Lecture II

Transfection of naked DNA

Chemical vehicles for plasmid vectors

17.10.2011

Every disease can be (potentially) treated with gene therapy, because every disease has a genetic background .

It means that each disease is due to the **misfunction of the genes** which are either mutated or not sufficiently active to overcome the problems or they are too active and cause the problems!

There is no diseases in which the genes are not involved!

Information about gene therapy

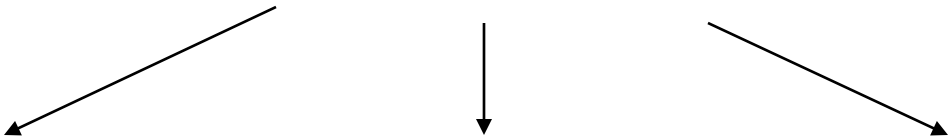
European Society of Gene & Cell Therapy

http://www.esgct.eu/

American Society of Gene & Cell Therapy

http://www.asgct.org/

Gene therapy



Enhancing

enhancement
of gene expression

Acquired diseases

Substituting

delivery of
the missing gene

Inherited diseases

Suppresive

inhibition of
gene expression

Acquired diseases

Gene addition

Gene correction/alteration

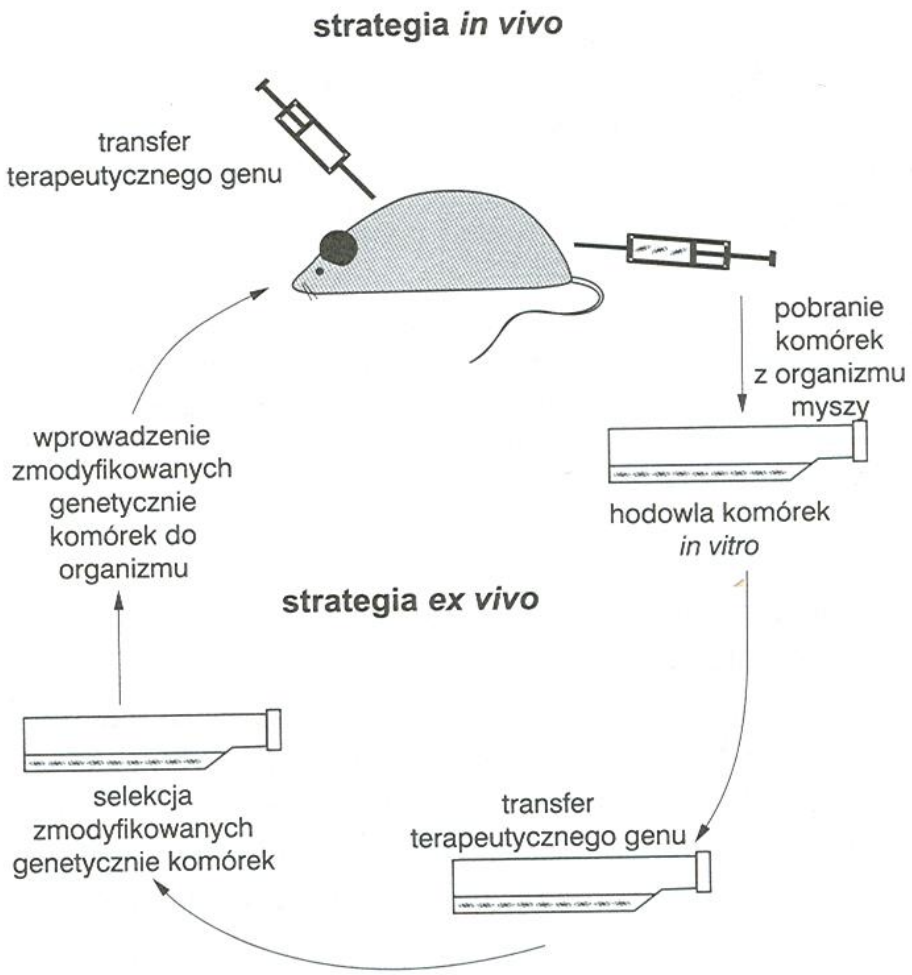
Gene knockout

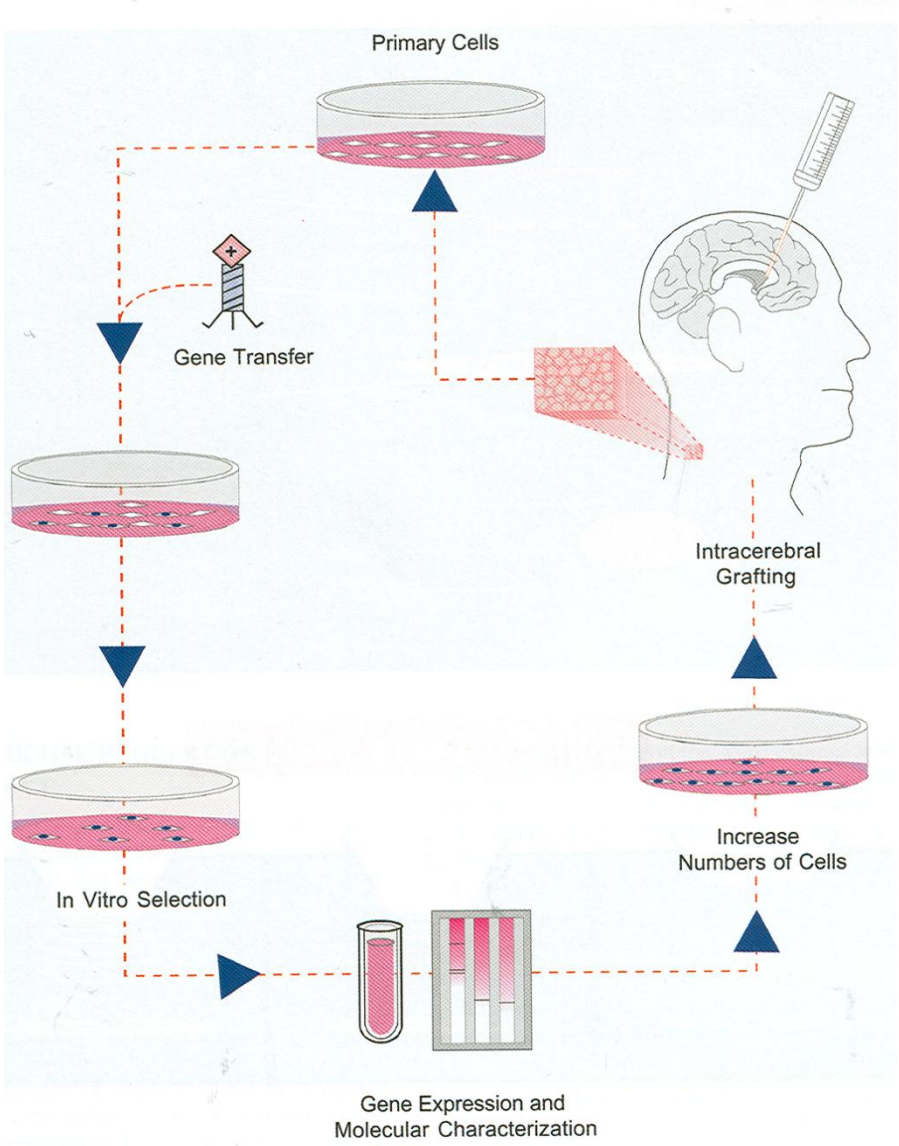
Types of gene therapy

-Somatic

- germ line

Strategies of gene therapy – in vivo and ex vivo





Nucleic acids can be introduced to the cells using

* physical methods (transfection)
* chemical methods (transfection)
* biological methods (transduction)

Other useful terms

Transformation of bacteria

Introduction of foreign genes into bacteria

Transfection

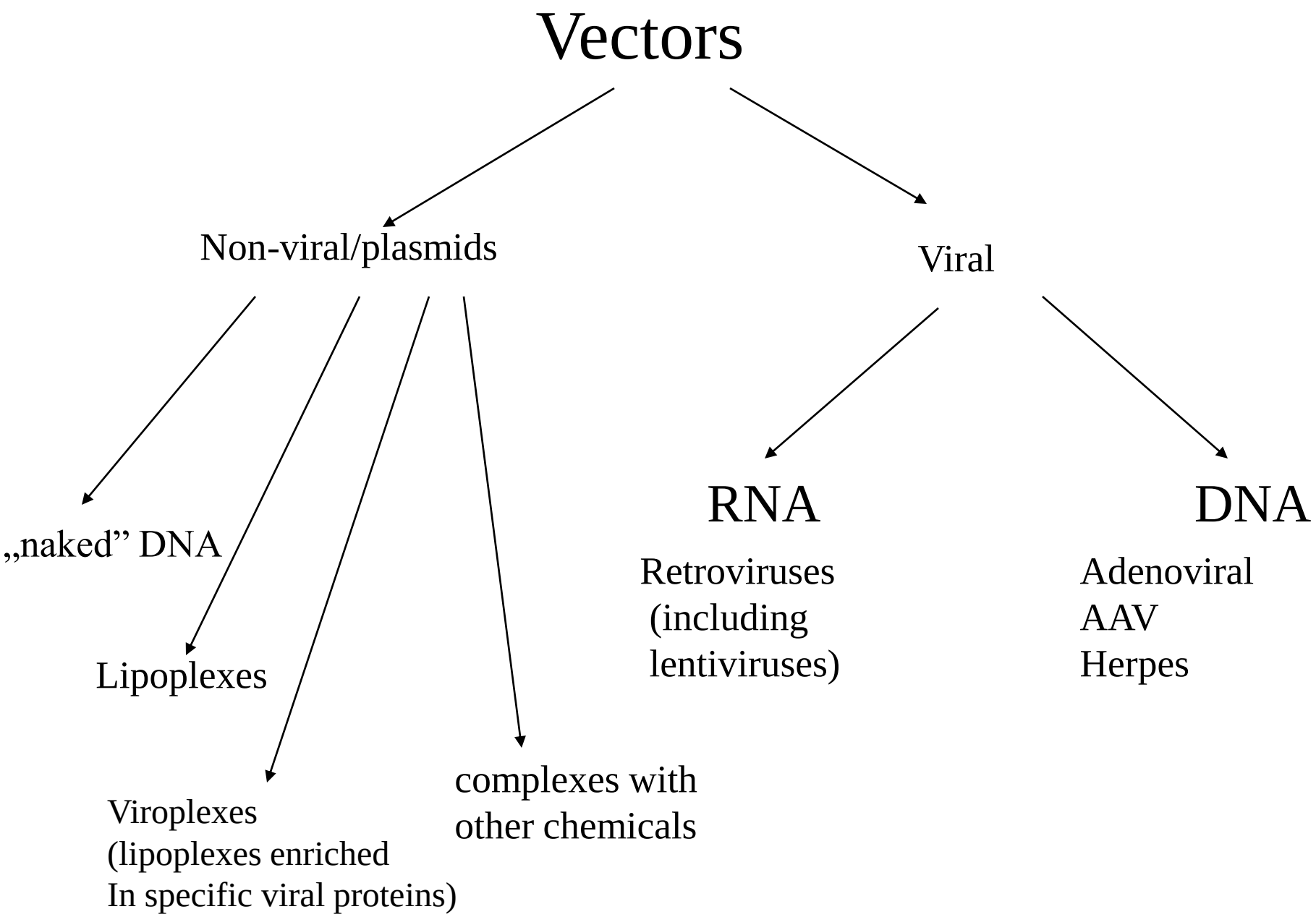
Introduction of foreign genes to eucaryotic cells by means of plasmid vectors (using physical or chemical methods)

Transduction

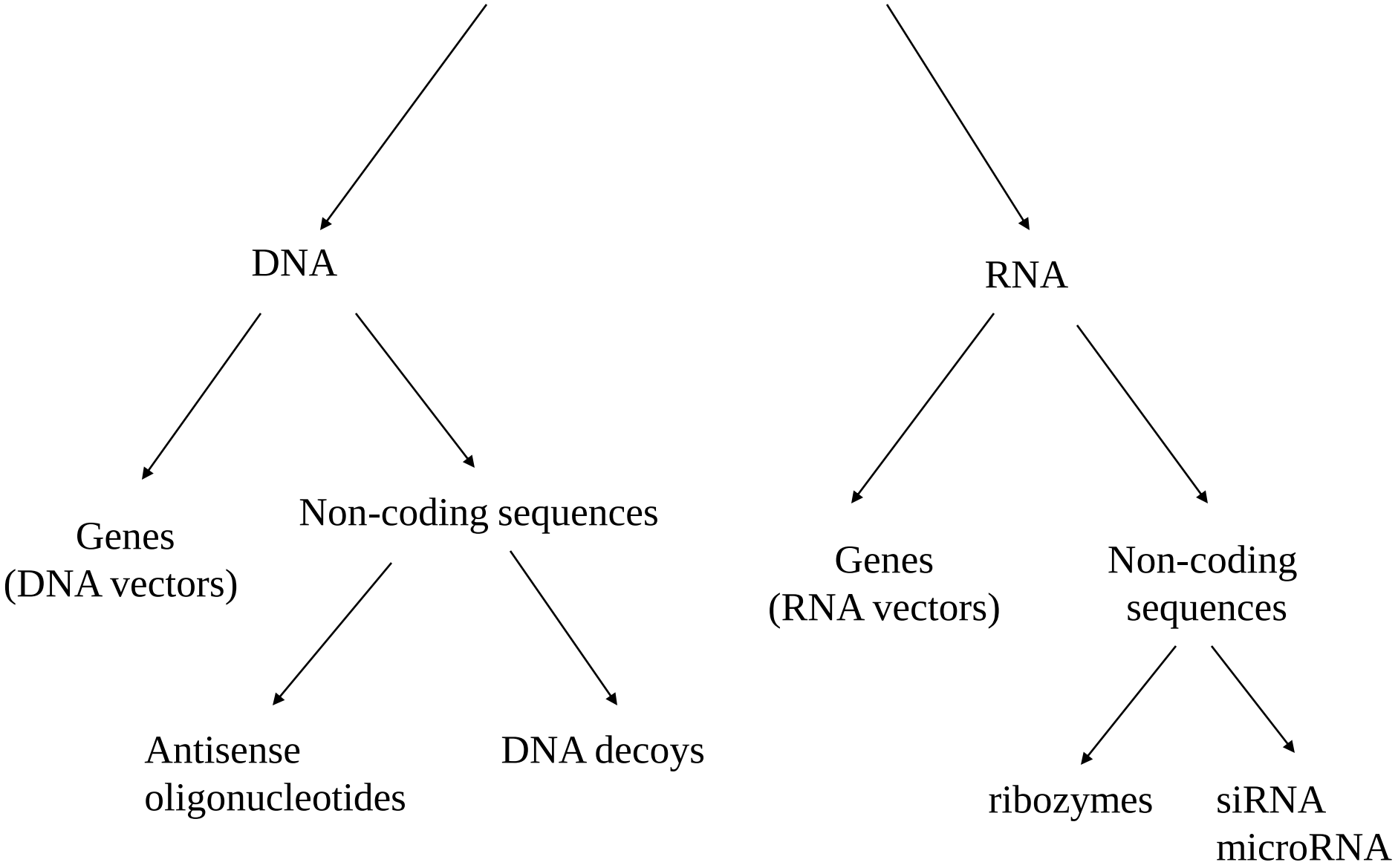
Introduction of genes by means of viral vectors (biological methods)

Molecular cloning

Production of numerous copies of a gene, by methods of genetic engineering, eg. transformation of bacteria with plasmid, PCR cloning


Vectors
Non-viral/plasmids
Viral
„naked” DNA
Lipoplexes
Viroplexes
(lipoplexes enriched
In specific viral proteins)
complexes with
other chemicals
RNA
Retroviruses
(including
lentiviruses)
DNA
Adenoviral
AAV
Herpes

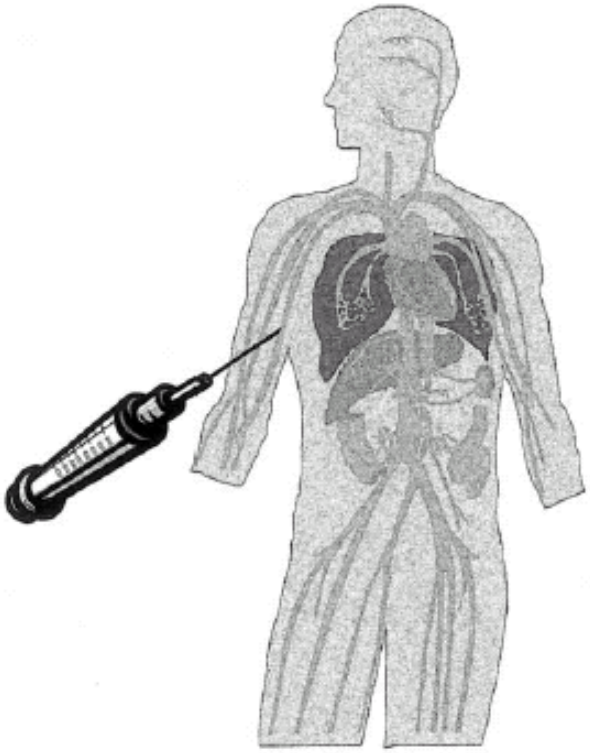
Therapeutic nucleic acids

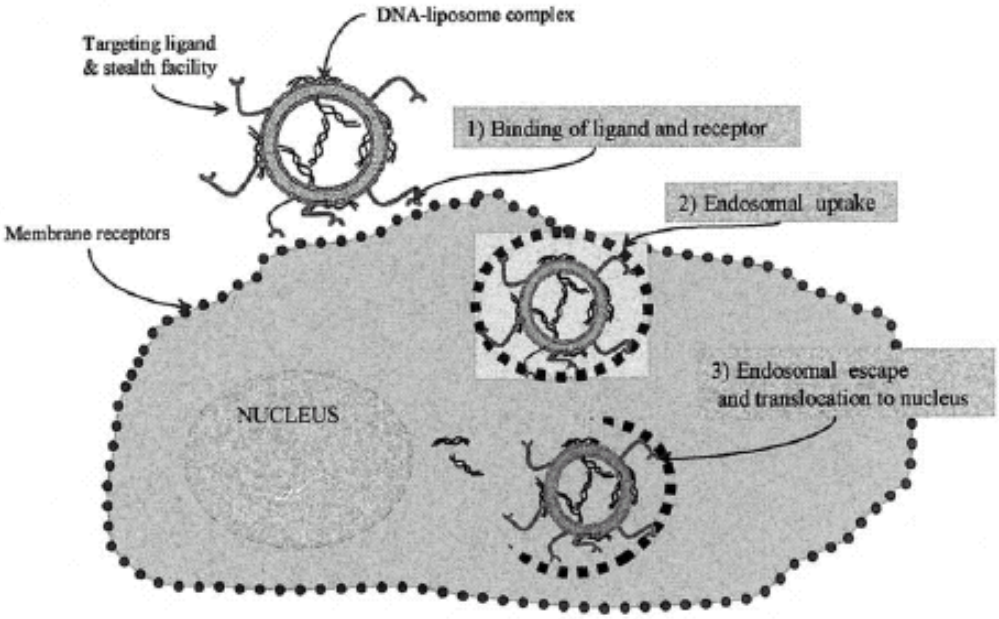


Bariers decreasing transfection efficiency in vivo

I. *Extracellular barriers*
1. Opsonins
2. Phagocytic cells
3. Extracellular matrix/mucus
4. Digestion enzymes

II. *Cellular/intracellular barriers*
1. Cell membrane
2. Endosomes/lizosomes
3. Nuclear membrane



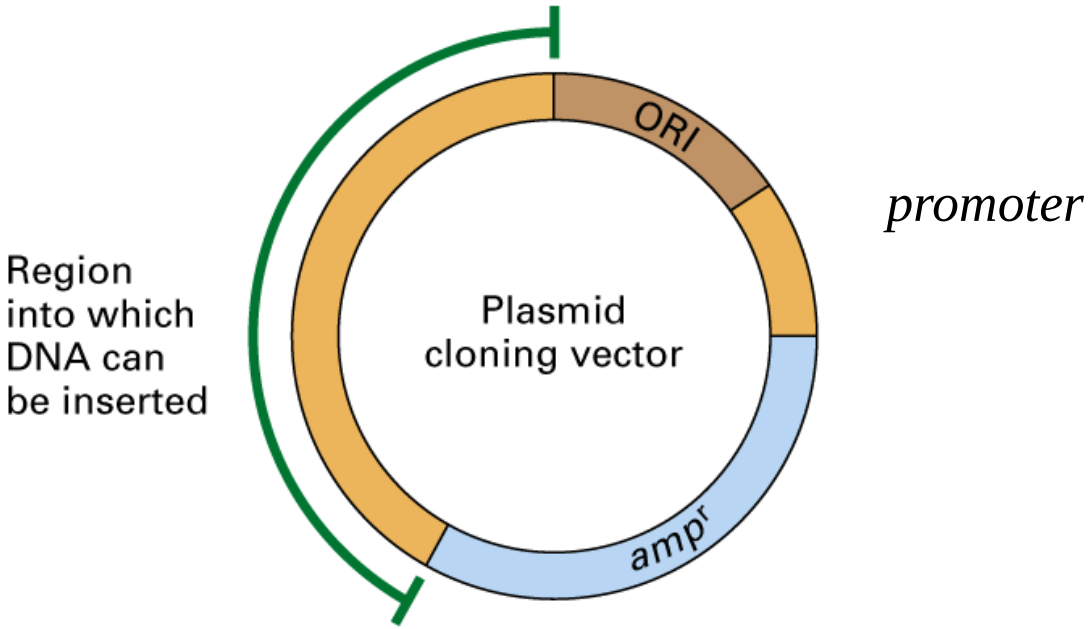


Plasmids

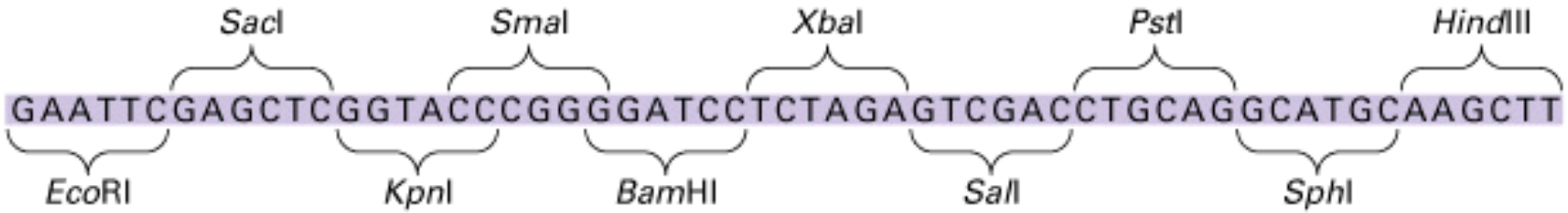
the main tools of gene therapy

Plasmids are always in the begining…

Organisation of a typical plasmid vector



(a) Sequence of polylinker



Plasmids are bacterial of origin, so they have to be modified to act in eucaryotic cells

Transient and stable transfection

Transient transfection

- In most cases plasmid DNA exists as an episomal element and is gradually degraded. Additionally it is lost during the replication of cells, thus the percentage of transduced cells gradually decreases. Therefore the effects of transfection are short-term.

