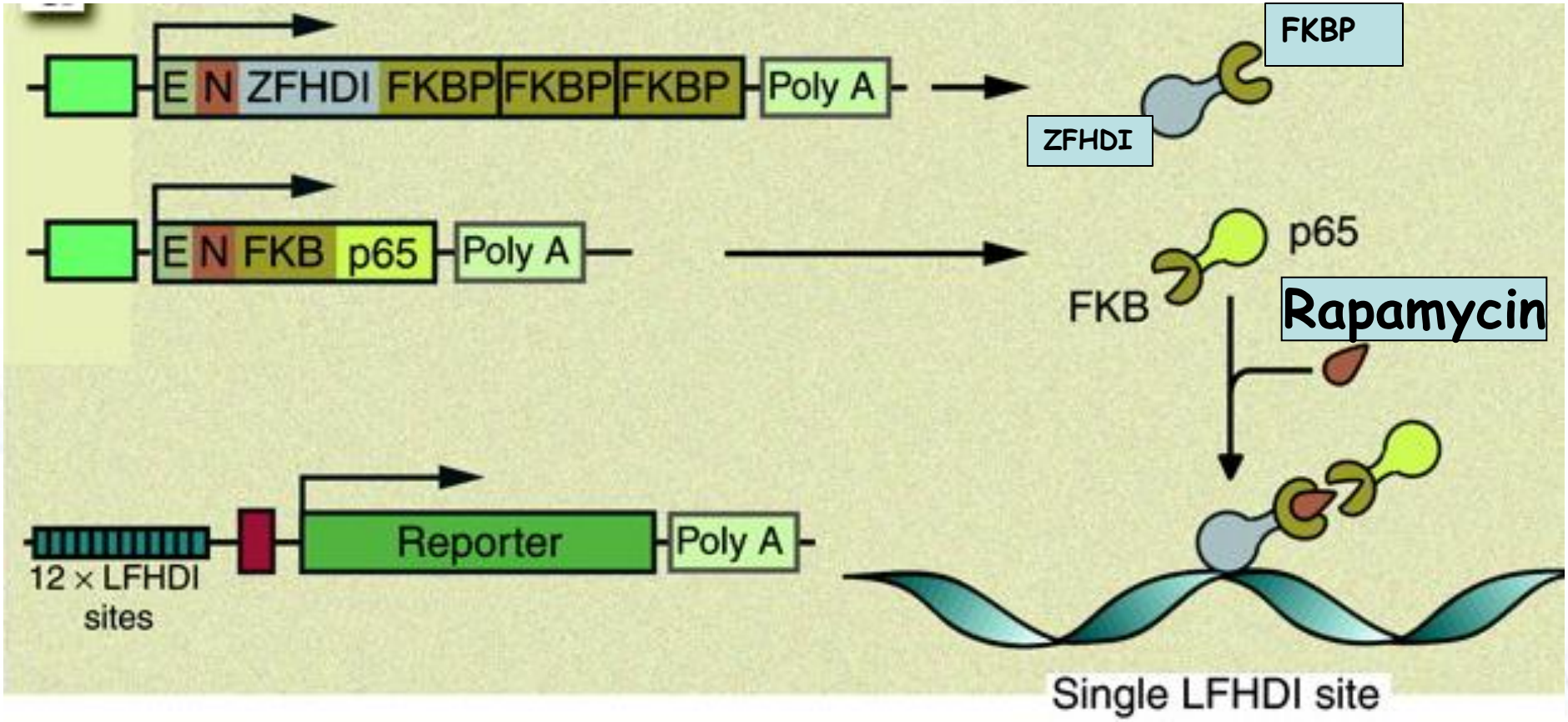
The SRL–FKBP–mTOR complex inhibits biochemical pathways that are required for cell progression and it blocks cytokine signal transduction



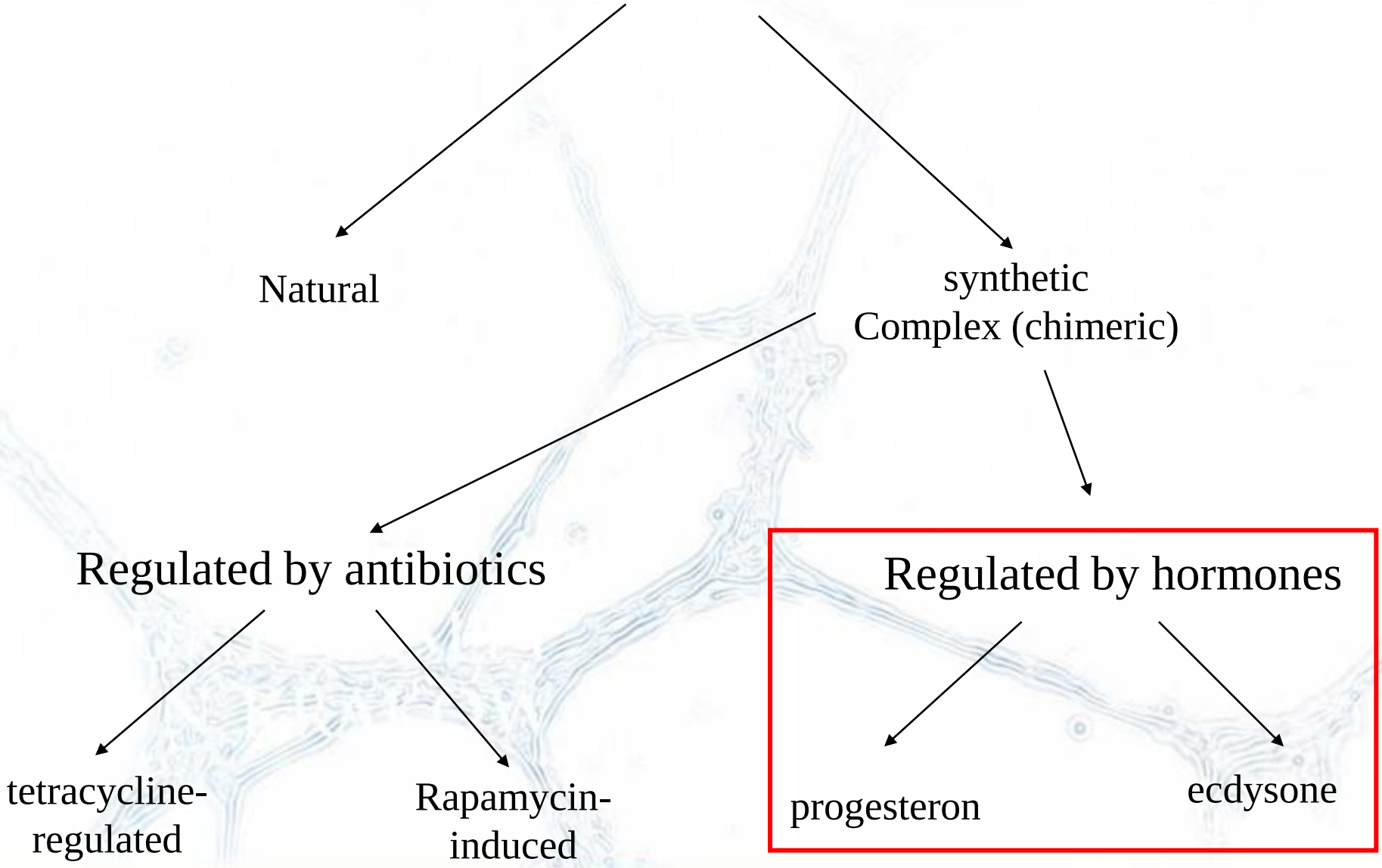
Mechanism of action of sirolimus (rapamycin)