Stable transfection

- Some vectors - especially retroviral vectors - introduce the transgene to the host genome. Such DNA replicates together with the rest of chromosome and is traded to the all doughter cells. Therefore, the effects of transfection are long-term.
- Integration can occur also for other vectors e.g. plasmids or adenoviral ones, but this process is very uneffective ranging from 1 per 100 to 1 to 1000 successfuly transduced cells.
- Transgene usually builds into more-less random site of genome.
- Site-specific integration using a homologous recombination mechanism is possible, but more diffucul and rarerly used.

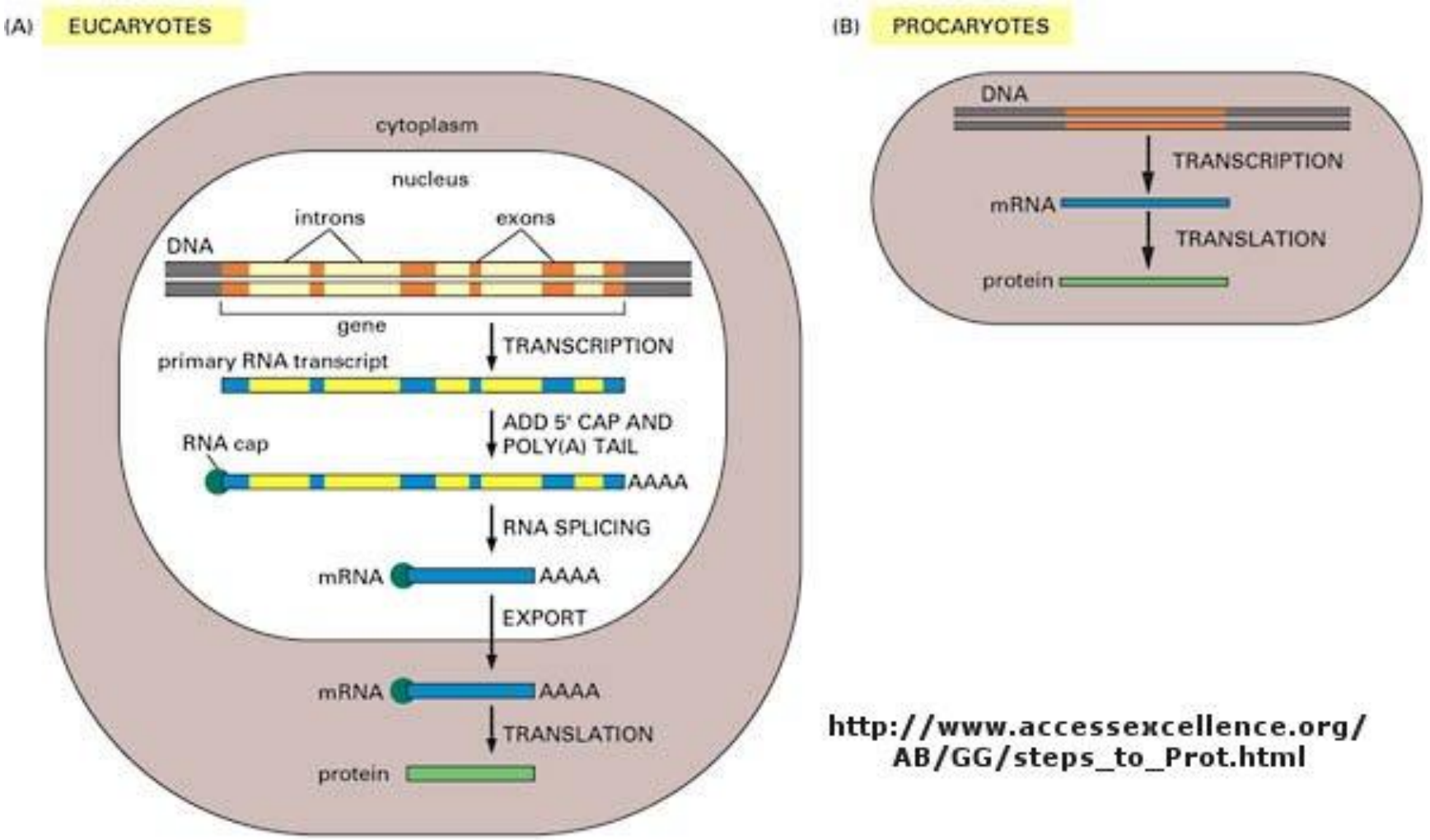
How to construct a plasmid with a desired gene, which we do not have...

Cloning on your own – use RT-PCR

Synthesize from scratch (on the basis of the sequence deposited in GeneBank

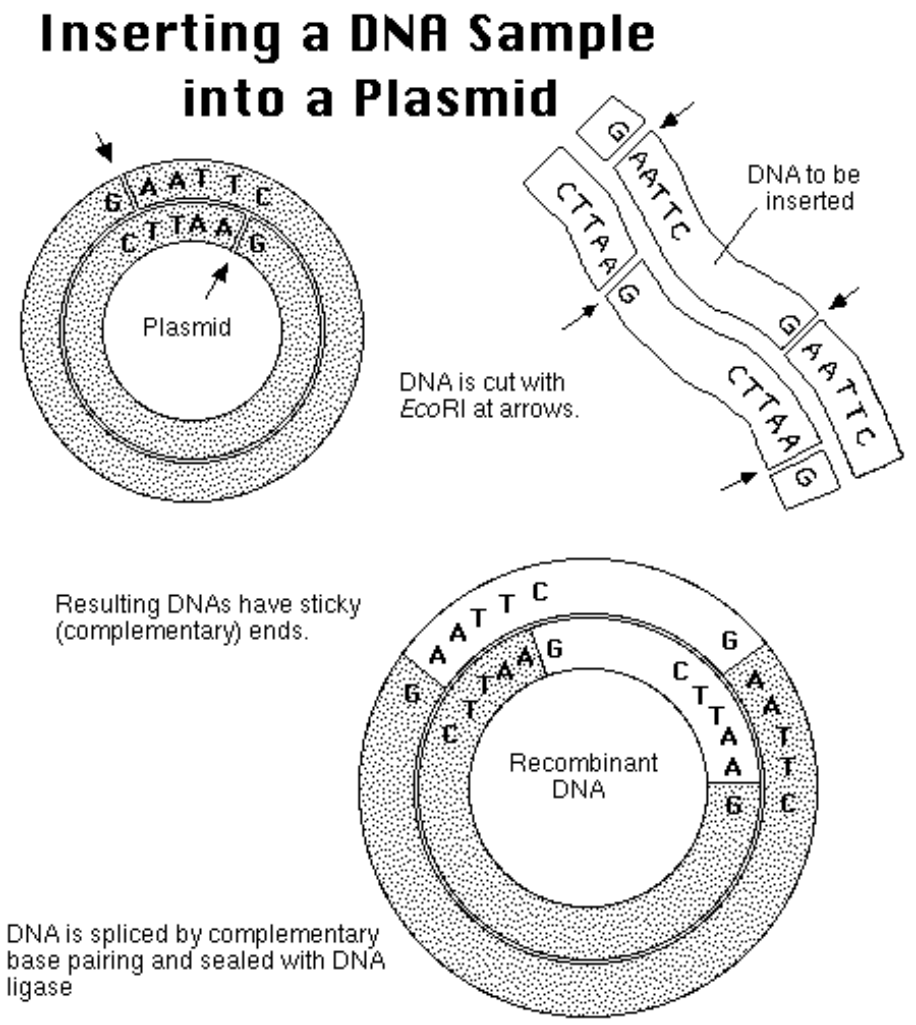
Other scientists – simply ask... – the most common way...

RT-PCR is a preferable way for gene cloning



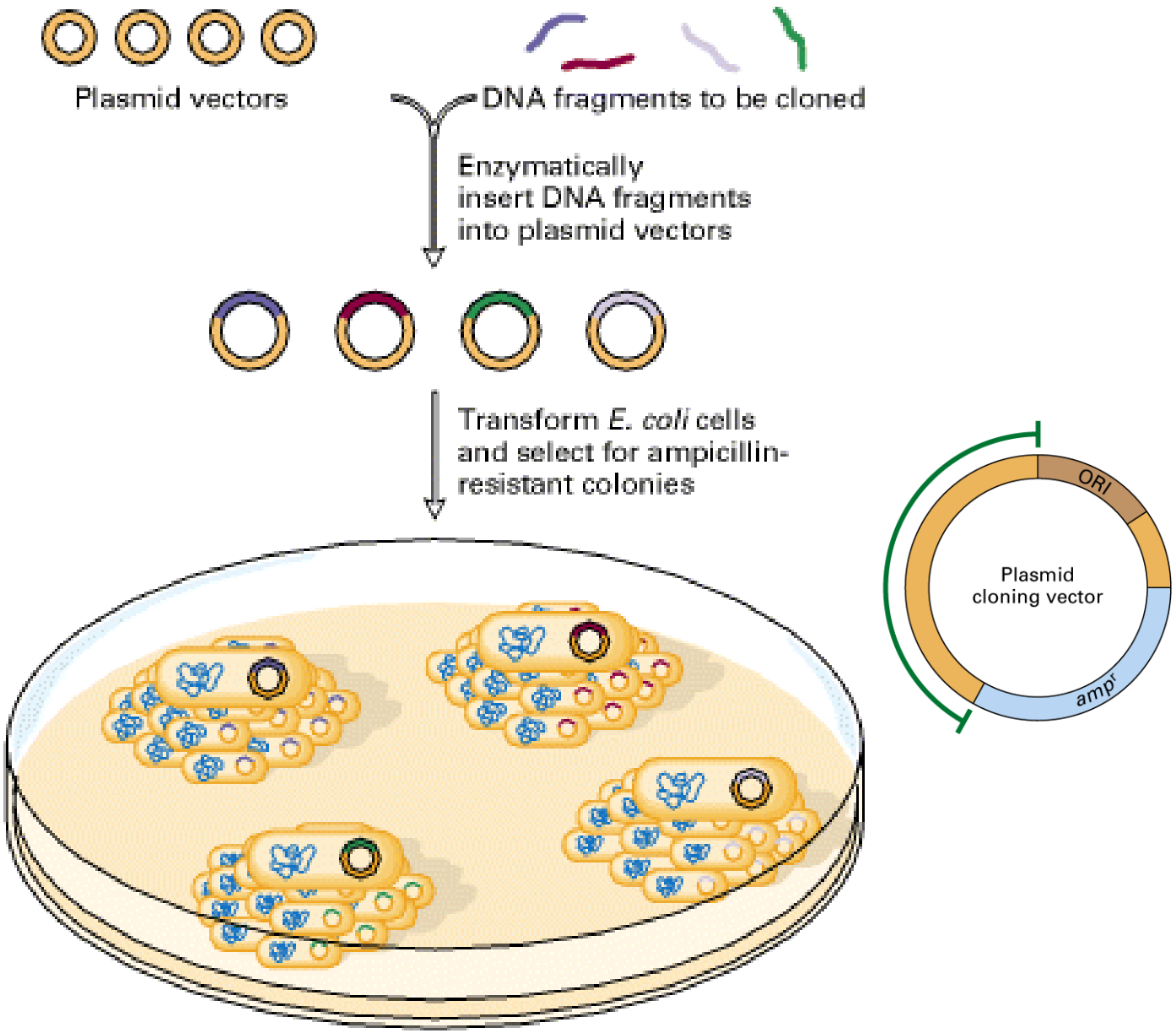
http://www.accessexcellence.org/AB/GG/steps_to_Prot.html

DNA cloning



A piece of target DNA can be inserted into a plasmid if both the circular plasmid and the target DNA have been cleaved by the same restriction nuclease in such a way as to create sticky ends. The newly created recombinant molecule is stabilized with the DNA ligase enzyme which repairs nicks in the backbone of the DNA molecule.

Isolation of DNA fragments from a mixture by cloning in a plasmid vector



Transformation of bacteria

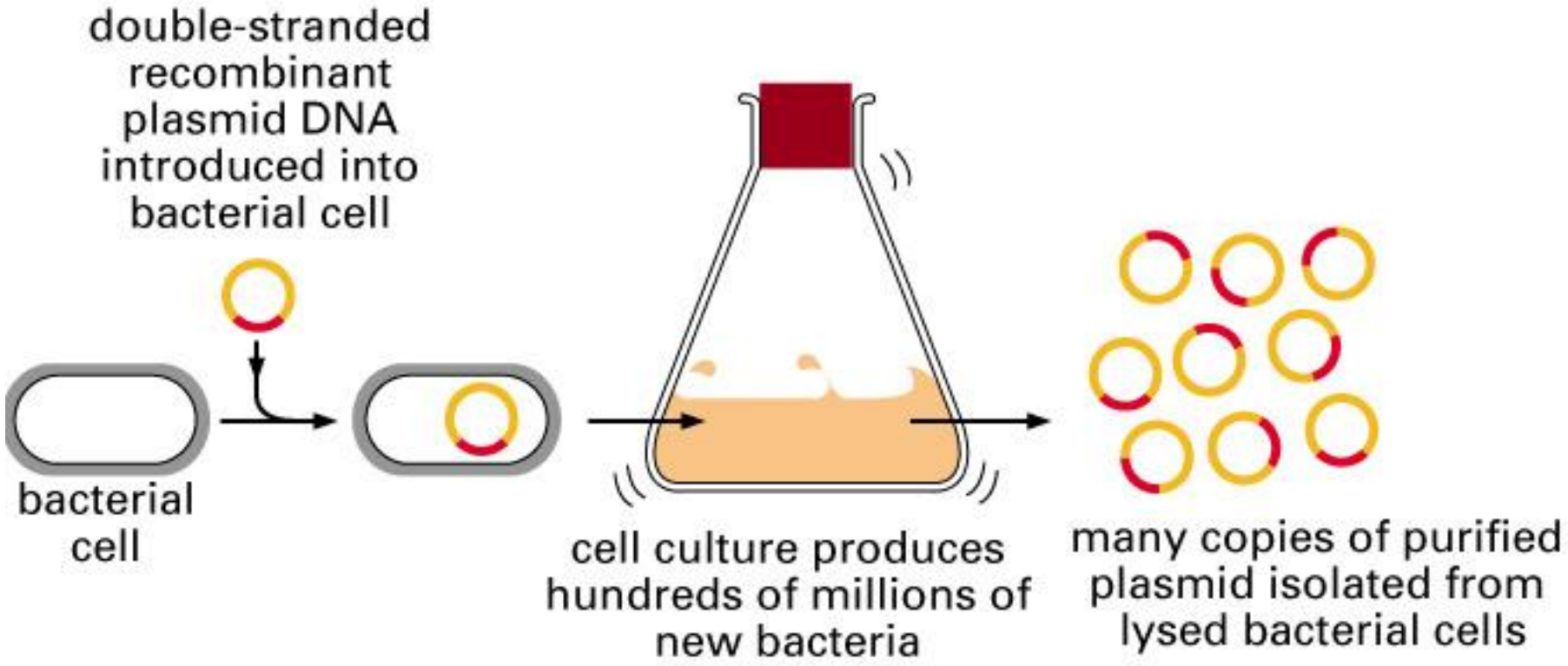
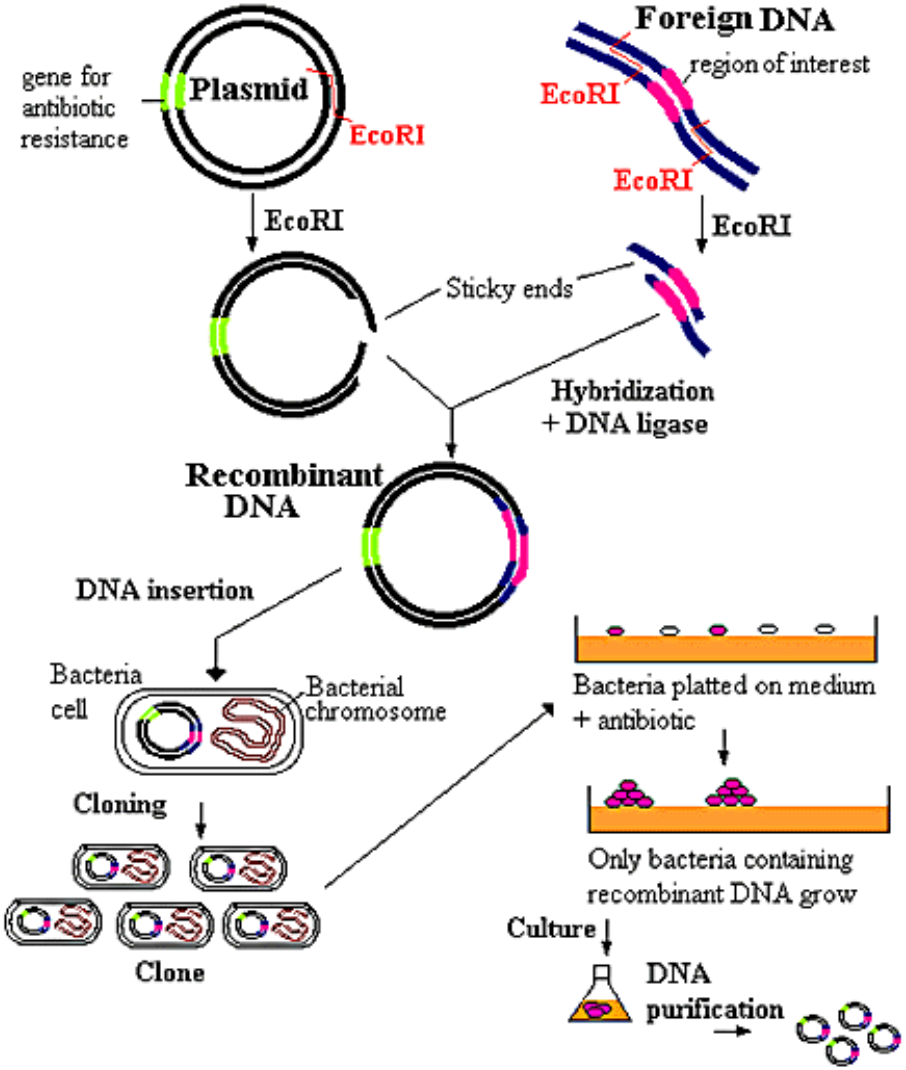


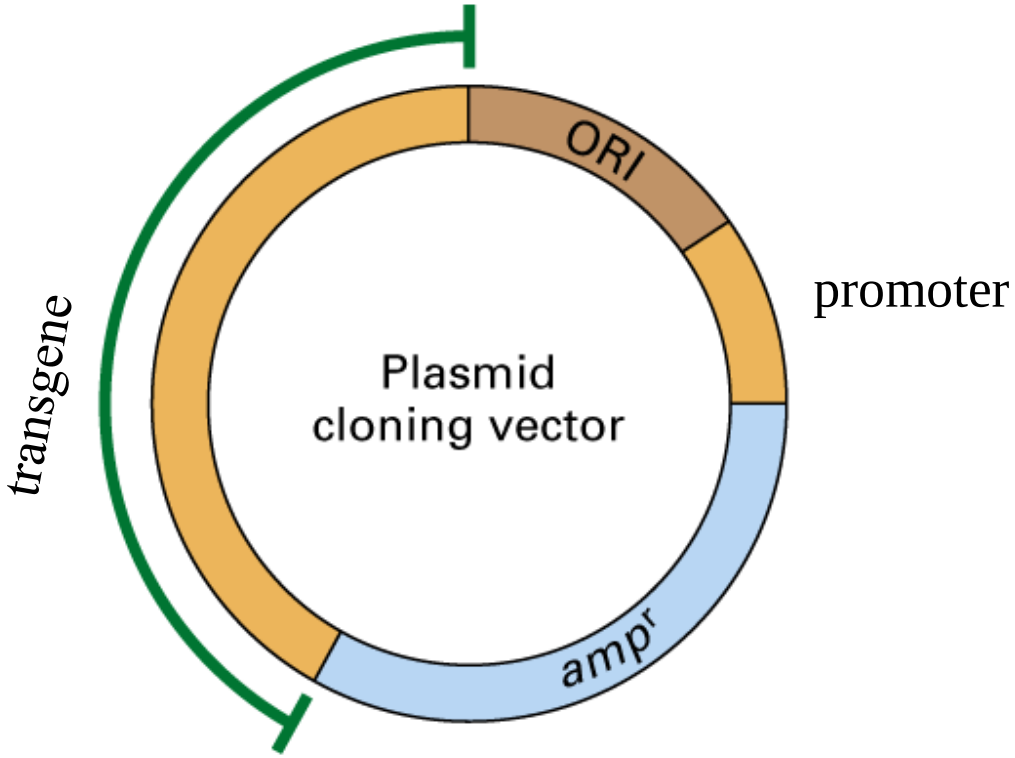
Figure 8–31. Molecular Biology of the Cell, 4th Edition.