Expert Reviews in Molecular Medicine © 2000 Cambridge University Press

Rapamycin regulatable system



Inducible promoters



steroid hormone receptor regulatory systems

- Steroid hormone receptors are the largest group of transcription factors in the mammalian proteome

-The endogenous ligands of steroid receptors cross epithelial barriers and plasma membranes easily

- Ligands bind to their receptors in the cytoplasm and these ligand–receptor complexes can then be translocated to the nucleus where they regulate gene expression

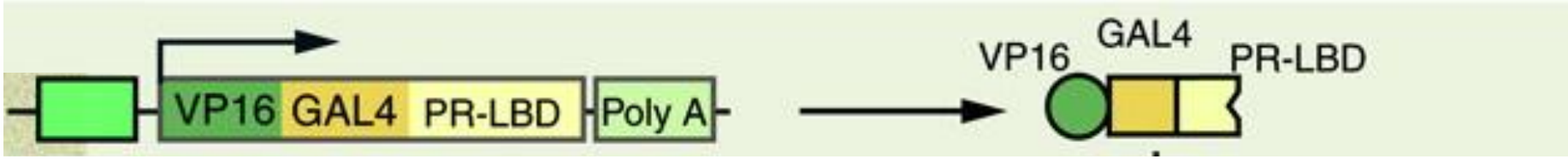
However

- Inducers or repressors of target genes may also modulate endogenous gene expression in cells.

- Additionally, physiological changes in natural ligand expression may affect expression of the target gene

progesterone receptor regulatory system

contains a gene that encodes the progesterone receptor with a C-terminal truncation, which prevents binding with progesterone, yet the truncated receptor retains the ability to bind with the antagonist mifepristone (RU 486)

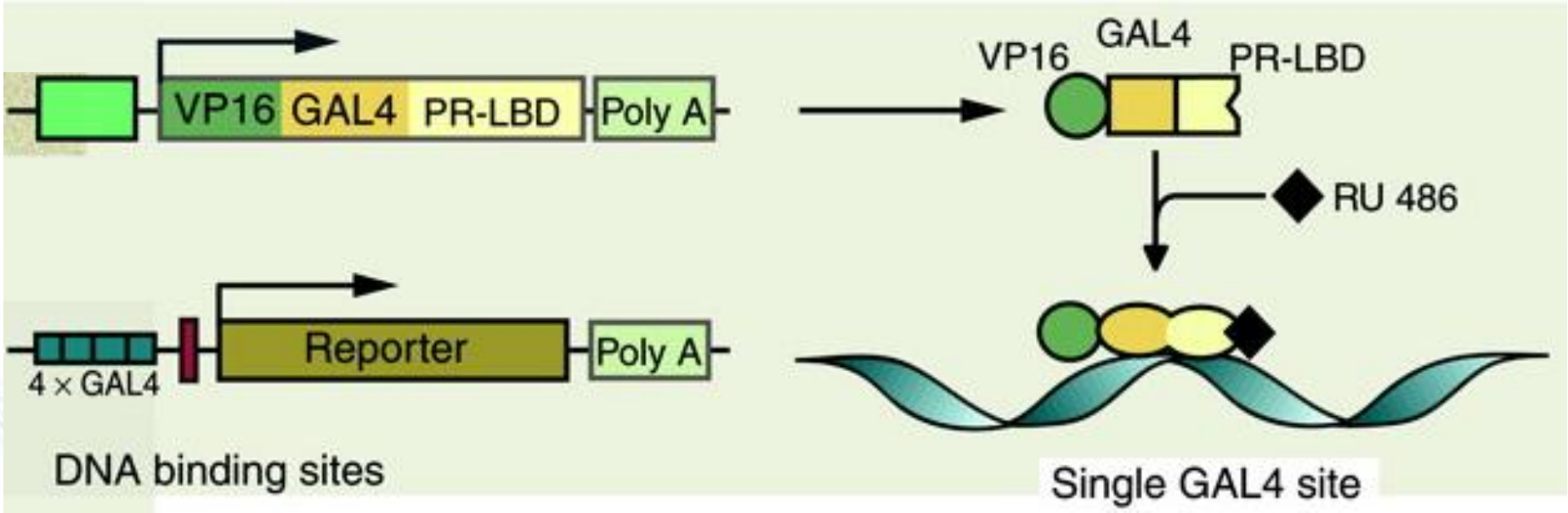


To improve the specificity of this regulatory system, the truncated progesterone receptor was fused with the Gal4 DNA binding domain and VP16 activation domain, an eukaryotic transactivator derived from HSV-1.

This chimeric protein induces transcription more than 10-fold *in vivo !!!*

(at a dose of mifepristone well below the threshold required to induce abortions in women)

Progesterone-regulatable system



PR-LBD - human progesteron receptor

RU 486 activates the transactivator by promoting the binding of the GAL4-DNA-binding domain to its consensus elements

Non-mammalian steroid hormone receptor

- Ecdysone receptor (EcR) is a steroid hormone receptor involved in triggering metamorphosis in *Drosophila melanogaster*

- Ecdysteroids have short half-lives - potent gene induction

- Ecdysteroids are relatively nontoxic and nonteratogenic in mammals and do not appear to affect mammalian physiology

However,

- Expression of insect proteins *in vivo* may induce an immune response eliminating transgene expression.

- The lipid solubility of steroid hormones results in slower metabolism and clearance from the body than highly hydrophilic drugs.

Ecdysone regulatable system

In Drosophila melanogaster:

- Ecdysone is an inducer of metamorphosis

- The system is composed of ecdysone, the ecdysone receptor (EcR) and the Ultraspiracle protein (USP)

In mammalian cells:

- After cotransfection with EcR and USP, when cells are exposed to ecdysone or its analogs like, ponasterone A or muristerone A, transcription of an ecdysone-responsive reporter gene is induced

- Upon addition of the hormone 1000-fold of induction has been reported

Ecdysone regulatable system

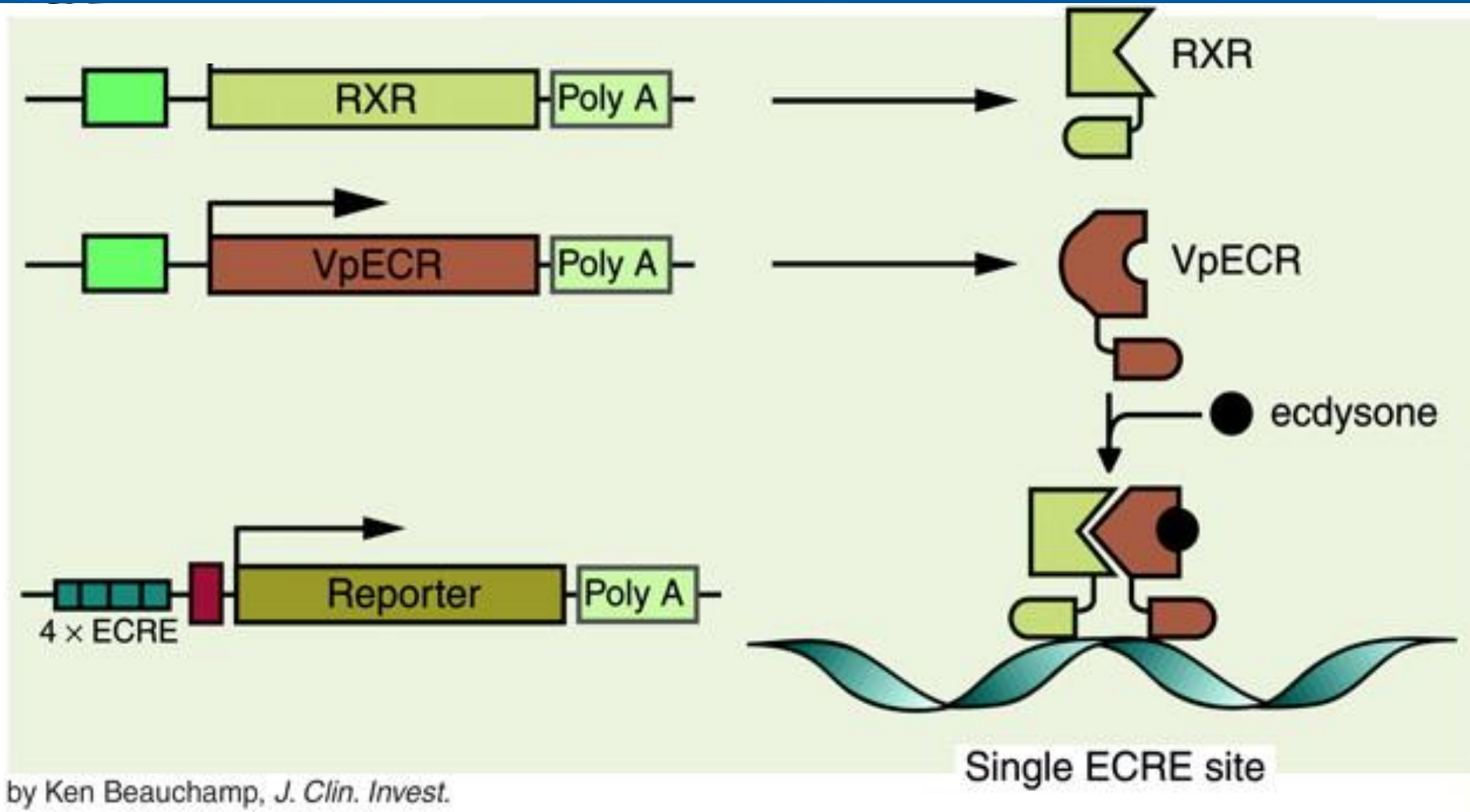
In mammalian cells the sensitivity of the system is improved by:

(1) USP has been replaced with its mammalian homolog the retinoid X receptor

(2) EcR has been truncated at the amino terminus end and fused to the VP16 activation domain