DNA cloning



Cloning into a plasmid

How to make a plasmid working in mammalian cells?



Expression of foreign genes in eucaryotic cells requires the eucaryotic promoter in a plasmid vector

1. Viral promoters : CMV, SV40
2. Eucaryotic promoters
   - constitutive:
     - non-selective: β-globin
     - tissue specific:eg. Tie-2 (in endothelial cells;
       MHC – myosin heavy chain – in cardiomyocytes)
   - inducible
3. Complex

There will be more about promoters during one of the next lectures

What’s more is required?

At 5’ end -

The **Kozak consensus sequence**, **Kozak consensus** or **Kozak sequence**, is a sequence which occurs on eukaryotic mRNA and has the consensus (gcc)gccRccAUGG, where R is a purine (A or G) three bases upstream of the start cadon (AUG), which is followed by another 'G'.

The Kozak consensus sequence plays a major role in the initiation of the translation process. The sequence was named after its discoverer, Marylin Kozak

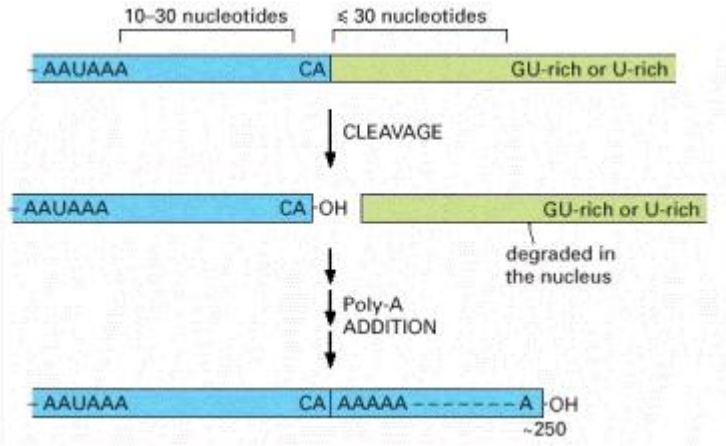
mRNA 5’–ACCAUGG–

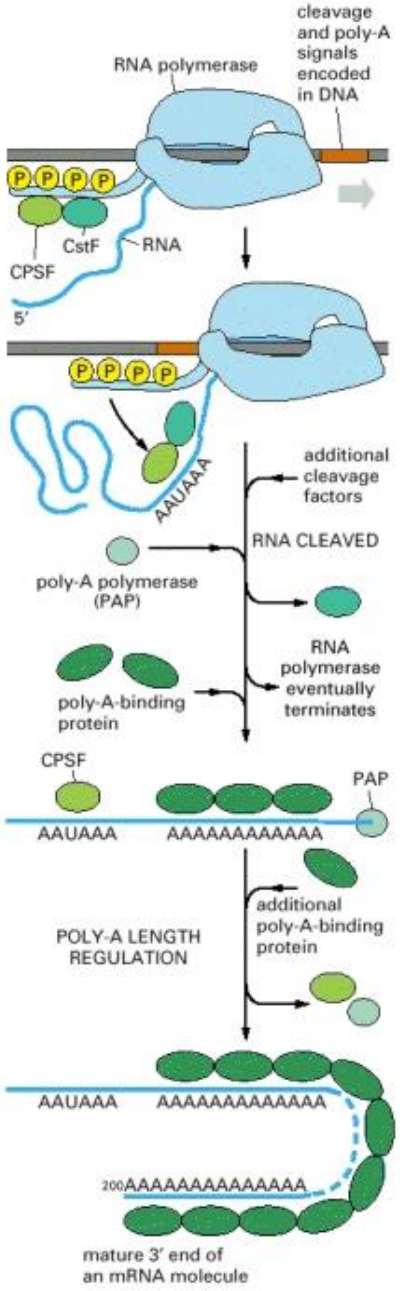
The A preceding the AUG seems to be the most important nucleotide affecting initiation efficiency

At 3’-end

Poly-A is required for expression of genes in mammalian cells

Consensus nucleotide sequences that direct cleavage and polyadenylation to form the 3′ end of a eucaryotic mRNA

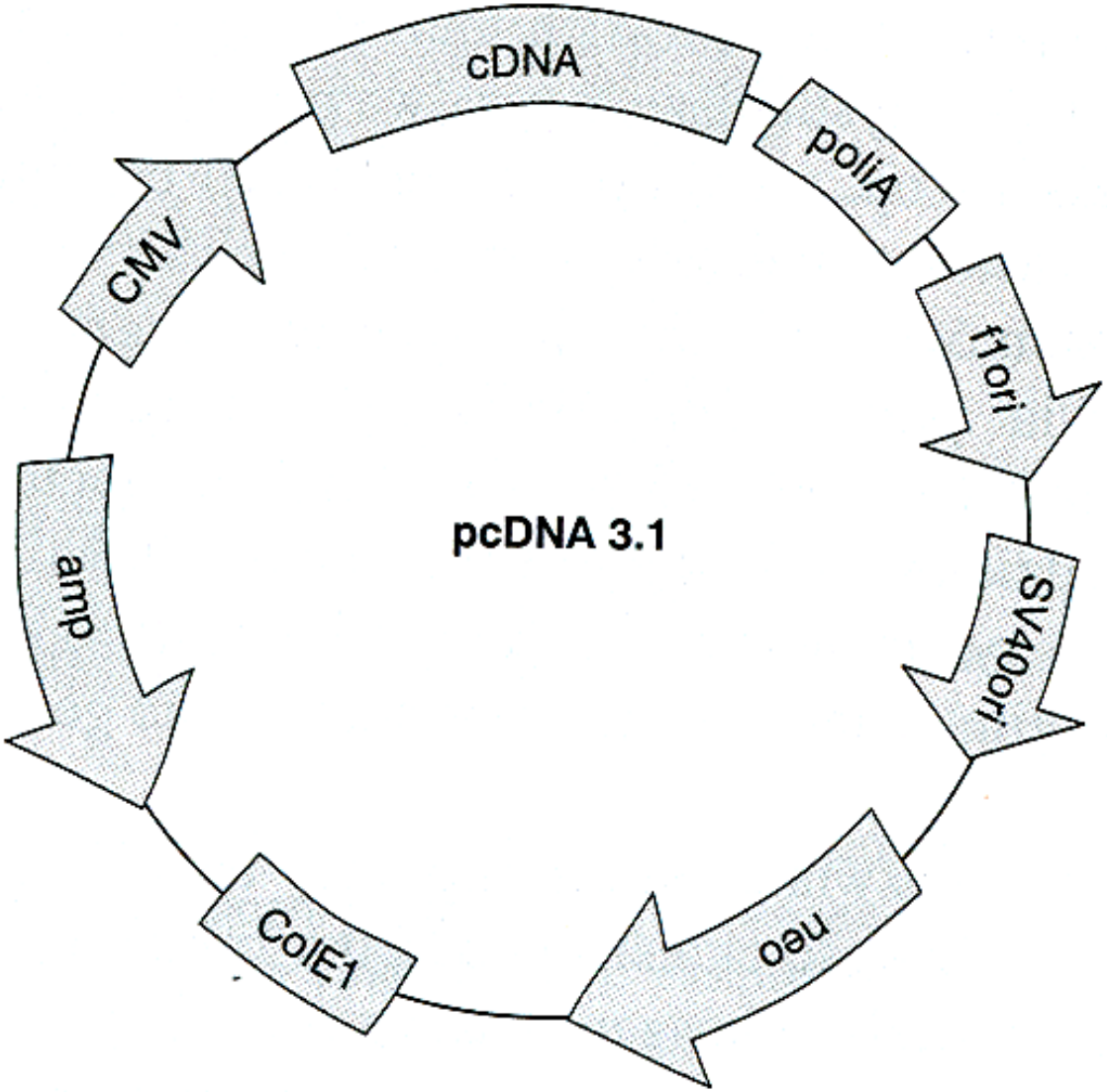




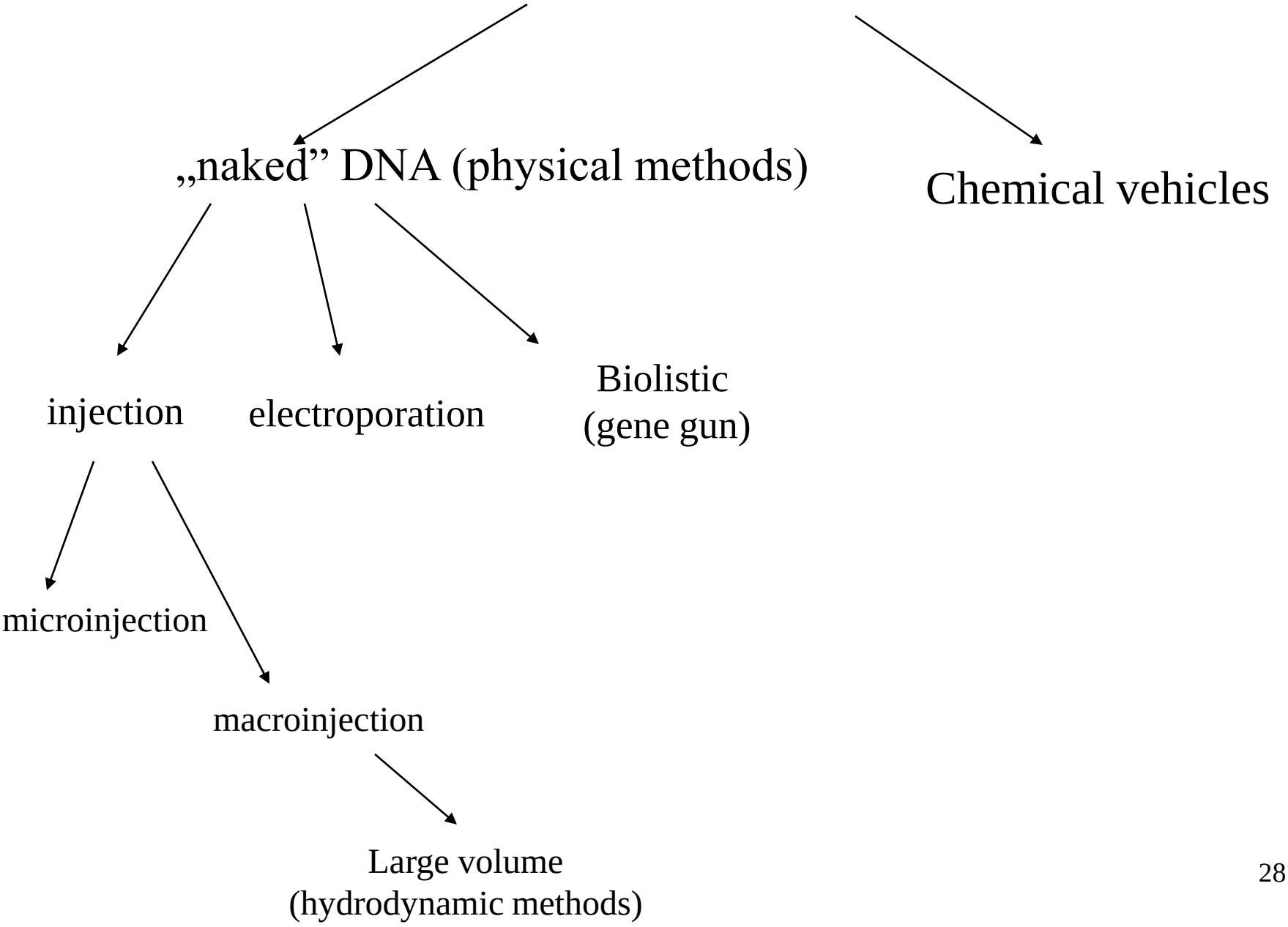
These sequences are encoded in the genome and are recognized by specific proteins after they are transcribed into RNA.

Molecular Biology of the Cell, 2002

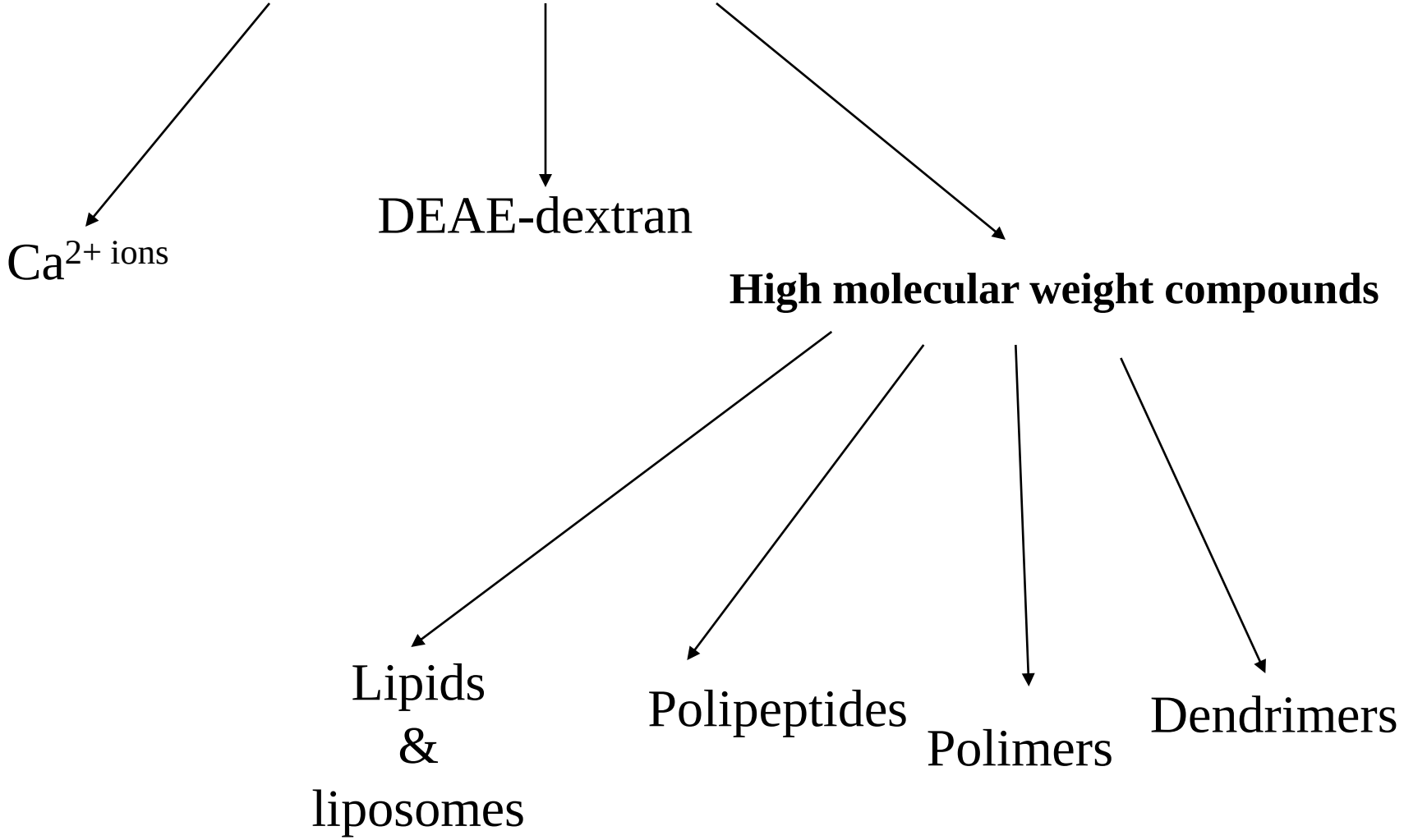
Mammalian expression plasmid



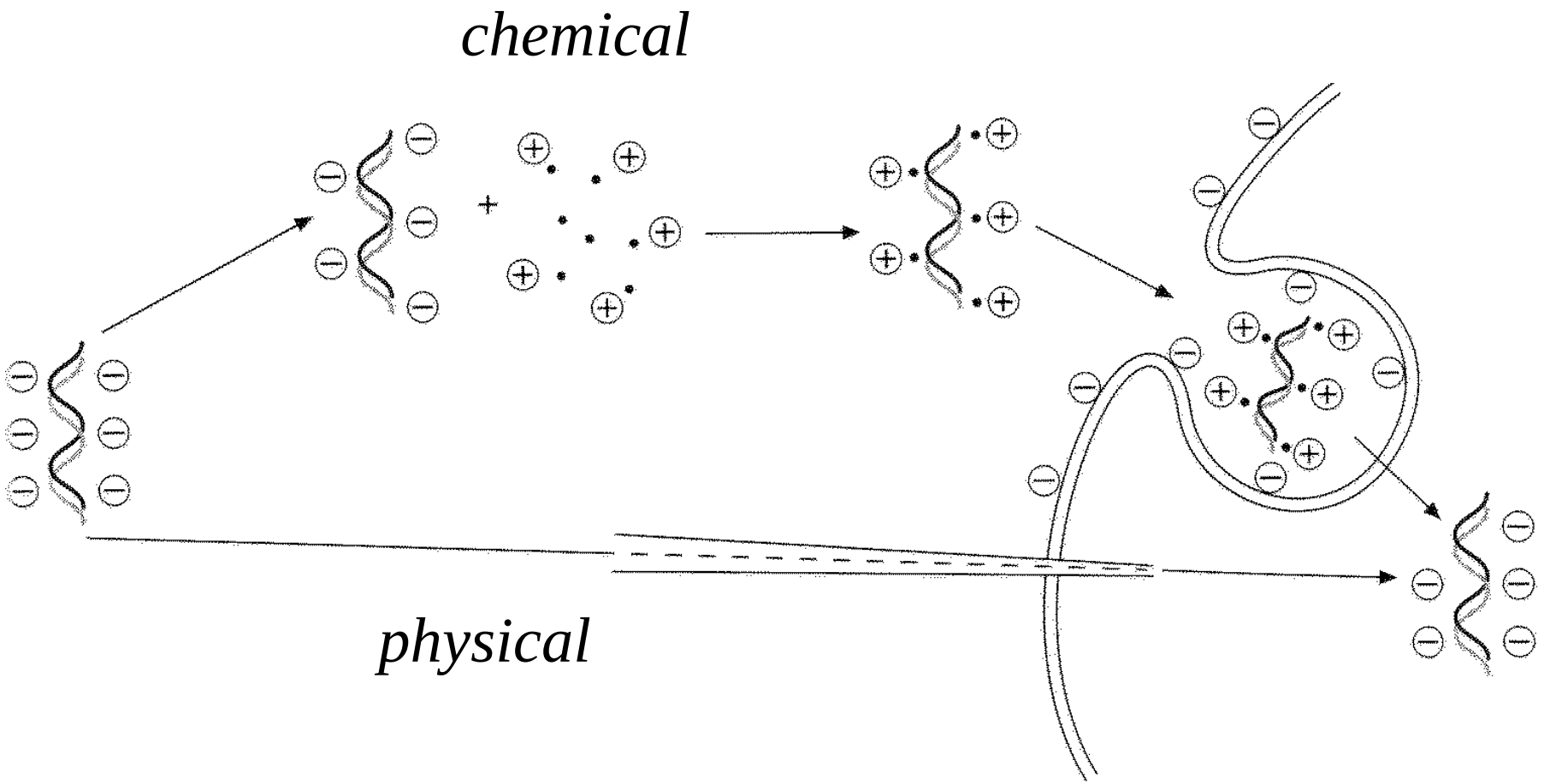
Transfections of plasmid DNA



Chemical vehicles for plasmid DNA



Chemical vs physical transfection



Barriers to be overcome by nucleic acid in the cells

A. Crossing plasmalemma

* Cell membrane has a negative charge, as DNA or RNA, which blocks attachment of nucleic acids to the surface of membrane.

* Both DNA and RNA are hydrophilic, which blocks the direct fusion with lipid plazmalemma.

B. Release from phagosomes (if nucleic acid is taken by phagocytosis).

C. Dissociation of nucleic acid from the chemical carrier (if chemical methods are used)

D. Transportation to the nucleus (can be improved by adding ORI sequence from SV40 virus to the nucleic acid or adding to the chemical vehicle a protein with nuclear localization signal, NLS, e.g. SV40 large T antigen).

Most often, the rate limiting steps are B and D.

Delivery of nucleic acids by endocytosis or direct fusion

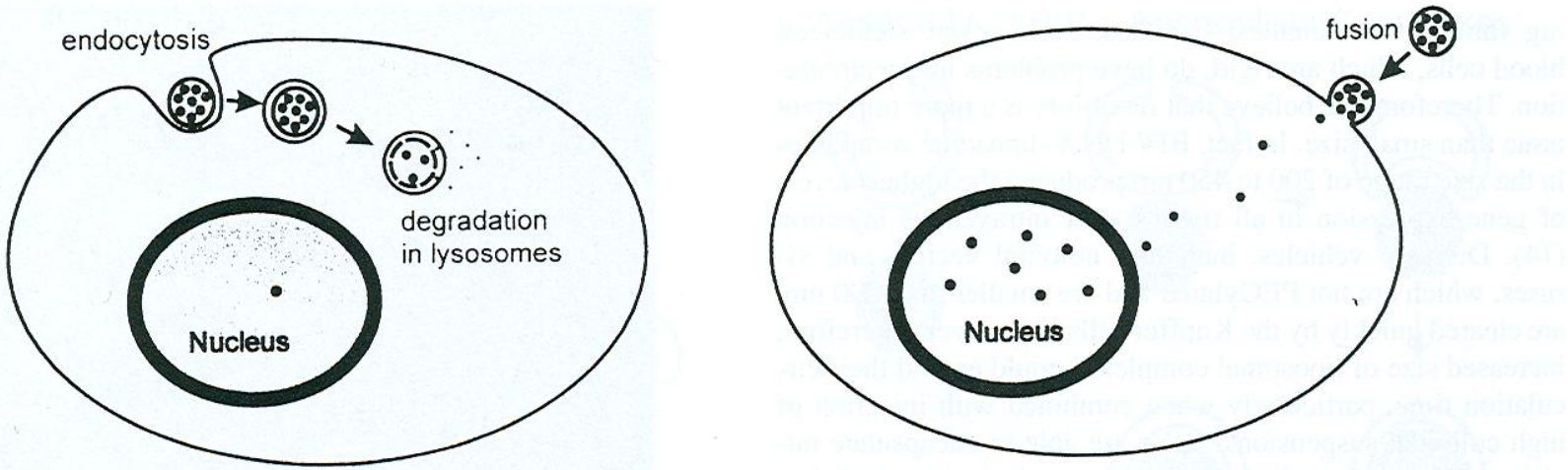
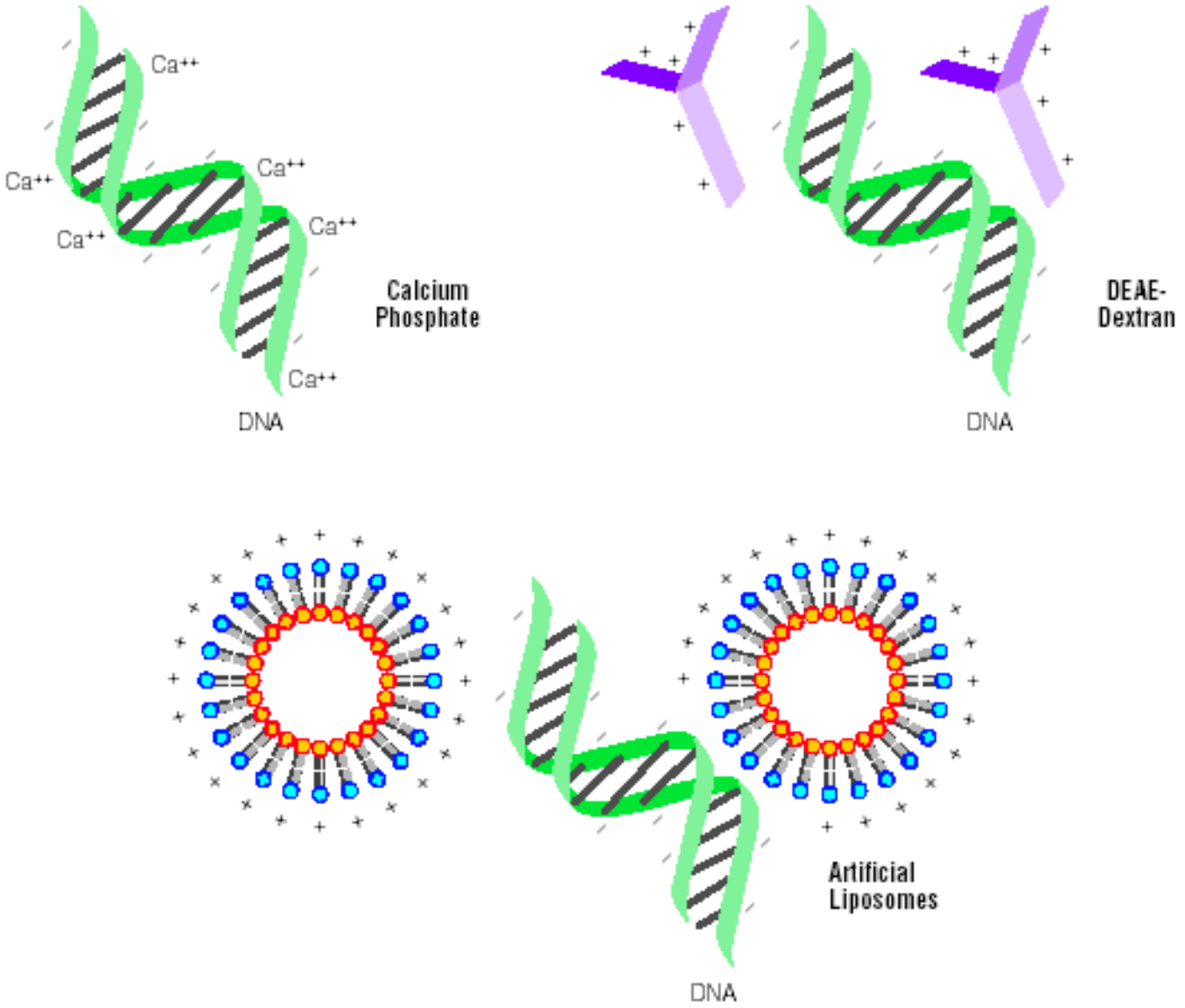


Figure 6 Mechanisms for cell entry of nucleic acid–liposome complexes. Two major pathways for interaction are by endocytosis or by direct fusion with the cell membrane. Complexes that enter the cell by direct fusion allow delivery of more nucleic acids to the nucleus because the bulk of the nucleic acids do not enter endosomes.

Types of chemical support

1. Protection of DNA from nucleases
2. Targeting vectors to specific cell types
3. Enhancing DNA delivery to cytoplasm and nucleus
4. Enabling long-term and/or controlled delivery of DNA

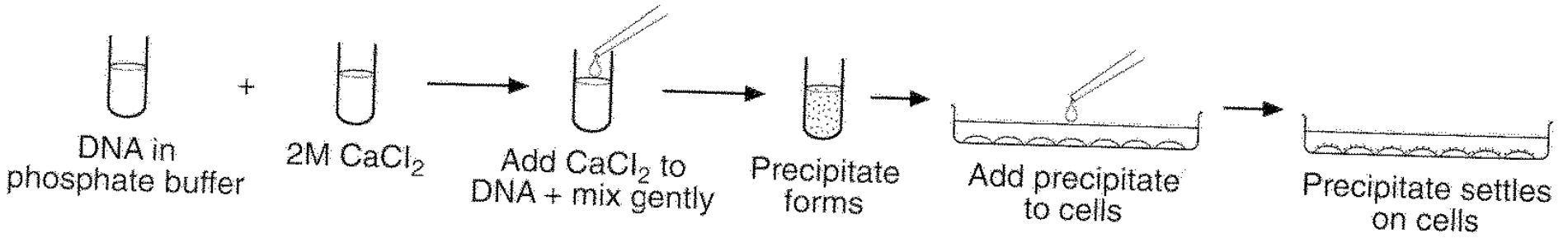
Chemical vehicles for in vitro transfection



Calcium phosphate-facilitated transfection

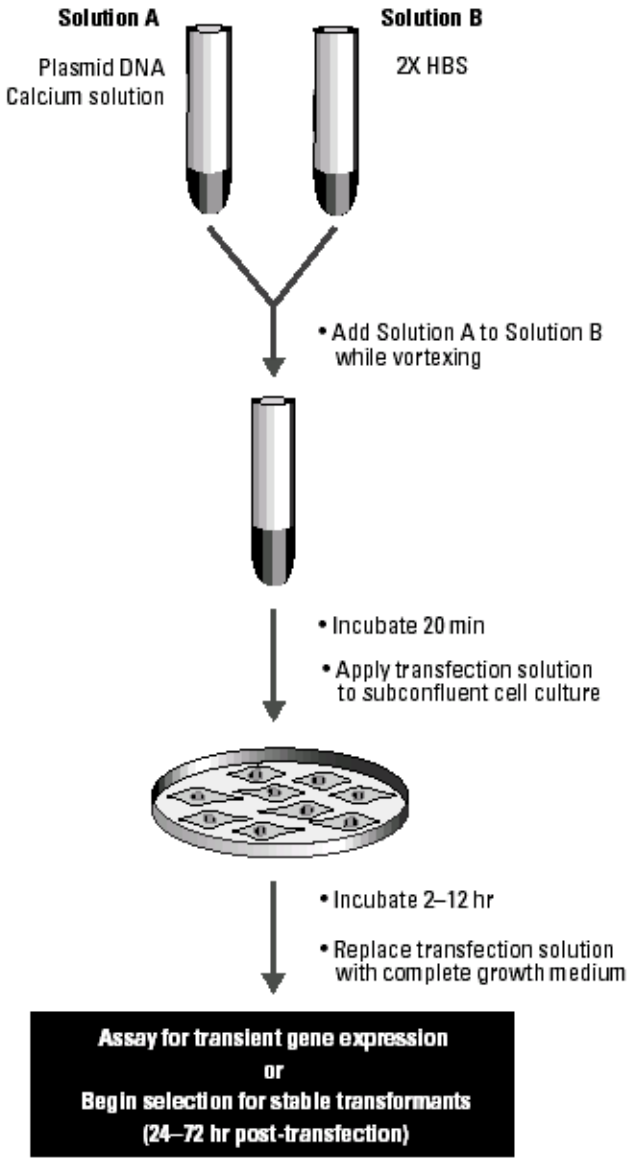
- inexpensive
- simple
- suitable for a range of cell types

Calcium-phosphate mediated transfection



R.W. Twyman – Gene transfer to animal cell. BIOS Scientific Publishers 2005

CalPhos Mammalian Transfection Kit



BD Biosciences

Conditions for calcium phosphate transfection

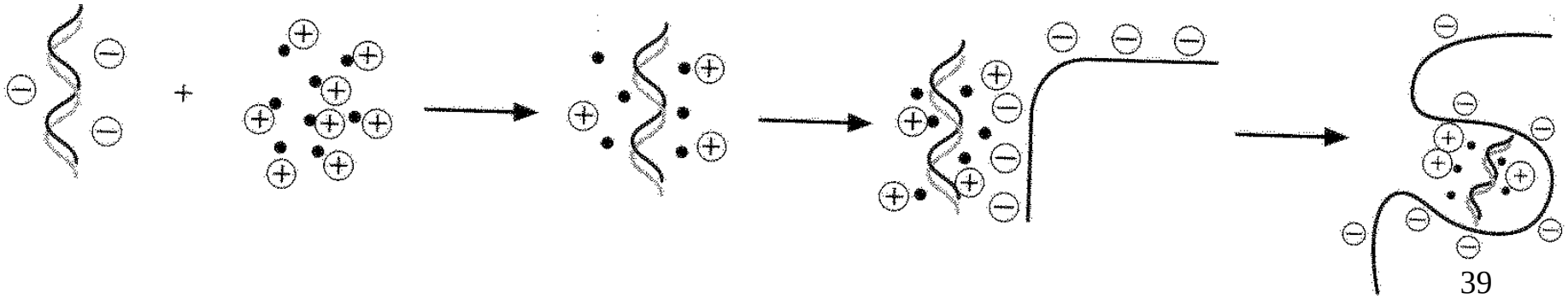
1. Method is efficient in a quite narrow range of conditions
2. Formation of large precipitates (crystals) should be avoided; small particles are better

3. Efficiency depends on
   a) DNA concentration
   b) temperature – phosphate is solved better in lower temperatures
   c) time of formation of precipitate before addition to cells
   d) pH – above 6.9 the complexes are not formed; pH 7.0 – complexes are small and delicate; pH above 7.0 – large complexes;
   but ... – opinion varies

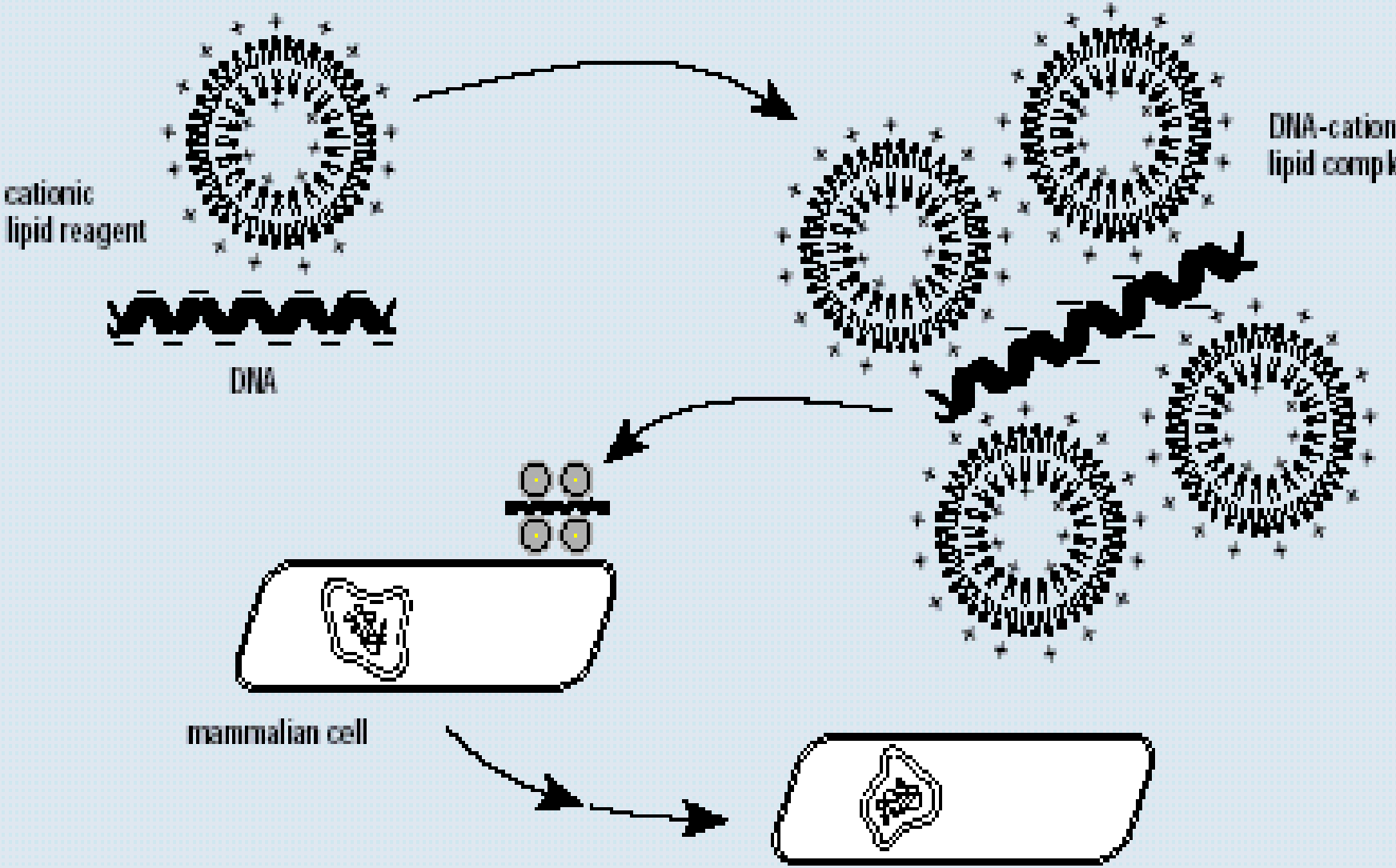
4. Important – DNA must be diluted in water! Tris buffer changes pH and decreases the transfection efficiency

DEAE-dextran
diethylaminoethyl dextran

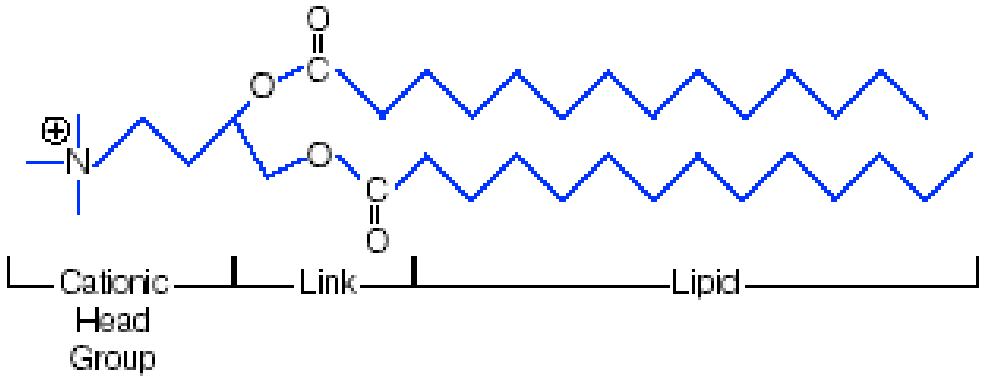
1. Cationic polimer
2. Allows transient transfection
3. Works well with some cell types
4. Transfection is possible with smaller amounts of DNA than used with Ca^{2+}
5. Low efficiency, variability



Cationic liposomes

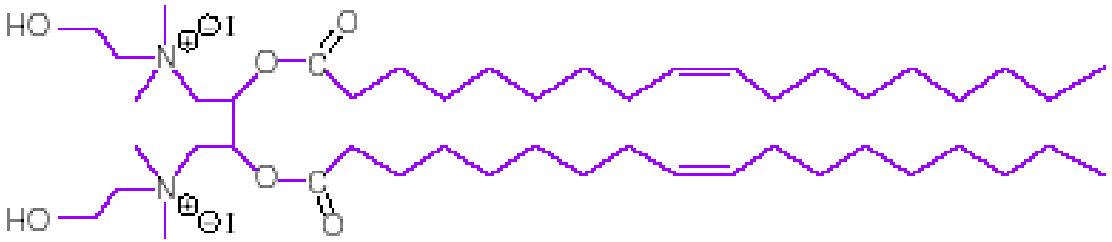


Cationic liposomes



DOTMA

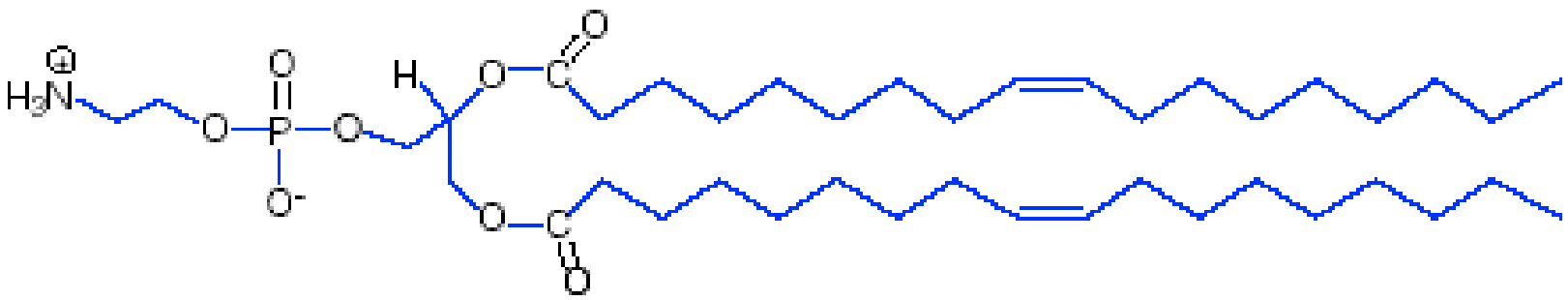
General structure of synthetic cationic lipid