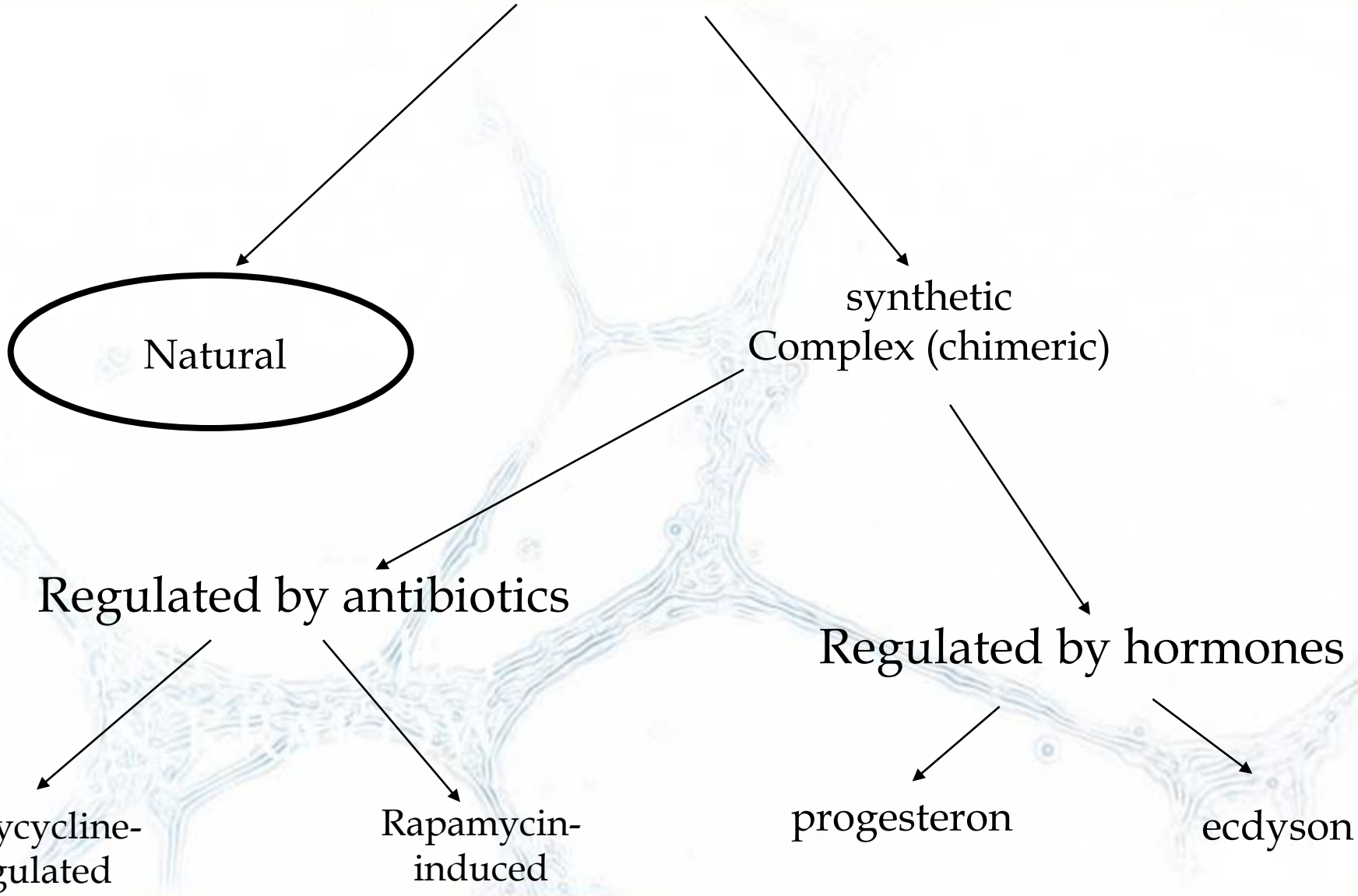
(3) Mutations in the DNA-binding domain to improve response

Ecdysone regulatable system



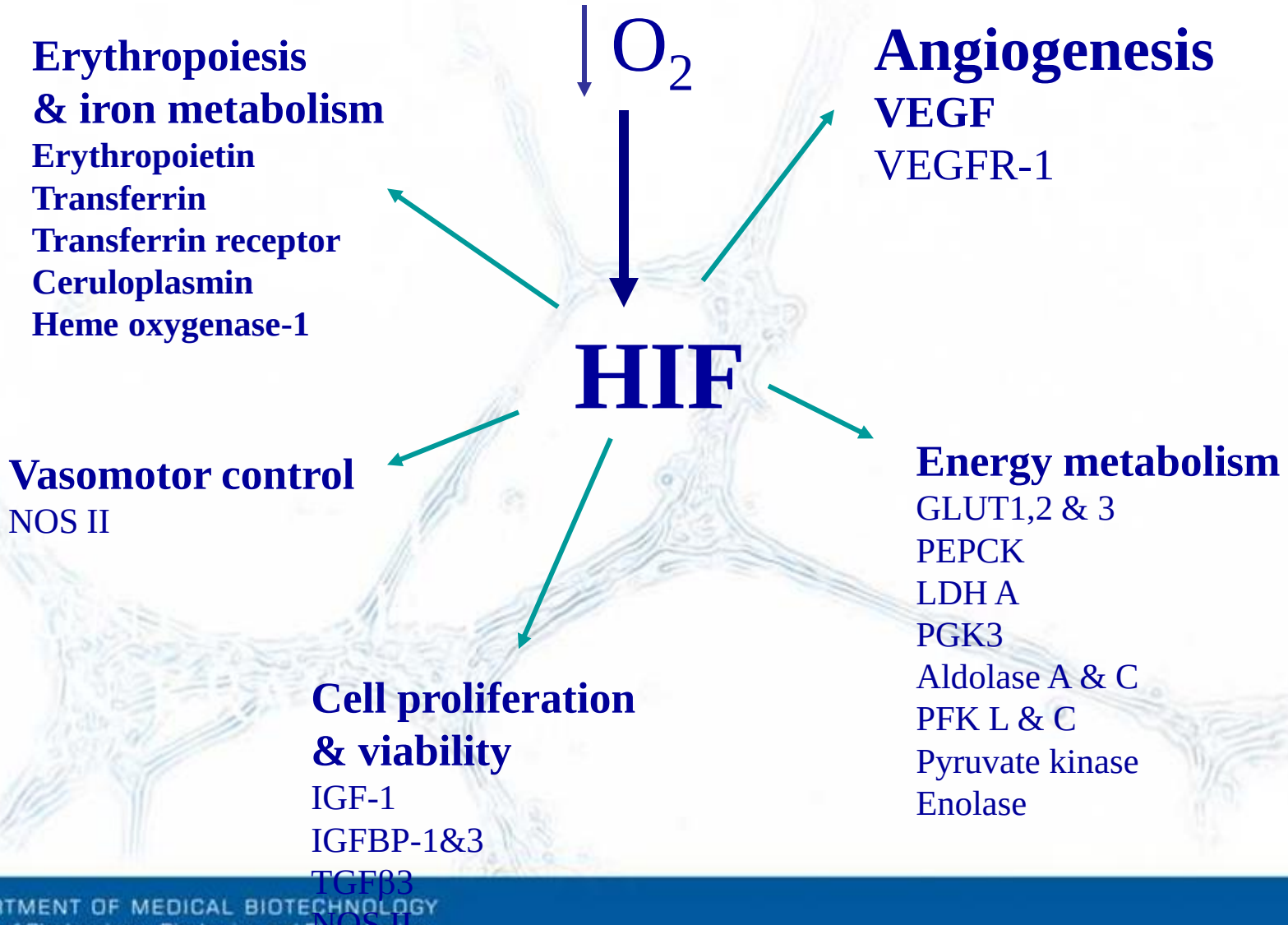
VpECR - modified ecdysone receptor
RXR - retinoid X receptor