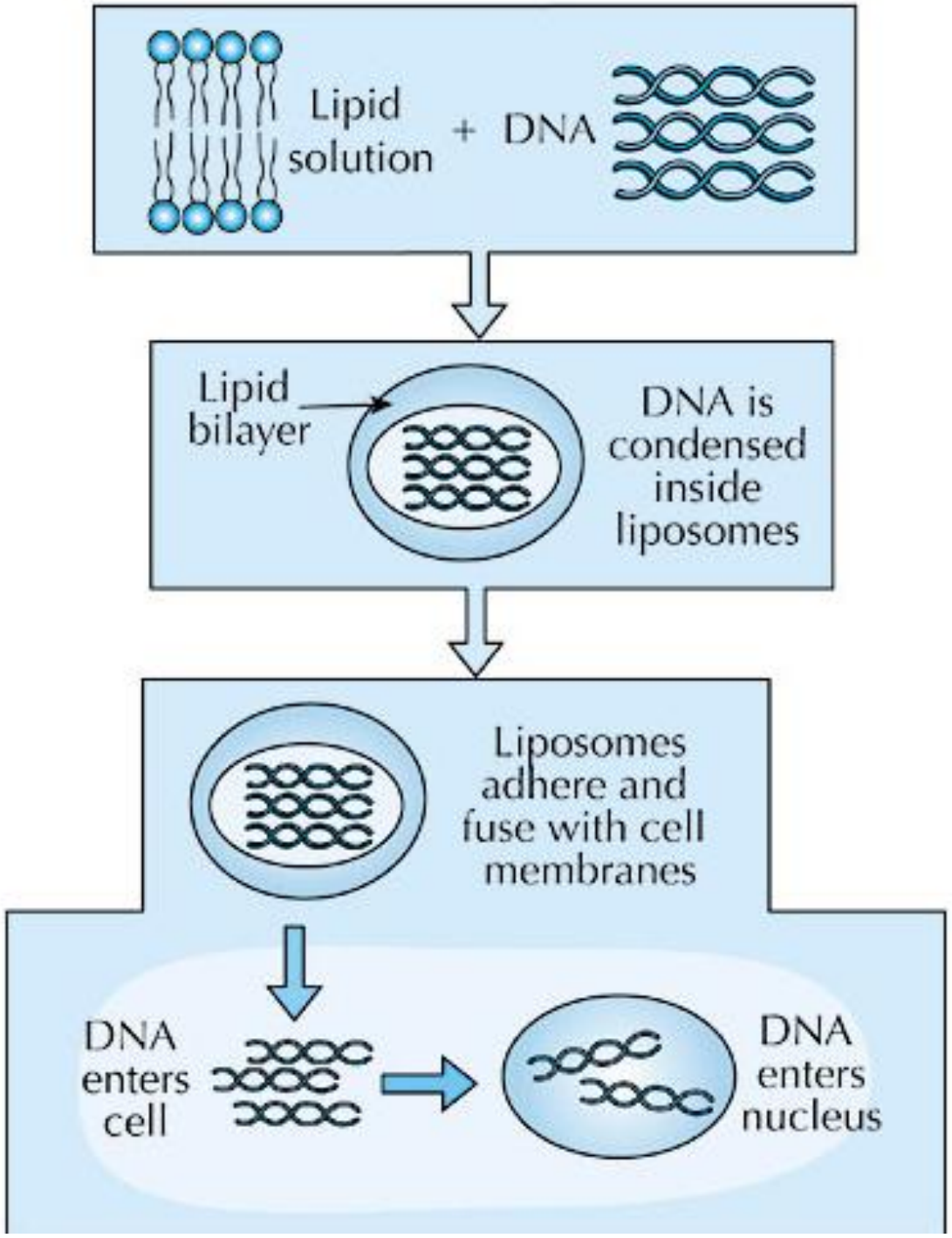
Tfx™

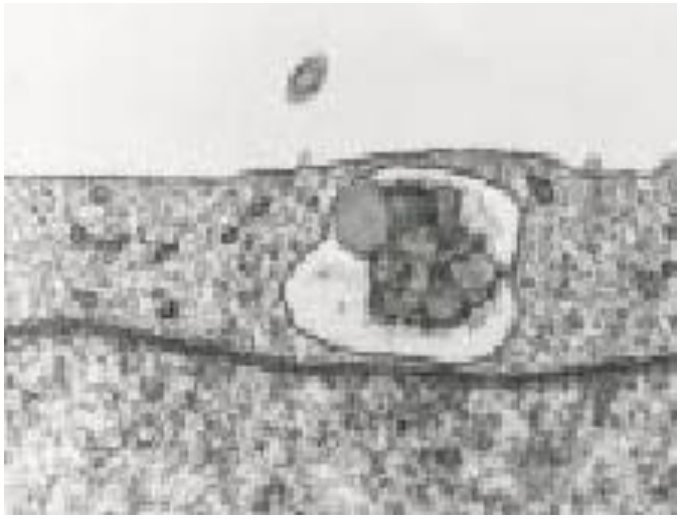
DOPE (L-dioleoyl phosphatidylethanolamine)

Neutral phospholipid

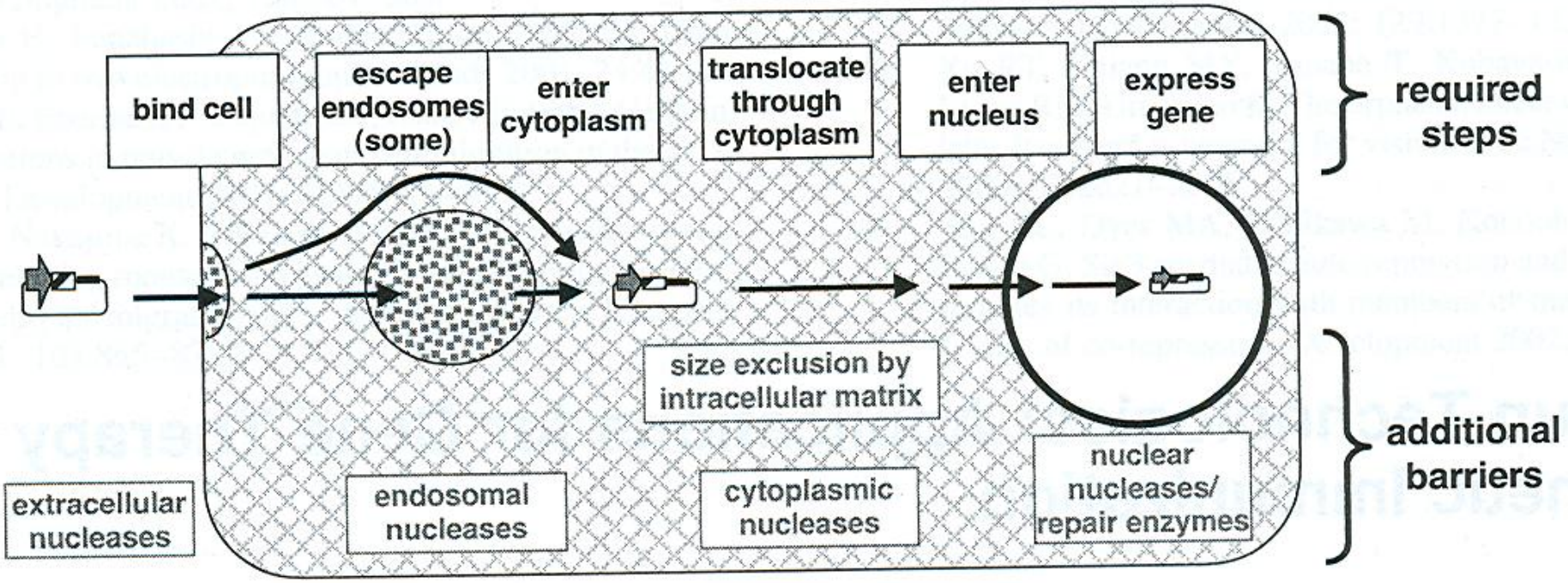


- facilitates fusion with cell membrane
- facilitates release from endosome


Lipid solution
+ DNA
Lipid bilayer
DNA is condensed inside liposomes
Liposomes adhere and fuse with cell membranes
DNA enters cell
DNA enters nucleus



Barriers for efficient delivery of naked DNA to the nuclei



Ways of protection from intracellular degradation

1. Chloroquine - increases pH in endosomes
2. Omission of endosomes: subunits of toxins *Diphteria i Pseudomonas*
3. PEG – stabilizes plasmid DNA and protects from nucleases -
   - prevents from immune system
4. Nuclear targeting
   a) passive transfer to nucleus – during cell cycle
   b) PEI – synthetic polimer – protects DNA in cytoplasm, facilitates nuclear entry
   c) viral nuclear localization signal, NLS

Nuclear localisation sequences

Table 3 Nuclear Localization Sequences

Protein	Sequence	Location (position)
SV-40 large T Ag	PPKKKRKV	N-term (132/708)
Adenovirus E1A	SCKRPRP	C-term (239/243-289)
HIV *rev*	RRNRRRRW	Internal (45/116)
Mat α2 (yeast)	NKIPIKD	N-term (8/210)
Histone H2B (yeast)	GKKRSKA	Internal (35/131)
Xenopus nucleoplasmin	avkRPAATKKAGQAKKKkld	C-term (172/200)
Xenopus N1/N2	LVRKKRKTEEESPLKDKDAKSKQ	C-term (553/589)
Rat glucocorticoid-R	RKTKKKIK	Internal (517/930)
Human lamin A	SVTKKRKLE	Internal (442/664)
Human estrogen-R	IRKDRRG	Internal (261/595)
Human *c-myc*	PAAKRVKL	C-term (327/439)
	RQRRNELKRSF	C-term (374/439)

Nuclear localisation signal (NLS)

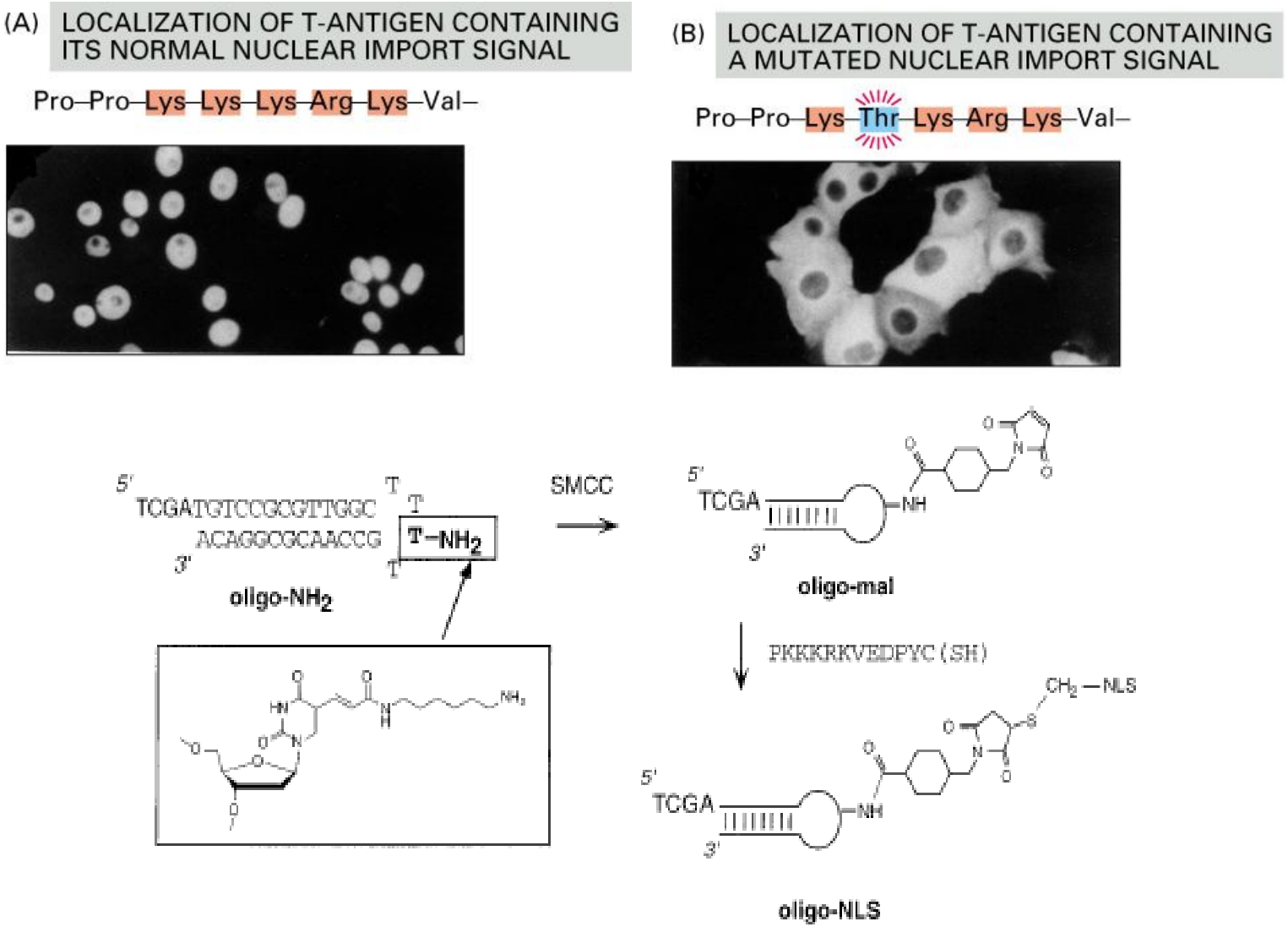
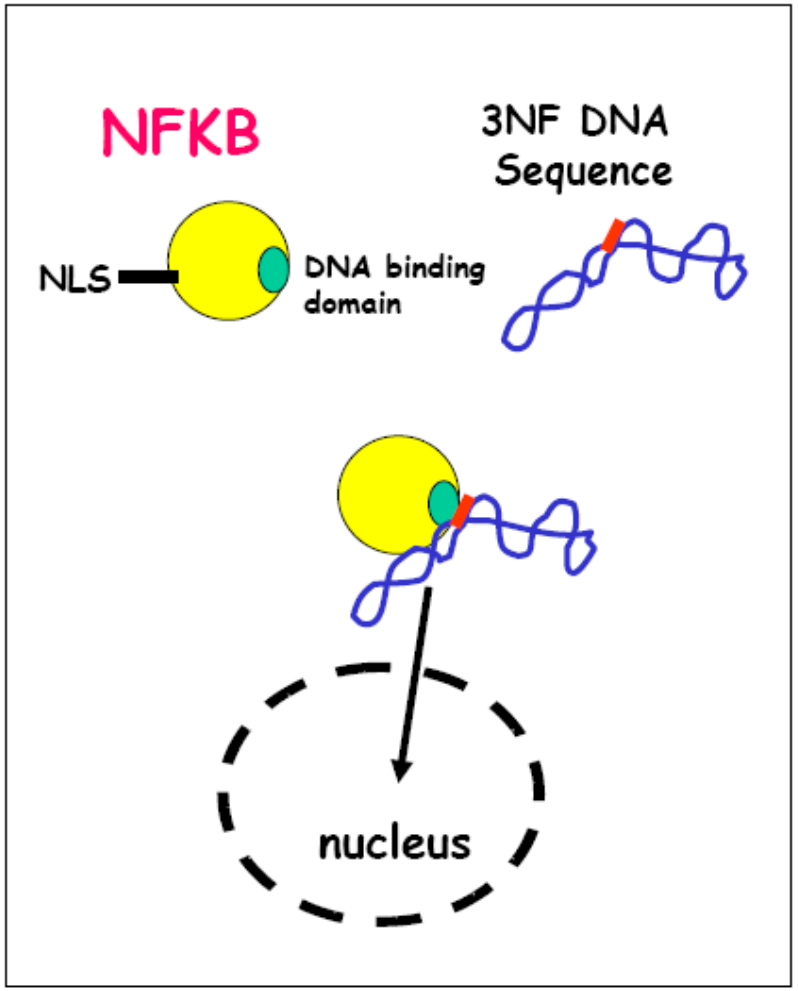


FIG. 2. Reaction scheme for the chemical coupling steps leading to the oligonucleotide-peptide conjugate (oligo-NLS). A hairpin oligonucleotide with a free alkylamino group in the T_4 loop (oligo-NH_2) was reacted with the heterobifunctional crosslinker SMCC to give a thiol-reactive maleimide oligonucleotide (oligo-Mal), which was in turn reacted with the C-terminal cysteinamide residue of the NLS dodecapeptide.

Nuclear localisation signal – subunits of transcription factors



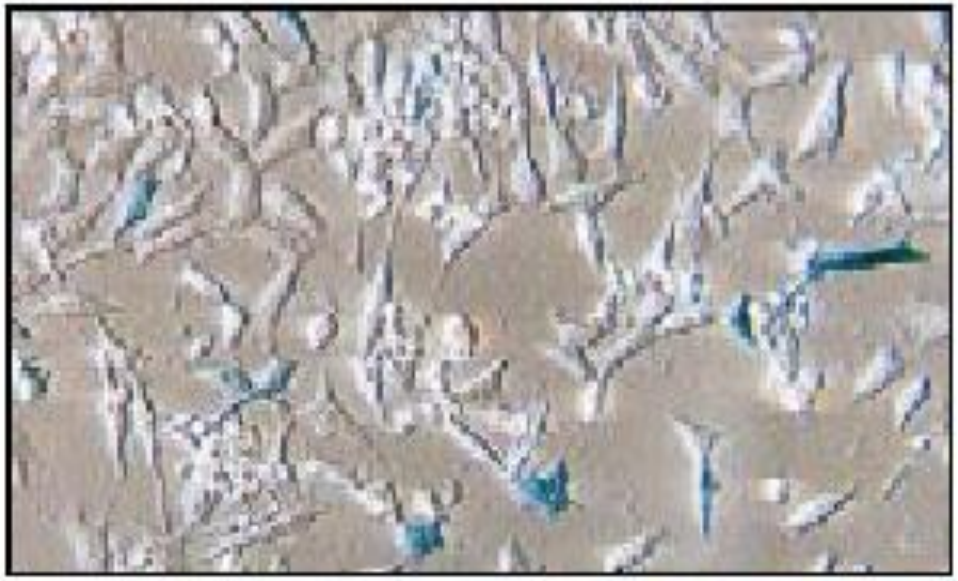
Chantal Pichon
Maryvonne Ardourel (Univ Orléans)
Brigitte Hartmann (IBPC CNRS, UPR 908013, Paris

Lipotransfection – in vitro conditions

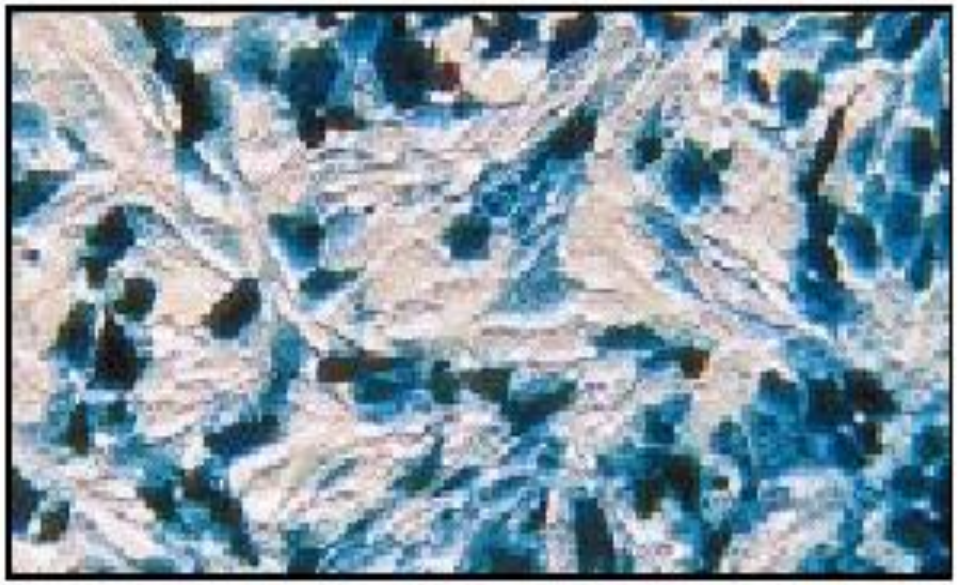
1. Quality of DNA – protein, RNA and other chemicals-free
2. Cell types
3. Liposome types
4. Phase of the cell growth (confluency)
4. Optimal proportions of DNA and liposomes
6. Culture conditions – lack of serum

Optimalisation of the transfection conditions

Transfection efficiency – comparison of methods

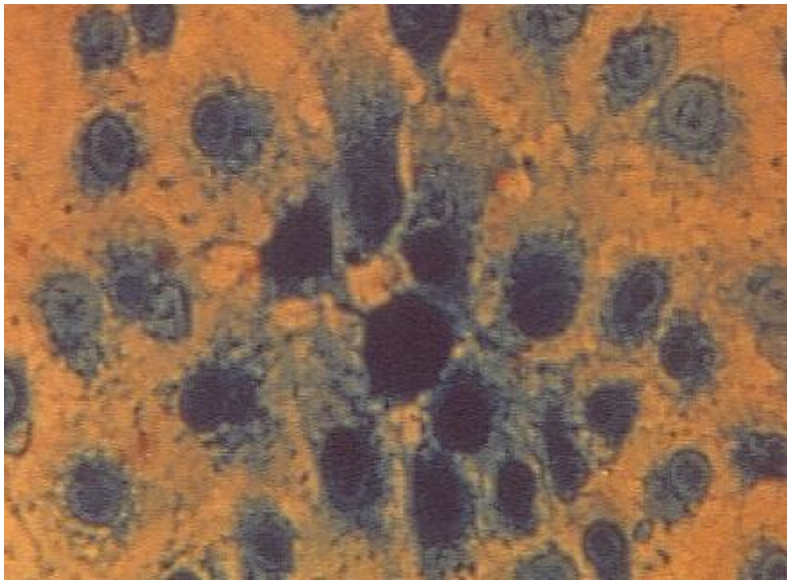


Ca^{2+} - transfection

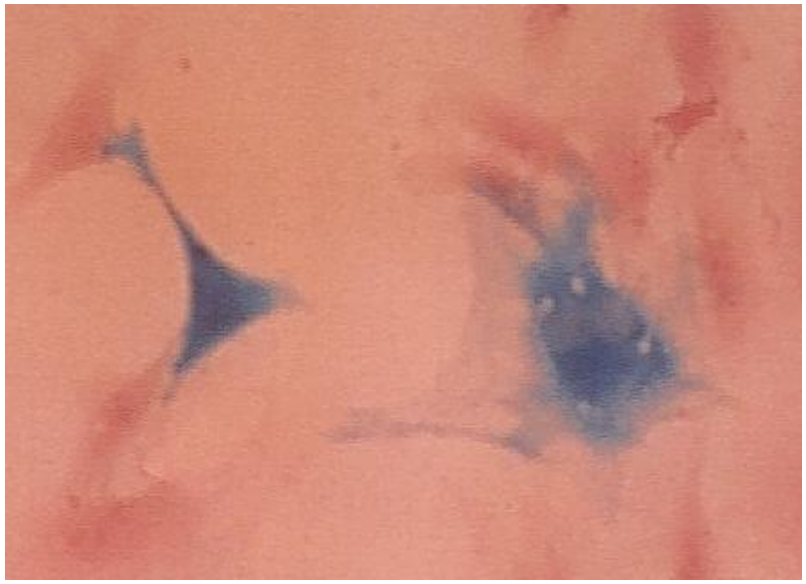


Lipotransfection

Lipotransfection efficiency depends on cell types

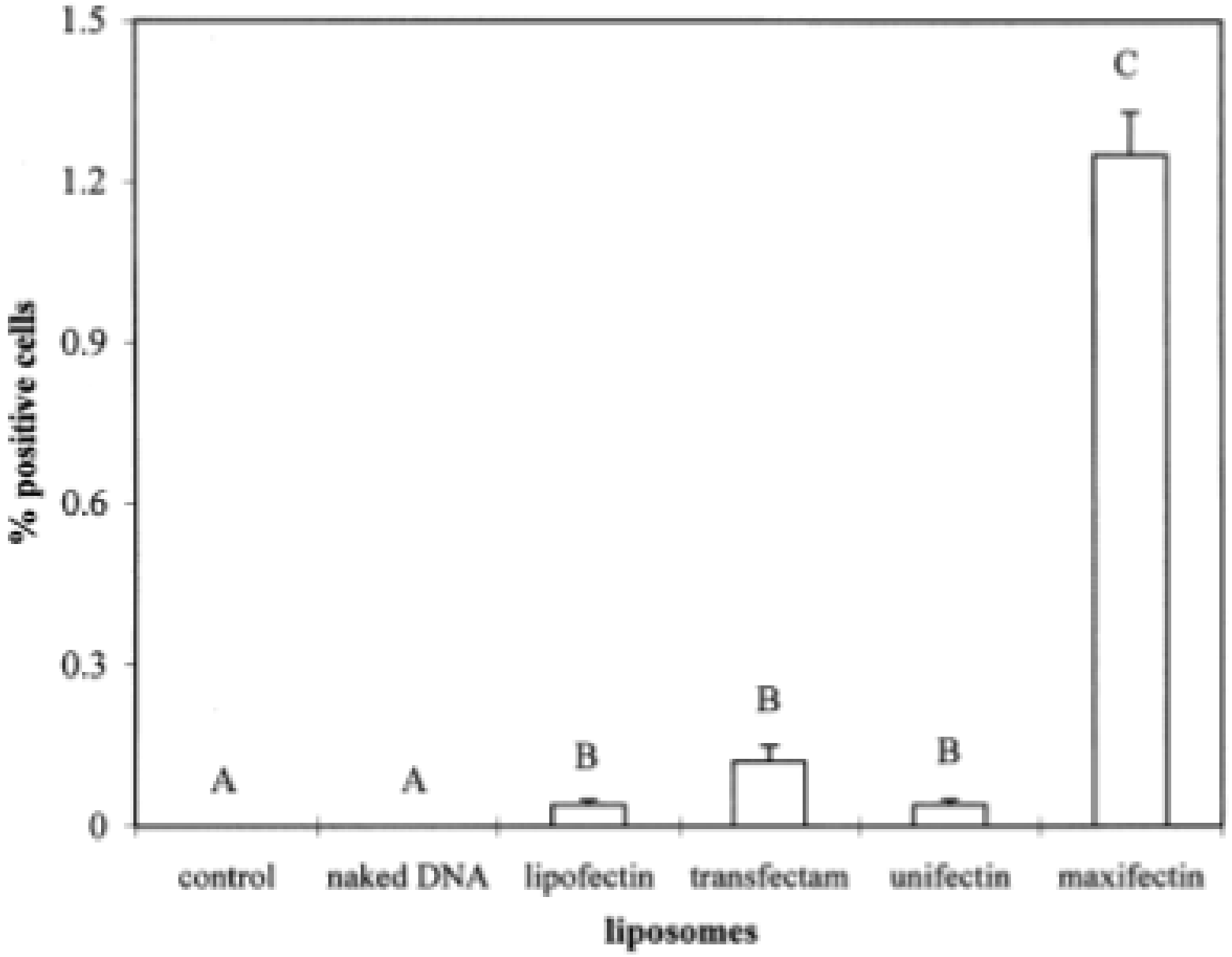


Fibroblastas COS-7
(*monkey kidney cells*)



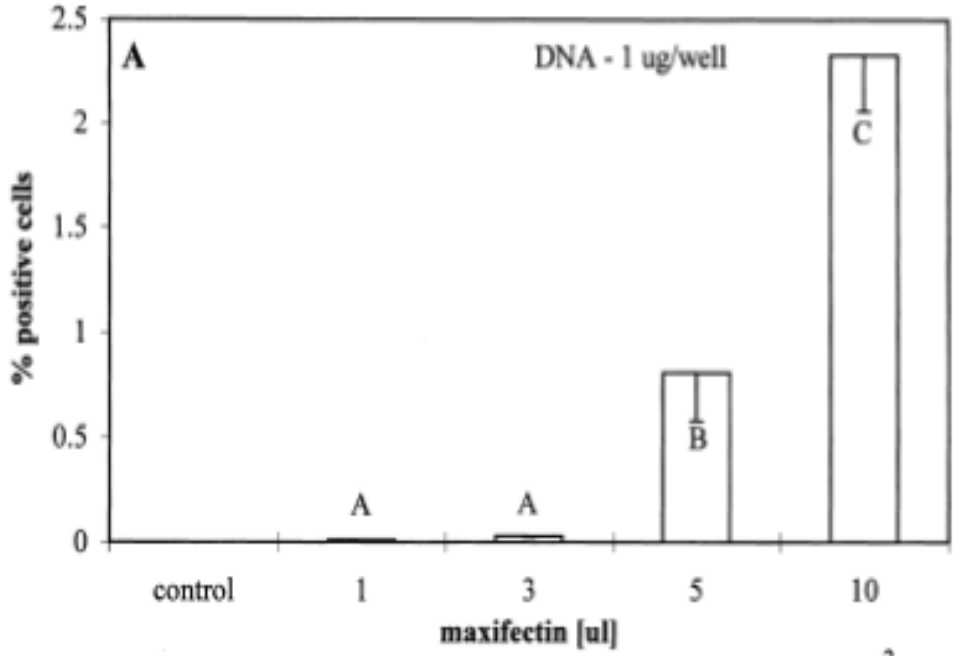
Aortic vascular smooth muscle cells

Transfection efficiency depends on the type of liposome



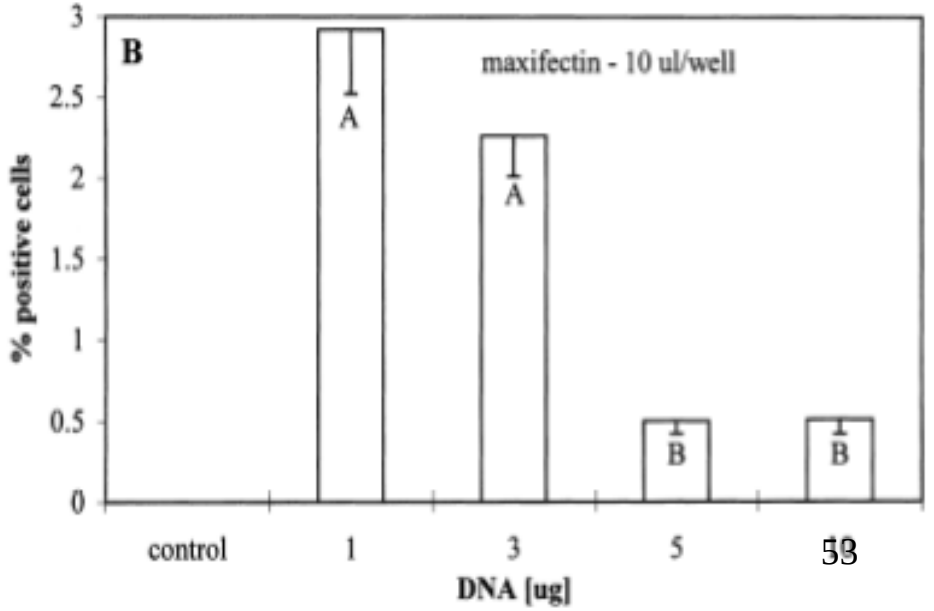
Jozkowicz & Dulak, Clin Chim Acta 1999

Transfection effciciency depends on DNA and liposome ratio



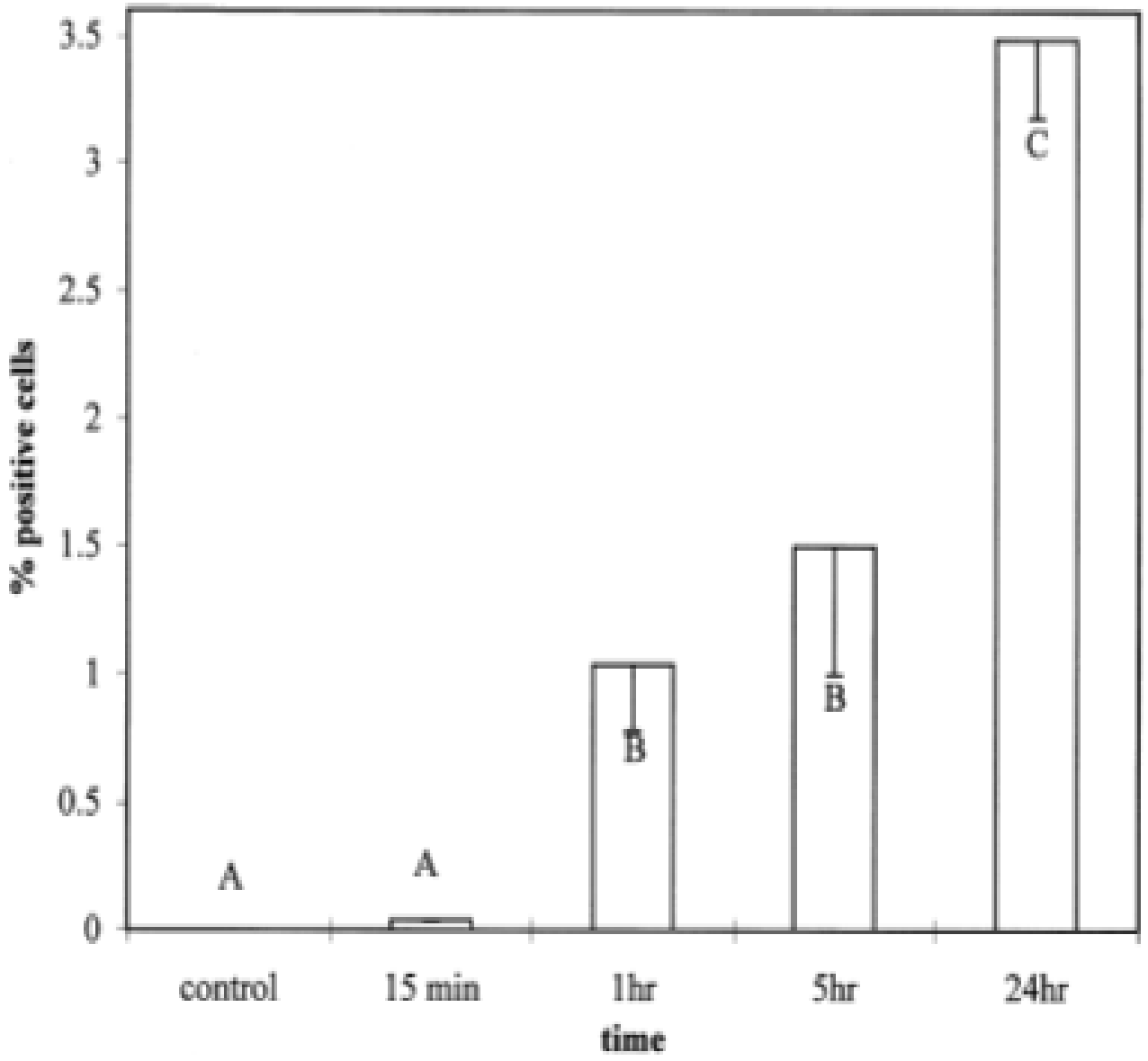
The same amount of DNA,
Variable amount of liposome

The same amount of liposome
Variable amount of DNA



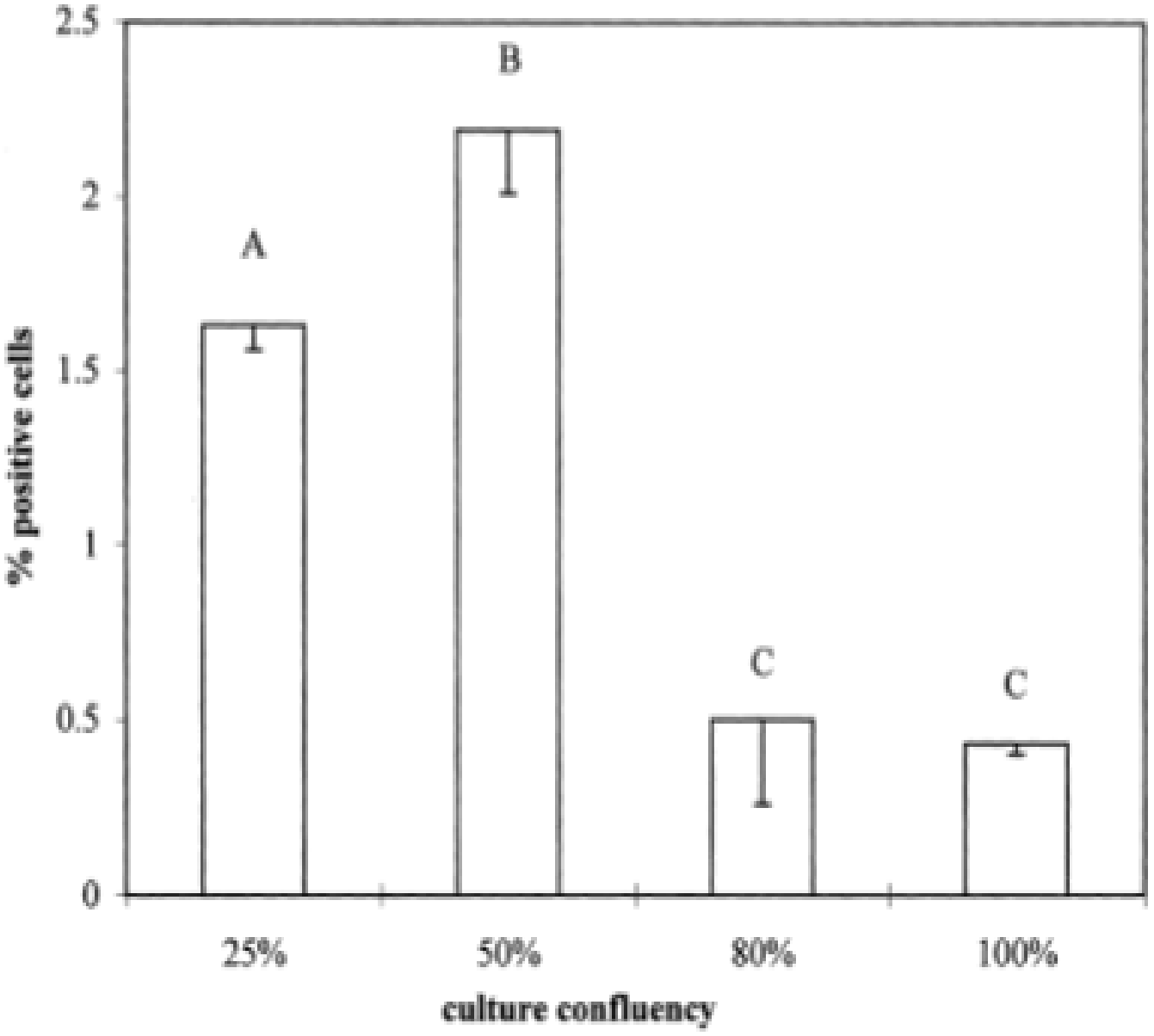
Jozkowicz & Dulak, Clin Chim Acta 1999

Transfection effeciency and time of incubation of lipoplexes with cells



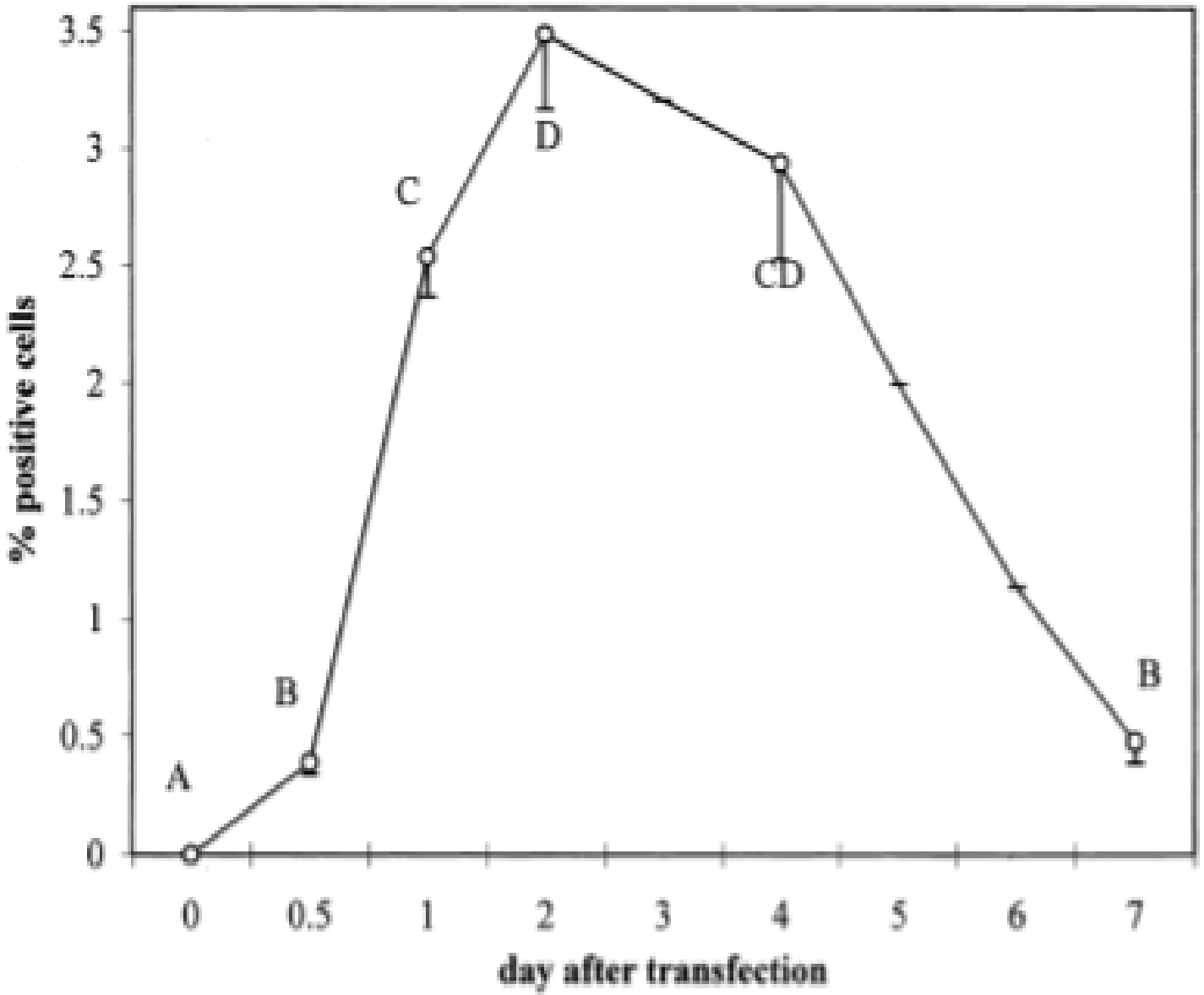
Jozkowicz & Dulak, Clin Chim Acta 1999

Transfection efficiency and cell confluency on a dish



Jozkowicz & Dulak, Clin Chim Acta 1999

Duration of expression



Jozkowicz & Dulak, Clin Chim Acta 1999

Liposomes – optimalisation

1. Improvement of electrostatic properties
    a) proper choice of components
        (eg. DOPE – enables exit from endosomes)
    b) variability in chemical structure of liposomes
    c) type and amount of neutral lipids
    d) addition of enhancers
    d) final charge and ratio to nucleic acid

Lipoplexes – problems with in vivo efficiency

1. Lack of stability
2. Low efficacy of transfection
3. Short-term expression
4. Unspecific interactions
5. Inductions of inflammatory response by:
   - components of liposomes
   - unmethylated CpG dinucleotides

Transfection efficiency of cationic liposomes

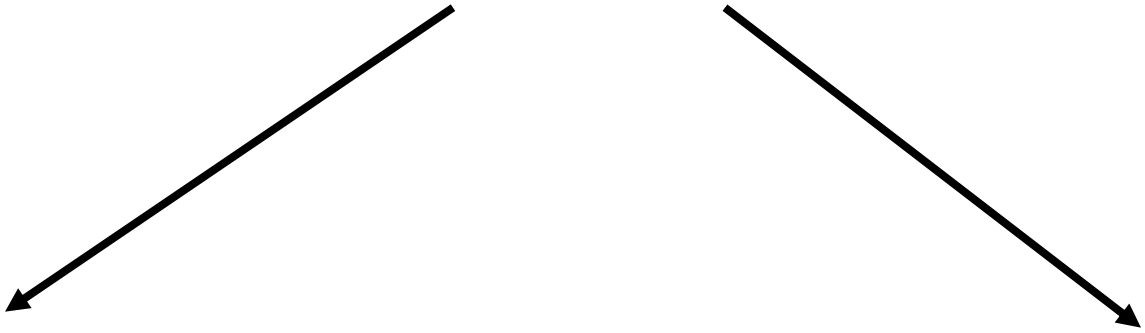
100%	1, 000,000 plasmids/cell transfected
30%	300,000 plasmid/cell in pellet
5%	50,000 plasmids/cell intracellular
0,3-0,1%	1,000 plasmids/cell intranuclear

History of development of gene transfer techniques

(1958 – Alexander et al. – purified polio virus RNA infects cells)

1962 – Szybalski &Szybalska – transfer of cellular DNA – calcium ions and spermin
1965 – Vaheri & Pagano – DEAE dextran
1973 – Graham & van der Eb – calcium phosphate (human adenovirus 5 DNA)
1979 – Mulligan – transfection of plasmid with rabbit β-globin gene to monkey kidney cells
1982 – Souther & Berg – selection of cells resistant to neomycin
1987 – Felgner – cationic liposomes
1987 – Wu & Wu – receptor-mediated transfection (polylysine)
1988 – Johnson – gene gun
1995 – Boussif et al.- poliethylenoimine
1996 – Tang i wsp. - dendrimers