Inducible promoters

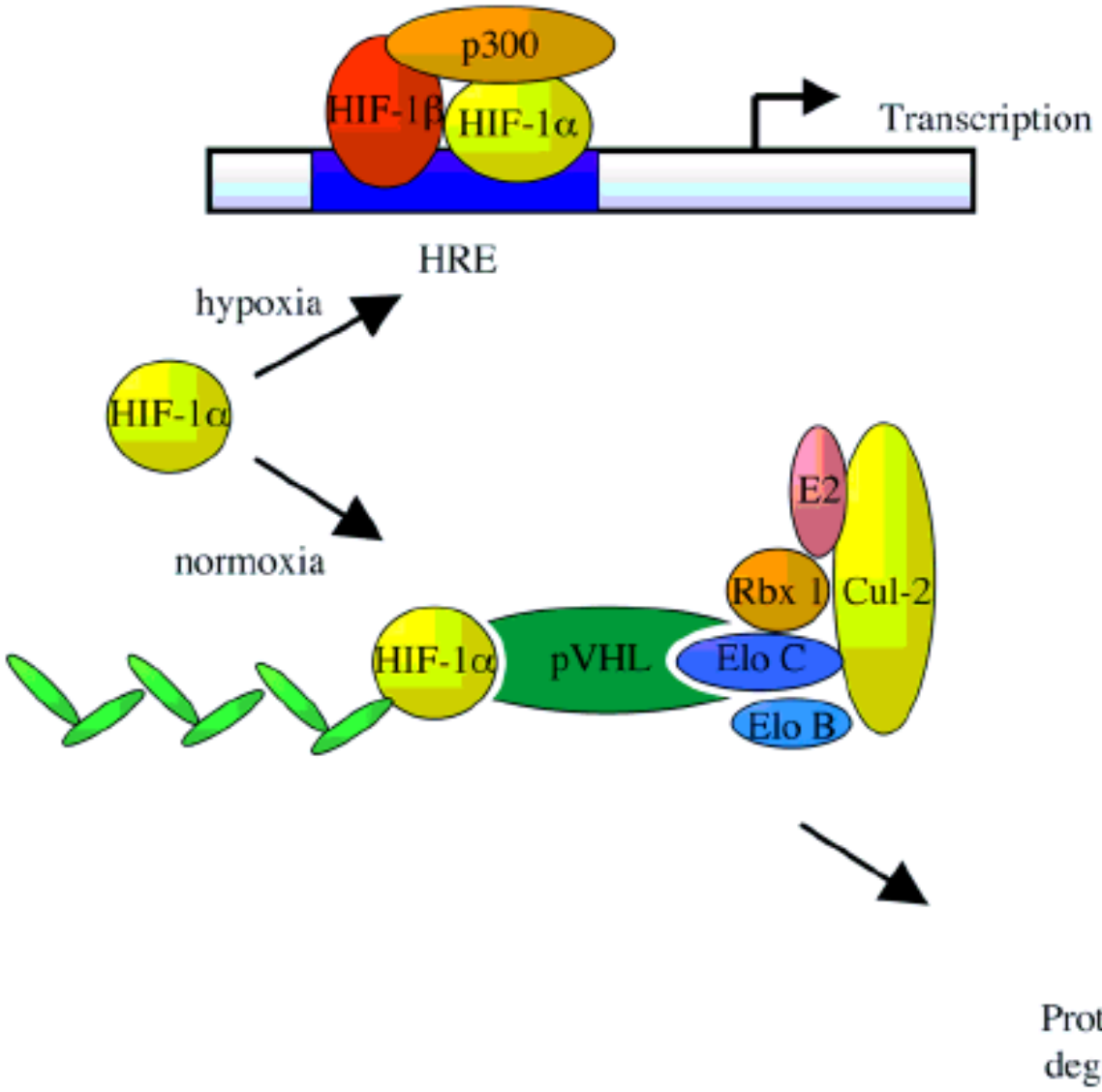


Cell-type specific gene regulation/ Regulation of gene under special conditions

Hypoxia inducible factor – a crucial mediator of hypoxia-induced gene expression