Lipotransfection



local **systemic**

Clinical applications of liposomes

1. Lung diseases – cystic fibrosis - easy delivery - aerosol
2. Tumors
3. Hemophilia
4. Cardiovascular diseases

Ligands improving transfection efficiency

1. Peptides which have the specific cellular receptors, eg. RGD – interactions with integrin receptors

2. Nuclear localisation signals
3. pH sensitive ligands – facilite exit from endosomes
4. Spheric stabilizing components
5. Viriosomes – HVJ (Sendai virus)

Receptor-targeted transfection

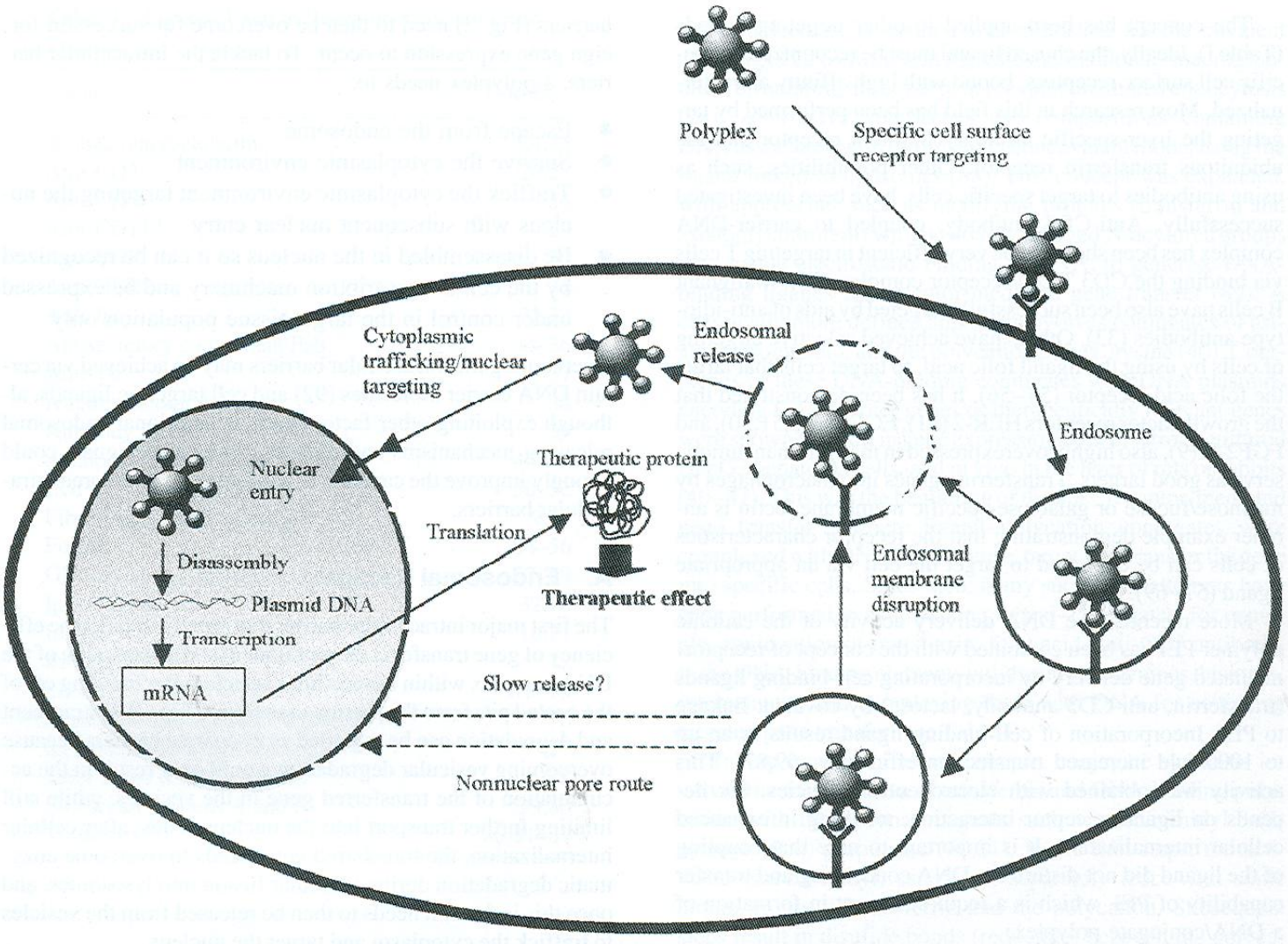
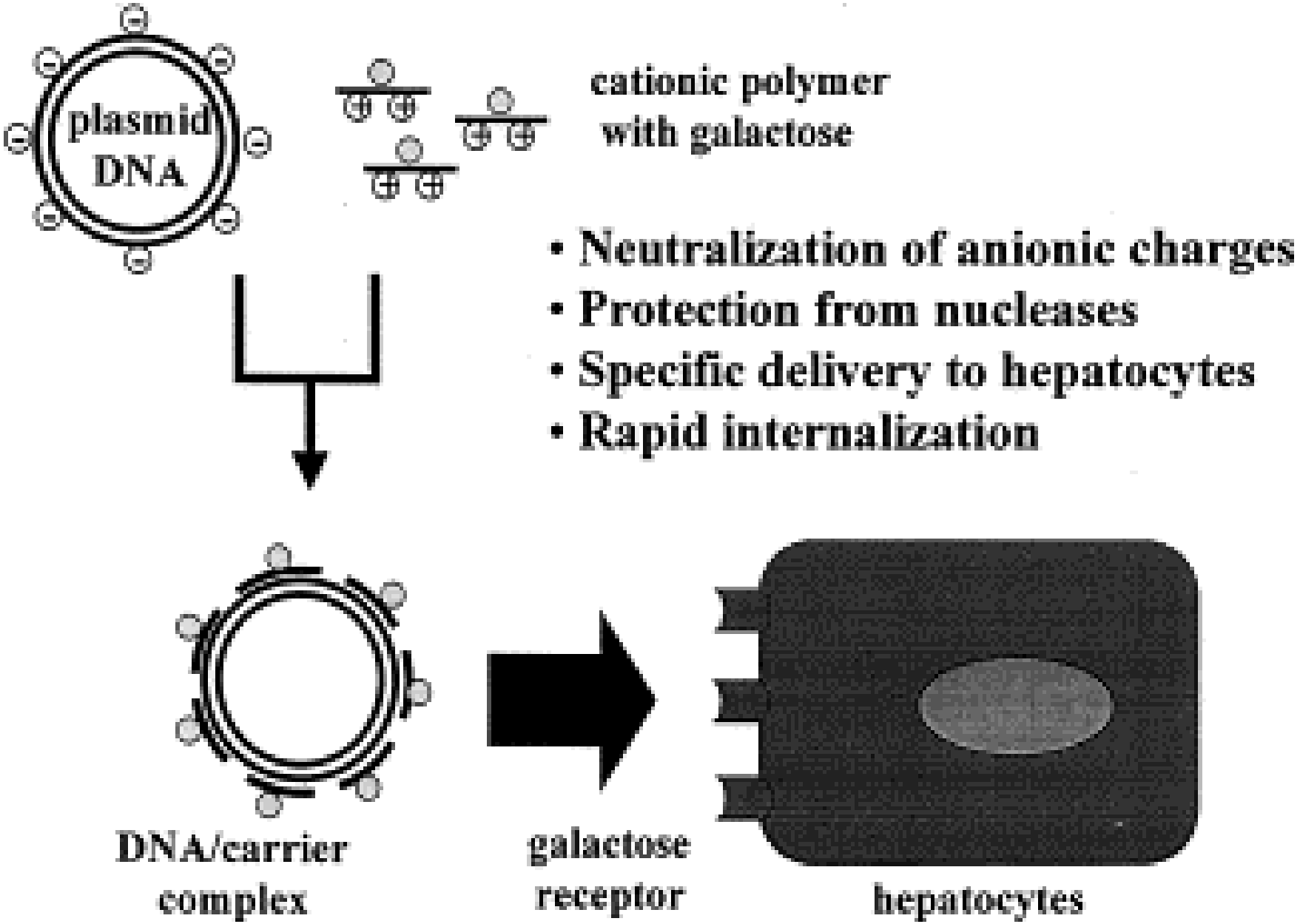


Figure 2 Intracellular release and trafficking of polyplexes.

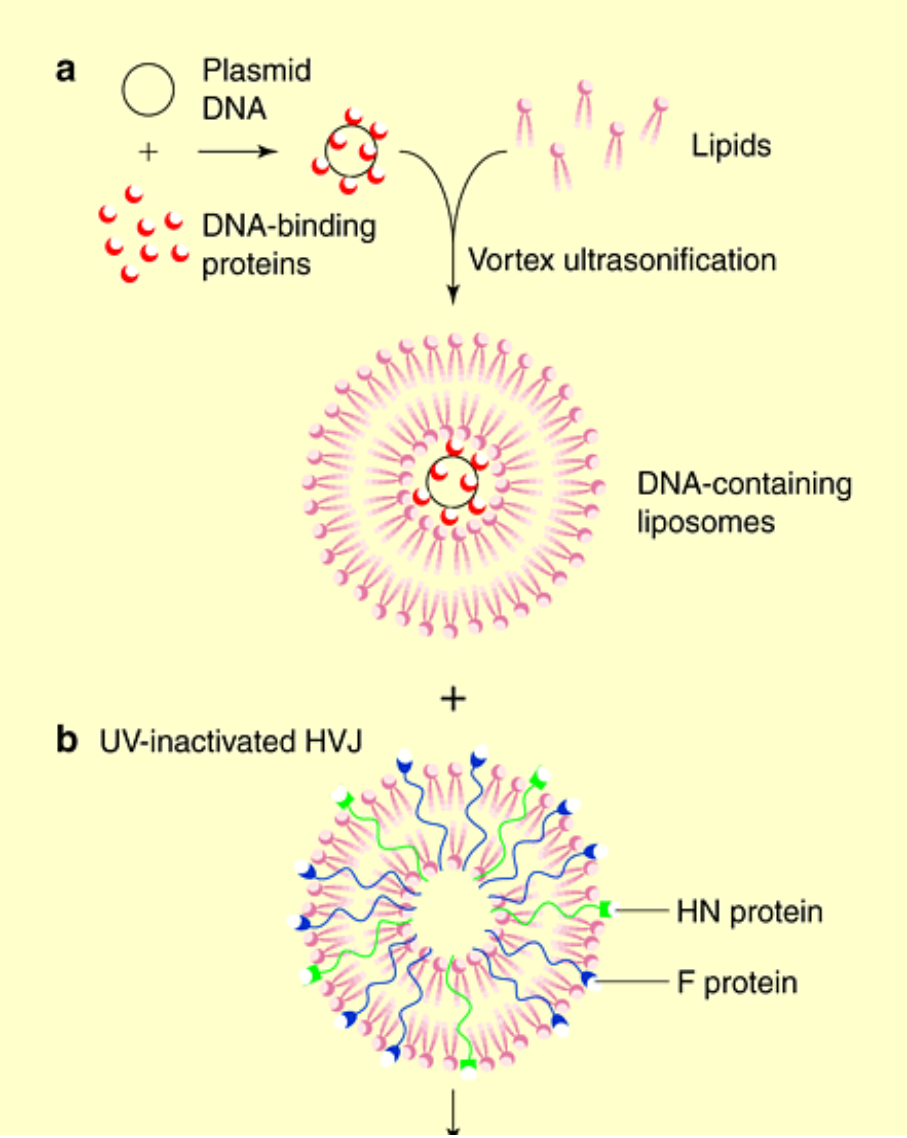
Table 1 Ligands Used in Receptor-mediated Gene Transfer

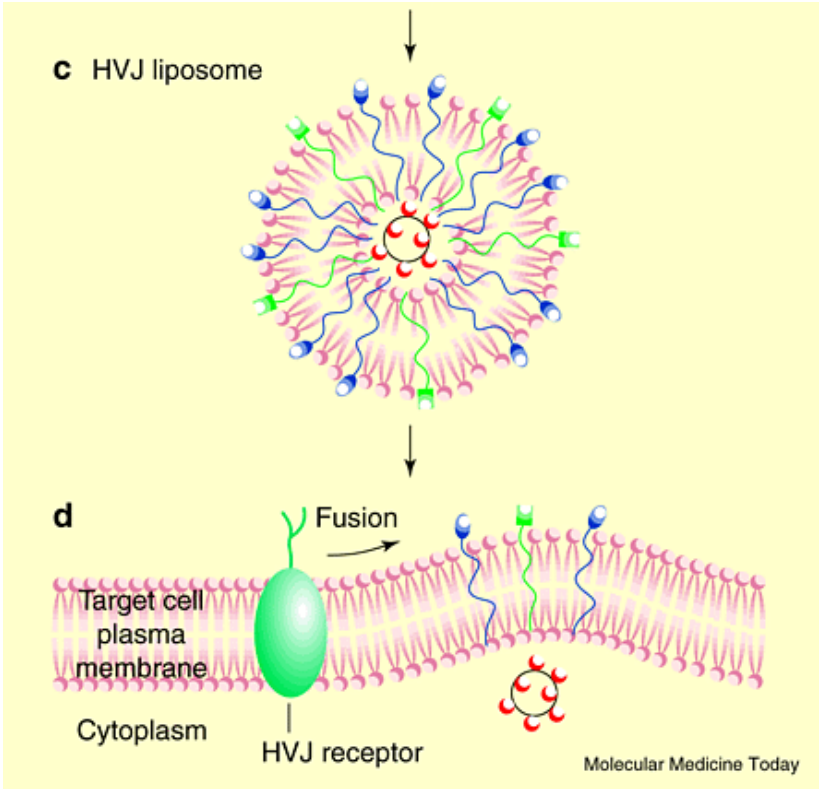
Ligands	Refs.
Alpha2 macroglobulin	24,25
Anti-CD3	26,27
Anti-CD5	28
Anti-CD117	29
Anti-EGF	30
Anti-HER2	31
Anti-IgG	32,33
Antisecretory component Fab	34–36
Anti-Tn	37
Antithrombomodulin	38
Antibody ChCE7	39
Asialoglycoproteins	40–49
EGF	50–52
Fibroblast growth factor 2(FGF2)	9,53
Folate	54–56
Glycosylated synthetic ligands	57–69
IgG (FcR ligand)	32,70
Insulin	10,71
Invasin	72
Lectins	73–75
Malarial circumsporozoite protein	76
RGD-motif (integrin binding)	77
Steel factor (CD117 ligand)	78
Surfactant proteins A and B	79,80
Transferrin	13,81–87


plasmid DNA
cationic polymer with galactose
• Neutralization of anionic charges
• Protection from nucleases
• Specific delivery to hepatocytes
• Rapid internalization
DNA/carrier complex
galactose receptor
hepatocytes

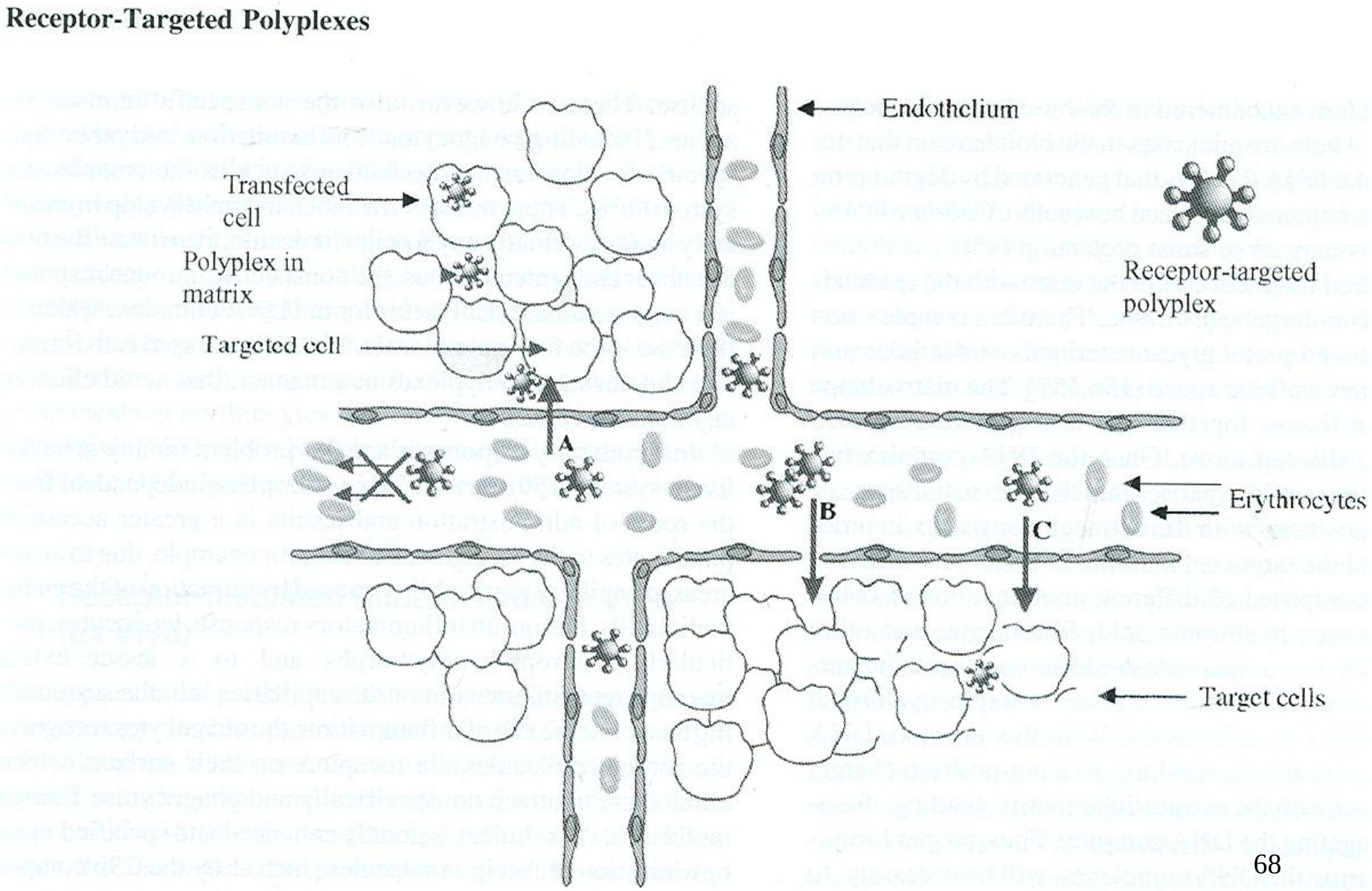
HVJ liposomes

Sendai virus = haemaglutinin virus of Japan

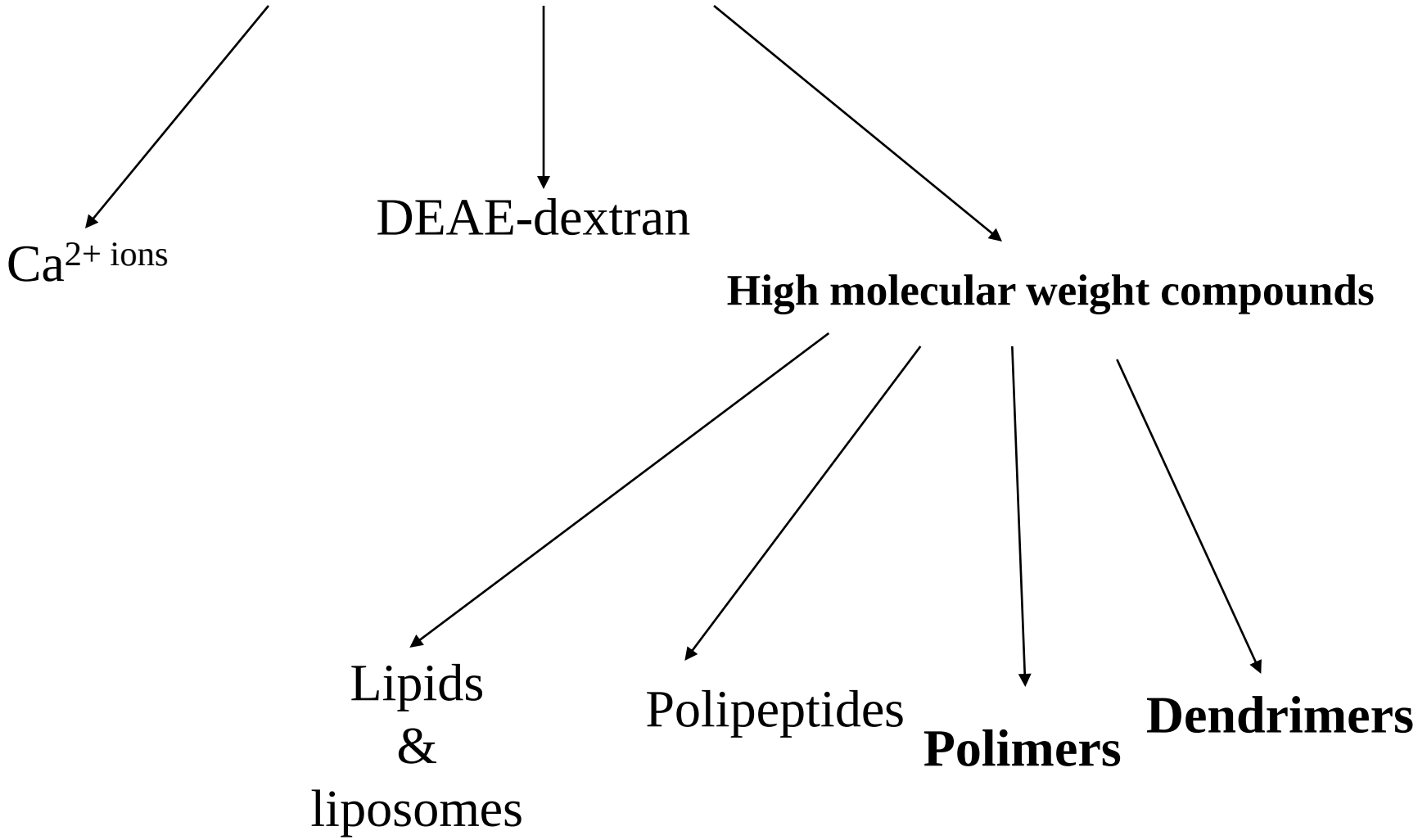




Targeted delivery allows to avoid barriers hindering gene delivery



Chemical vehicles for plasmid DNA



Polipeptide vehicles

- poli-L-lysine (PLL)
- poli-L-ornithine
- protamines

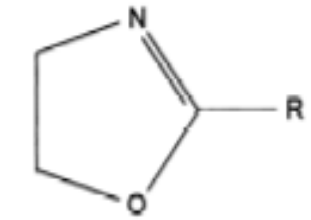
Complex vehicles

Gal4-invasin – complexes with PLL & DNA

Contains a domain of a yeast factor GAL-4 and invasin domain

(part of a protein of Yersinia pseudotuberculosis)

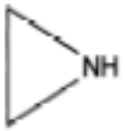
POLYETHYLENIMINE



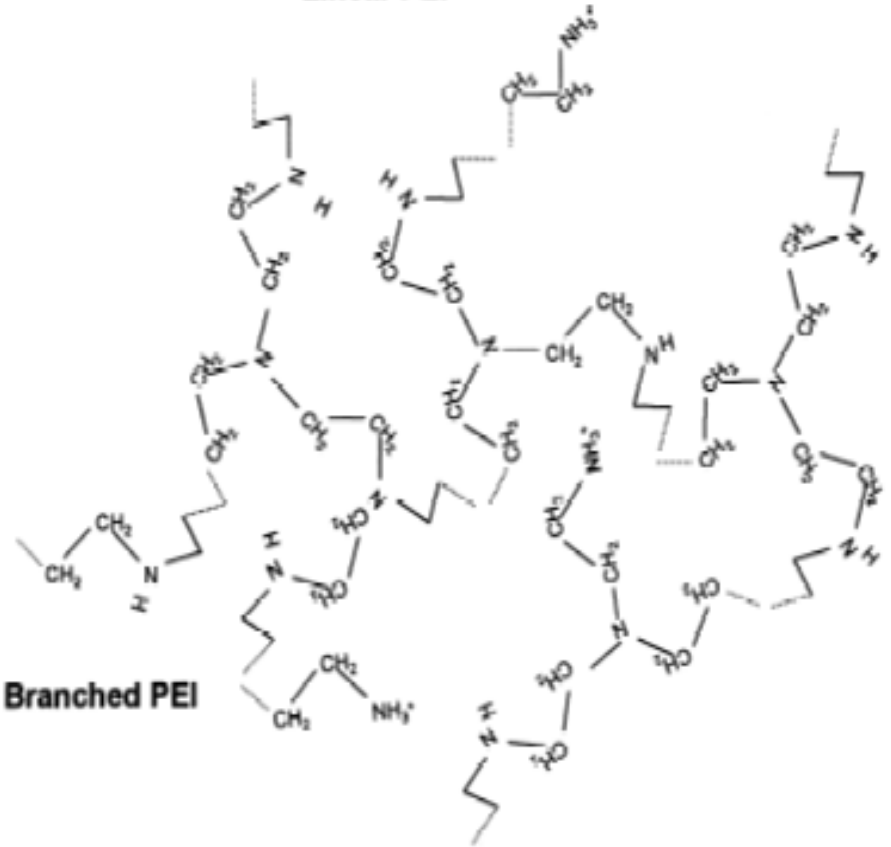
2-Substituted 2-Oxazoline

$H_3C\text{-}CH_2\text{-}NH\text{-}[CH_2\text{-}CH_2\text{-}NH]_n\text{-}CH_2\text{-}CH_2\text{-}NH_3^+$

Linear PEI*



Aziridine



Branched PEI

Fig. 1. Structures of PEI precursors and end products. *Aziridine can also yield linear PEI under certain conditions.

PEI - polyetyleneimine

1. Synthetic polication, MW ~ 22 kDA
2. Behaves like a proton sponge – concentration of ions in the endosome increases, water enters into the endosome, which is lysed and DNA is released to cytoplasm

Other applications of PEI

1. Receptor-dependent transfection – complex of PEI/DNA with inactivated adenovirus

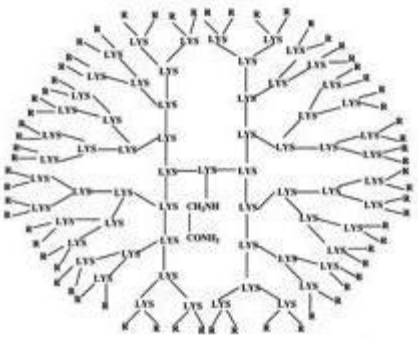
Dendrimers

Dendrimers represent one of several non-viral systems used for delivering nucleic acids into cells.

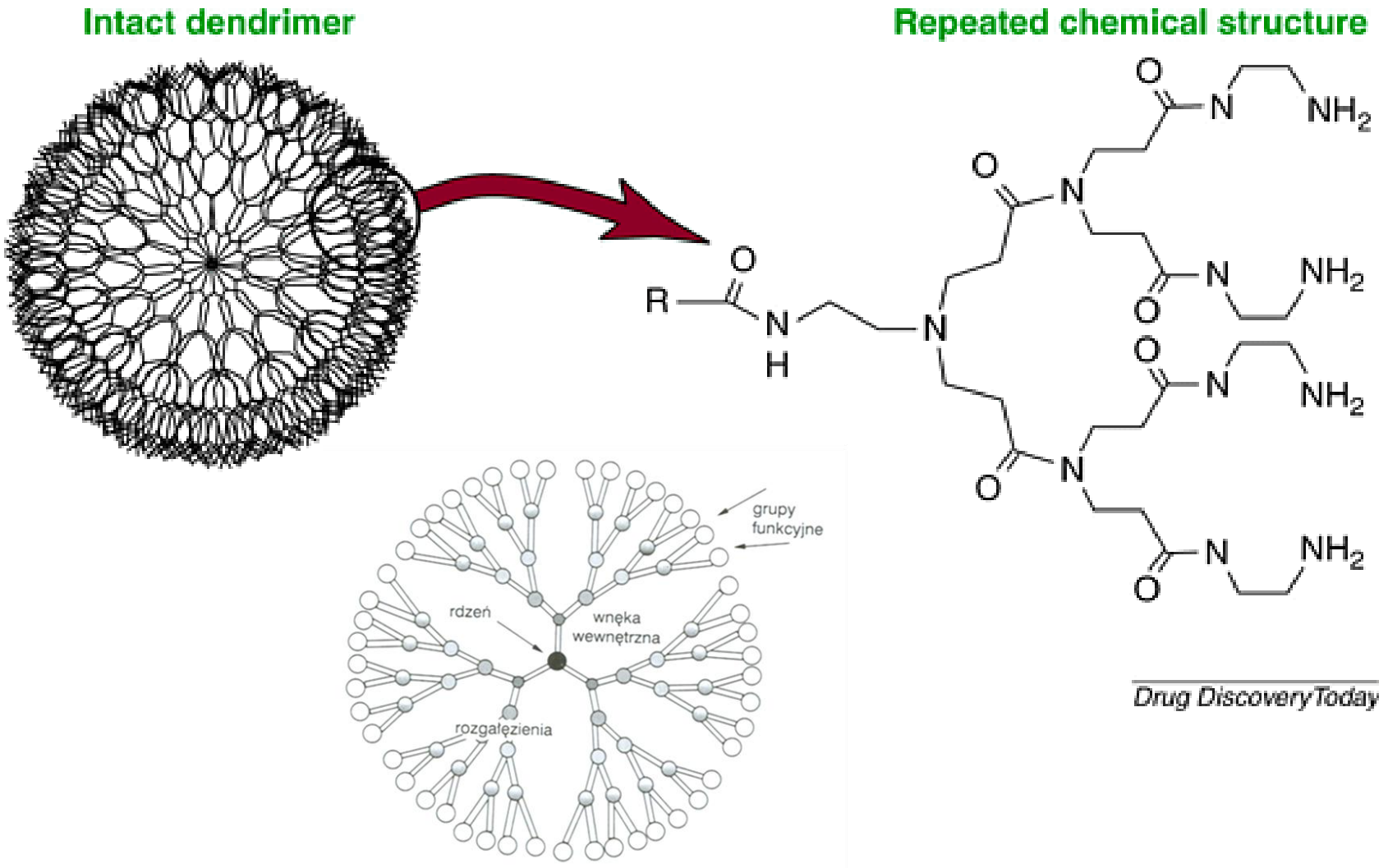
They are synthetic hyperbranched polymers which are highly soluble in aqueous solutions.

Dendrimers with positively charged terminal groups can bind DNA forming complexes , termed **dendriplexes** by analogy with similar complexes formed by liposomes and DNA called lipoplexes.

The DNA within dendriplexes is protected from cellular and restriction nucleases

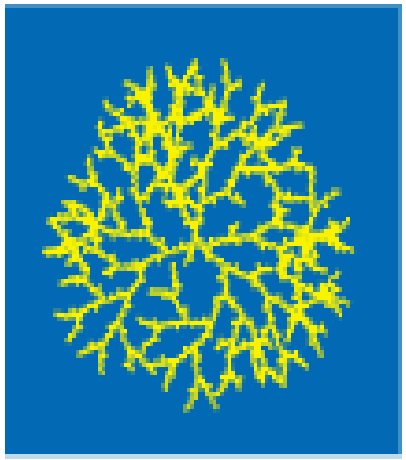


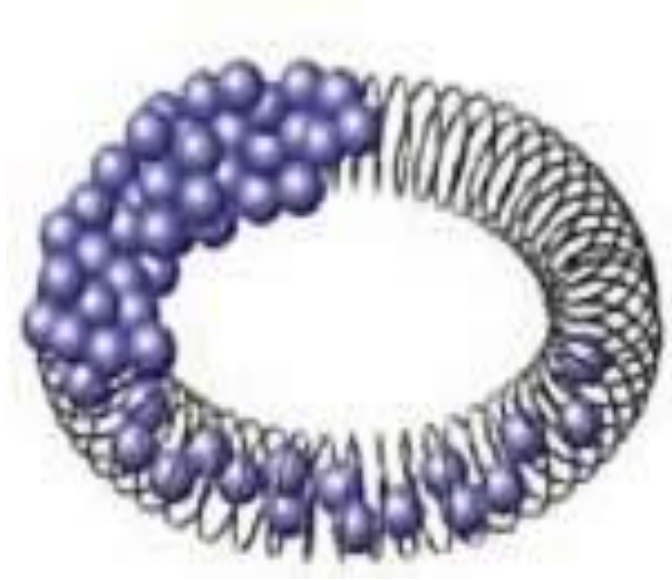
Poliamidoamid dendrimer



Poliamidoamid dendrimer

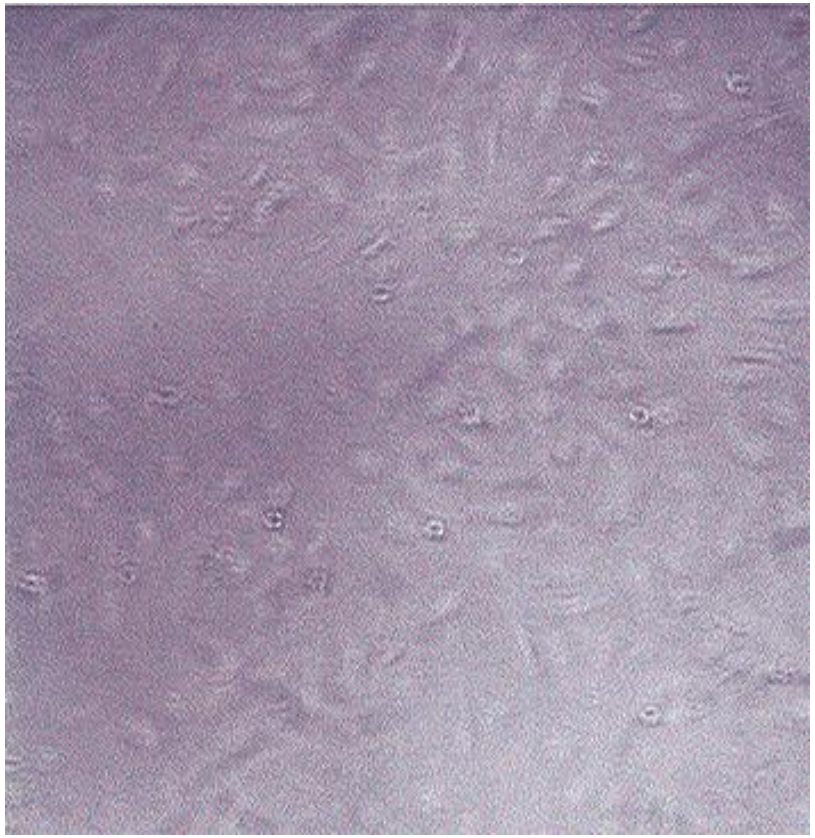
SuperFect Reagent consists of activated-dendrimer molecules with a defined spherical architecture (1). Branches radiate from a central core and terminate at charged amino groups which can then interact with negatively charged phosphate groups of nucleic acids

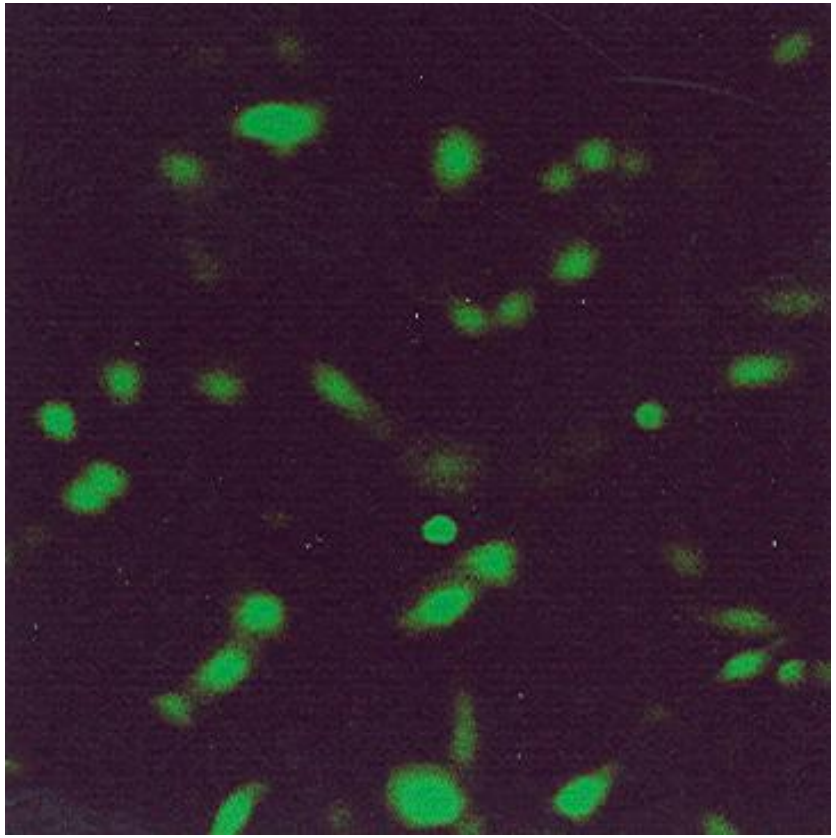




*Model of the **SuperFect–DNA complex**. SuperFect Reagent (purple balls) interacts with DNA (black) to form a ring-like (toroid-like) structure. The upper right section of the illustration shows naked DNA, the lower section shows the interaction between dendrimers and DNA inside the complex, and the upper left section shows the final complete coverage of DNA within the complex.*

Delivery of plasmid vectors to endohelial cells by means of Superfect





Human saphenous vein endothelial cells
GFP expression

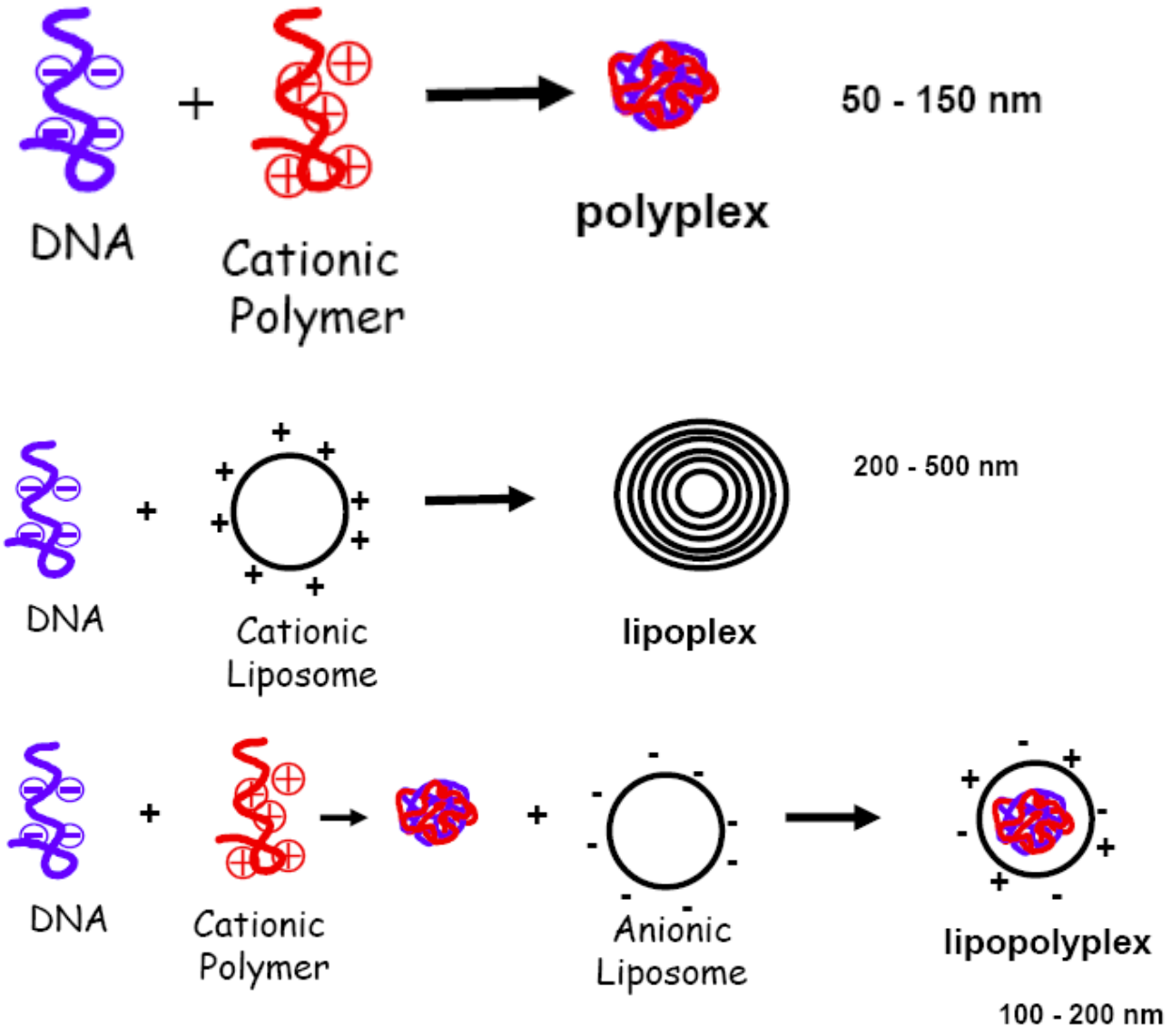
Cationic molecules facilitate the delivery of nucleic acids

1. Cationic lipids
   Usually formulated as cationic liposomes with a neutral co-lipid like
   DOPE or cholesterol

   Cationic liposomes form electrostatic complexes (so called lipoplexes) with pDNA.
At high positice charge ratios, lipids and pDNA undergo topological transformation
Into compact quasi-spherical vesicles of 200-300 nm in diamterem in which pDNA
And lipids adopt an ordered multilamellar structure

2. Other cationic polymers:
   - polylysine
   - polyethylenoimines
   - polyamidoamine
   - dendrimer
   - polyallylamine
   - methacrylate/methacrylamide polymers

Complexes of plasmids with various chemical vehicles have different size



Based on lecture of Dr Patick Midoux, 2007

Advantages of non-viral gene transfer

1. Decreased risk of insertional mutagenesis
2. Lack of the risk of viral infection
3. Limition of the response of organisms (immune and inflammatory)
4. Low costs and saving of time

Disadvantages of non-viral gene transfer

(Very) low efficiency of transfections in majority of tissues

Comparisons of various ways of transfection

	Non-viral	Retrovirus	Lentivirus	AAV	Adenovirus
Host tropism	Broad	Restricted	Broad	Broad	Broad
Construction system	Simple	Established	Difficult	Established	Established
Yield (titre)	High	Low	Low	Moderate	High
Transduction efficiency	Very low	Low	Low	Moderate	High
Capacity for transgene	Unlimited	4-5 kb	9 kb	4-5 kb	7-10 kb
Cytotoxic response	Low	Low	Low	Low	High
Duration of expression	Days	Long term	Long term	Long term	weeks-months

Wang Y. & Huang S. 2000. DDT 5: 10.