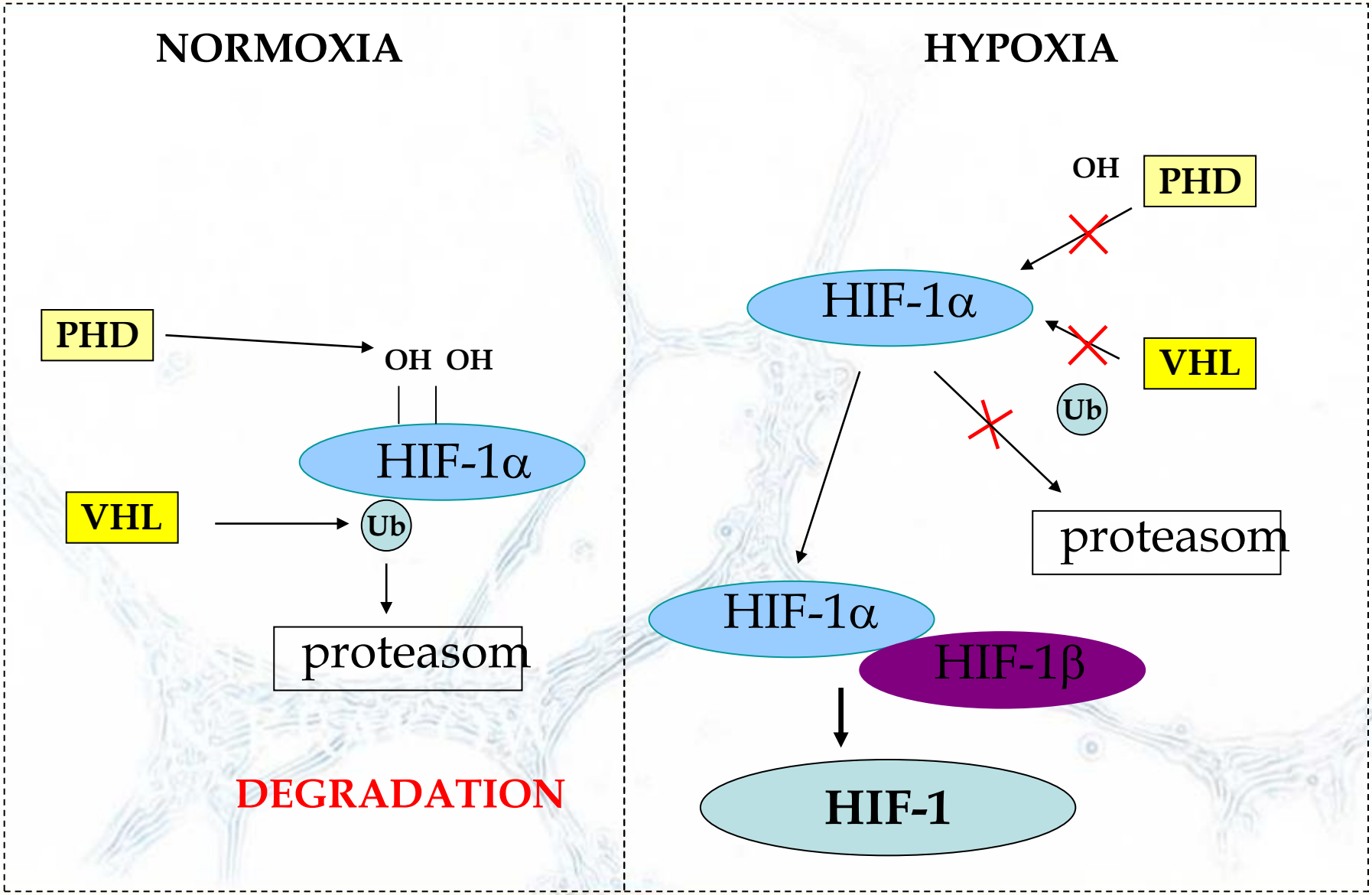


Activation and degradation of HIF-1α

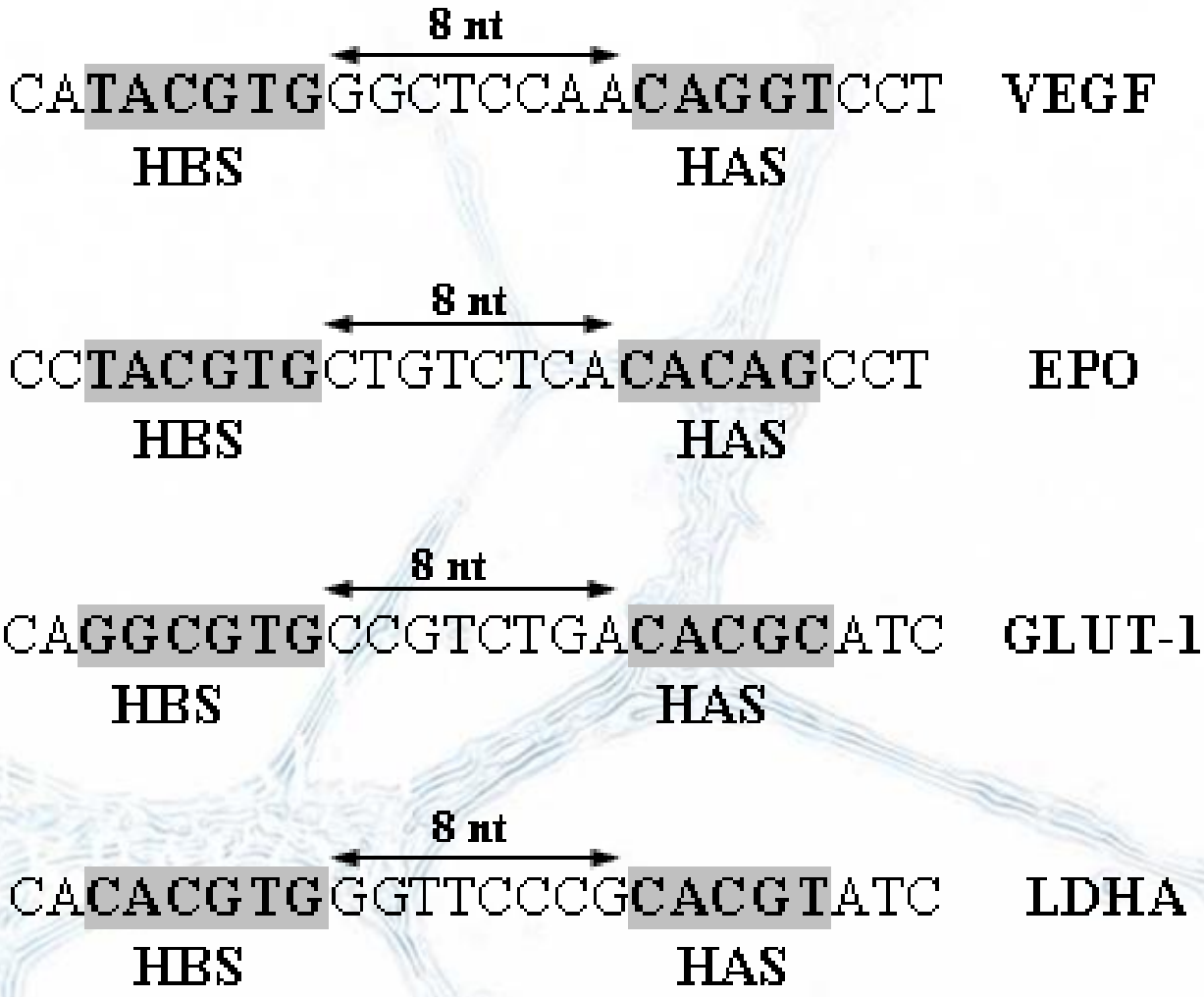


Mole et al., IUBM, 2001

Mechanism of gene regulation by HIF

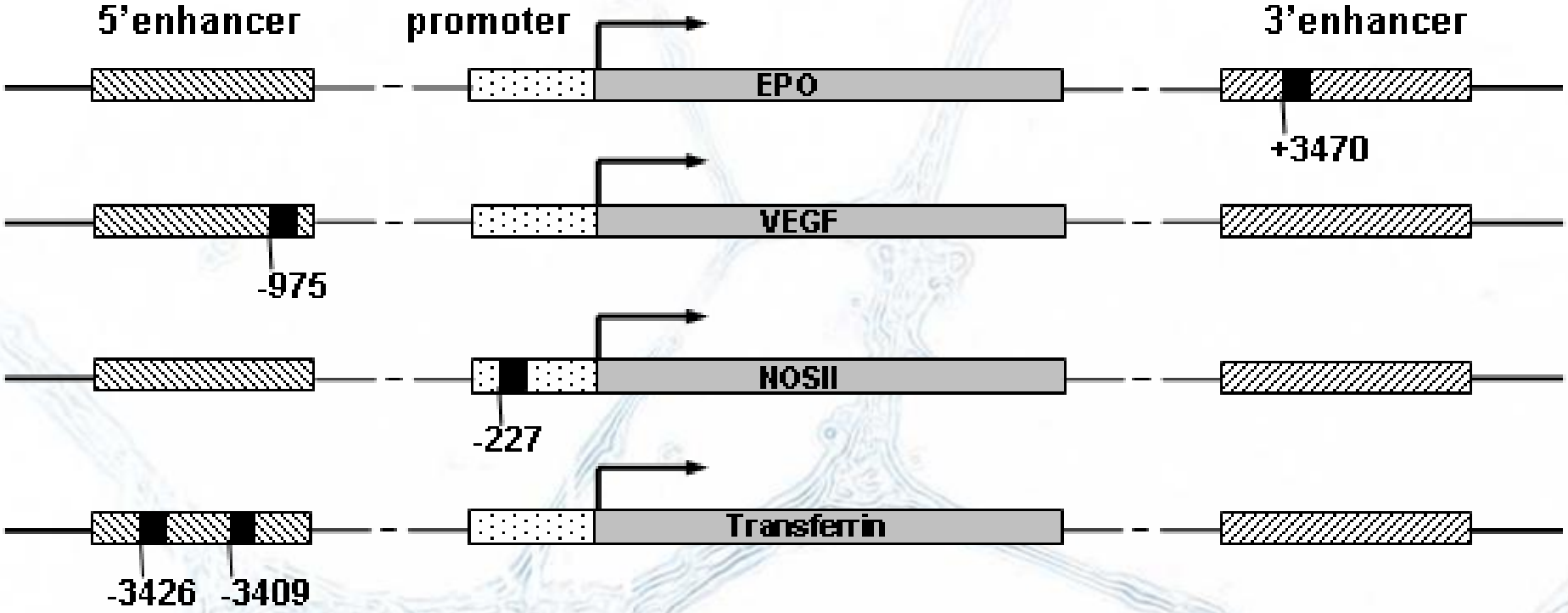


Various HRE

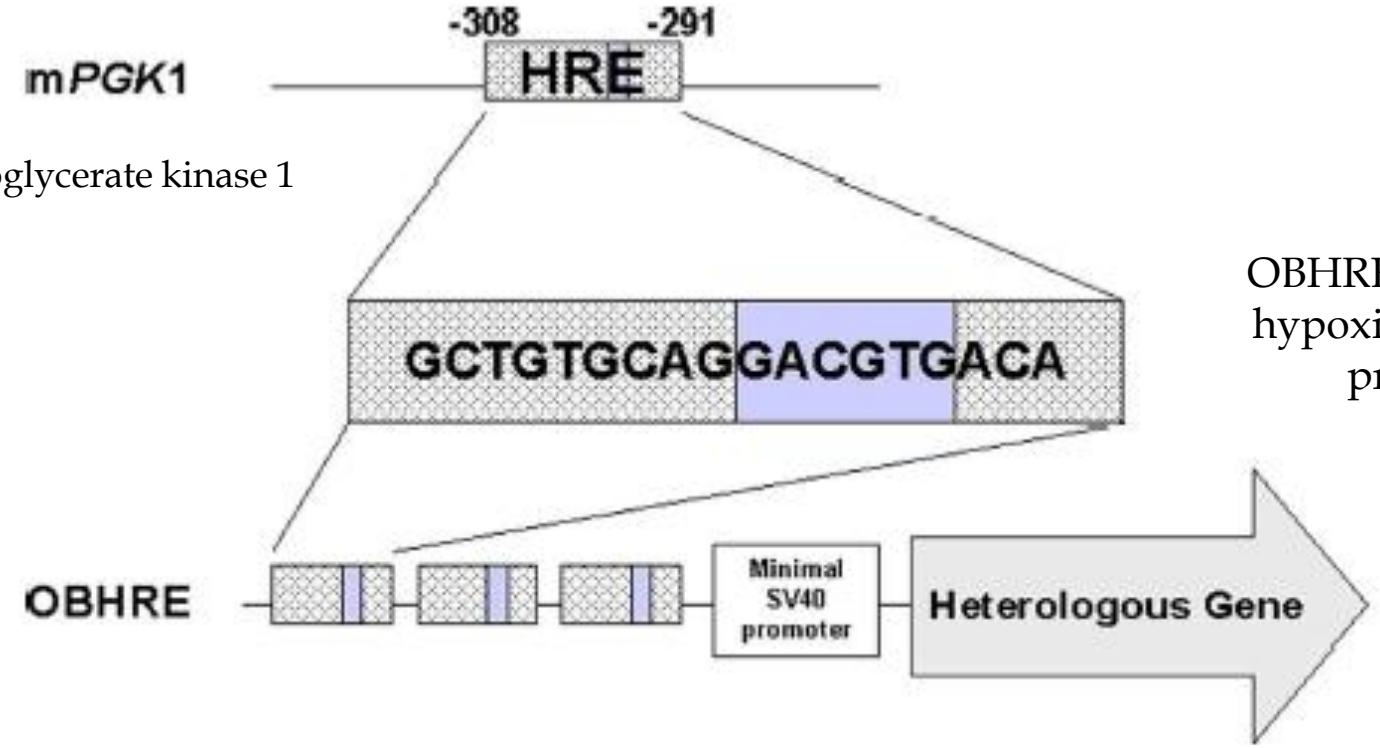


Zagórska & Dulak Acta Biochim Pol. 2004;51(3):563-85

HIF-1 binds to hypoxia responsive element present in regulating regions of many genes



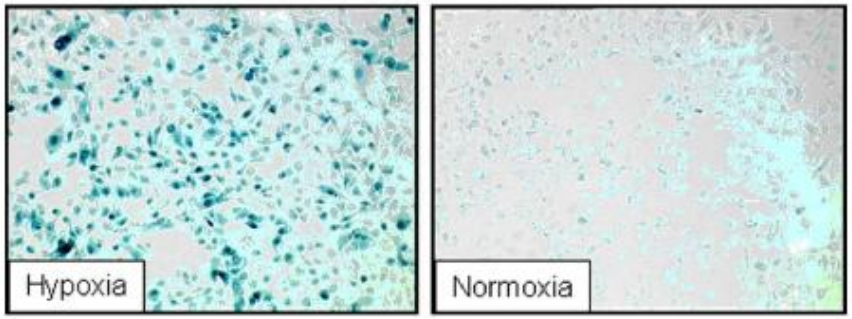
HRE – a hypoxia regulated sequence



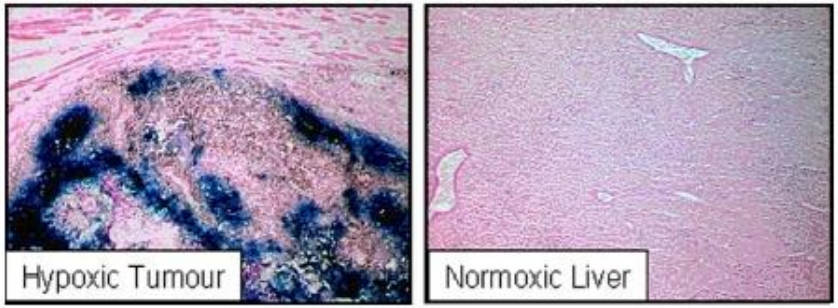
phosphoglycerate kinase 1

OBHRE - optimized hypoxia responsive promoter

OBHRE promoter *in vitro*



OBHRE promoter *in vivo*



Cell specific promoters

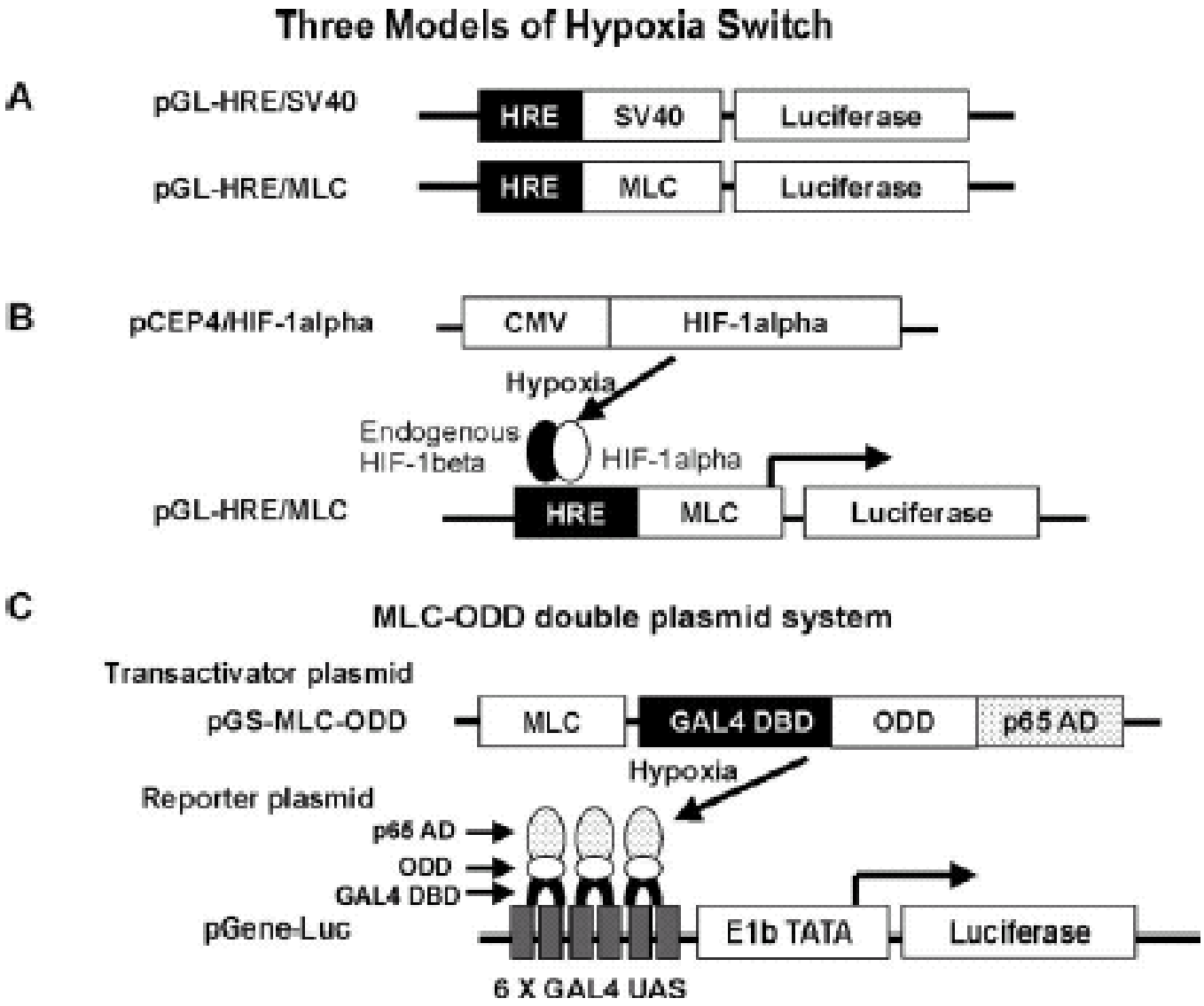
Keratin 14 – keratinocytes
Flk-1 (VEGFR-2) – endothelial cells
Tie-2 – endothelial cells

Brain specific
Liver specific
Heart specific
Kidney specific
Etc.

Cell-specific expression can be additionally increased by using the viral serotypes targeting specific cell types

How to regulate gene expression eg. in the heart

Hypoxia-dependent promoters



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Types of promoters used in gene therapy

1. **Constitutive**: viral
   eucaryotic: ubuiquitous
   cell-specific

2. **Inducible**:
   a) natural
   b) artificial: tetracycline-dependent
   rapamycin-dependent
   hormone-regulated

The systems can be used both in the non-viral and viral vectors

Table 1. General properties of different types of enhancer–promoters (EPs)

Type	Advantages	Disadvantages
Natural EPs	• Constitutive low or high levels • Tissue-, developmental-stage- and cell-cycle-phase-specific promoters for specific genes • Useful for long-term gene therapy	• Ubiquitously active EPs might lead to unwanted gene expression in non-targeted tissues • Untoward immune responses to transgene products, viral vectors and other adverse effects • Levels of transgene expression might not be optimal • Hard to fine-tune the activity of the promoter
Synthetic EPs	• Responsive to particular environmental signals, including patho-physiological signals • Might be useful for cancer therapy	• Endogenous genes might be affected by the same environmental signal(s) and thus side effects are produced if the signal is artificially generated
Inducible gene-expression systems	• Spatial, temporal and quantitative control of expression by a small molecule (drug) • Especially useful for expression of cytokines, chemokines and toxic gene products • Minimum safety risk due to stringent control • Doxycycline, mifepristone and rapamycin are clinically approved drugs	• 'Leakiness' might still be an issue for some systems • Artificial transactivators might be immunogenic • Effectiveness in humans needs to be validated

The optimal gene regulatory system should exhibit

(a) good regulation and induction kinetics,

(b) quick response to the administration or removal of the inducer,

(c) strong transgene regulation

(d) negligible cytotoxic or inflammatory responses associated with the regulatory elements within the switch system.

Gene Therapy