Heterologous systems

- Expression of recombinant proteins in cells where the proteins do not naturally occur
- Insulin first in *E. coli*
- Remember the drawbacks of expression in *E. coli*?

Other problems with *E. coli*

- Most proteins in *E. coli* expressed intracellularly
- Therefore, recombinant proteins expressed in *E. coli* accumulate in the cytoplasm
- Requires extra primary processing steps (e.g. cellular homogenization) and more purification (chromatography)

Other problems with *E. coli*

- Inclusion bodies
  - Insoluble aggregates of partially folded product
  - Heterologous expressed proteins overload the normal protein-folding machinery
  - Advantage- inclusion bodies are very dense, so centrifugation can separate them from desired material

Preventing inclusion bodies

- Lower growth temperature (from 37°C to 30°C)

- Use a fusion protein (thioredoxin) - native in *E. coli* – protein expressed at high levels and remains soluble

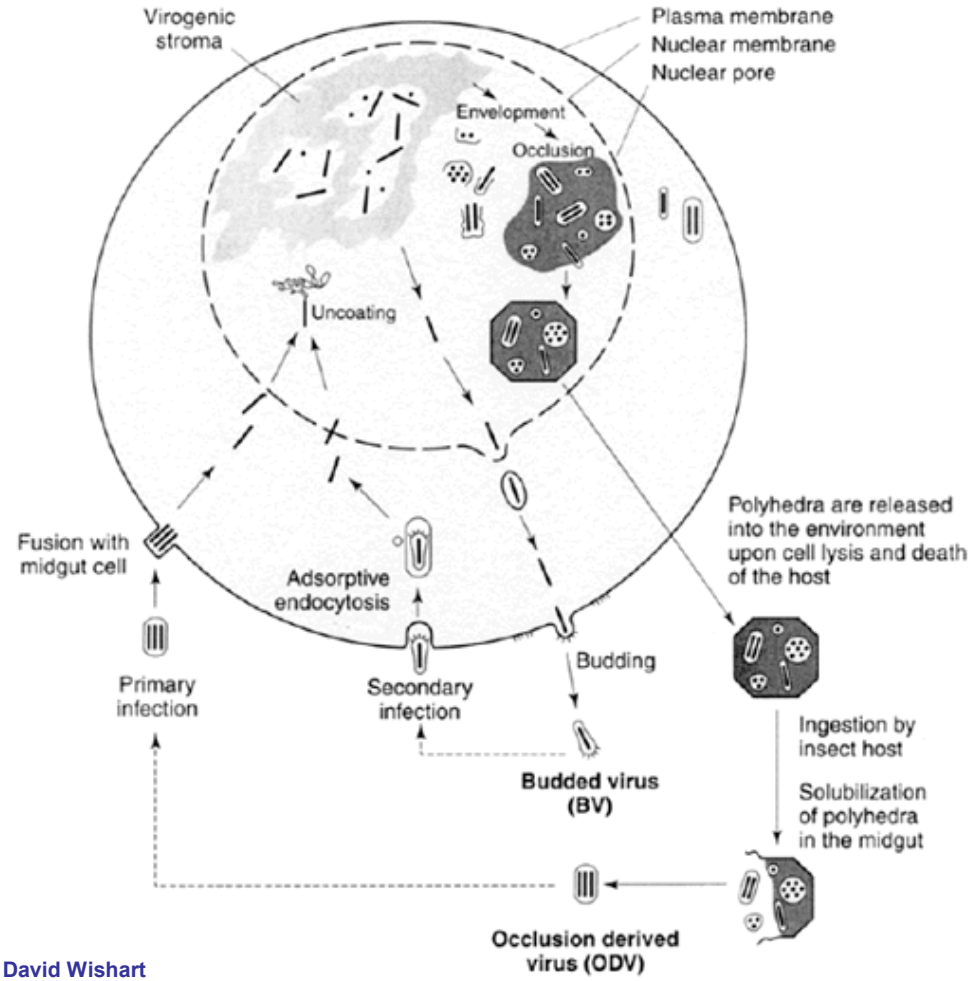
Problemi con *E. coli*

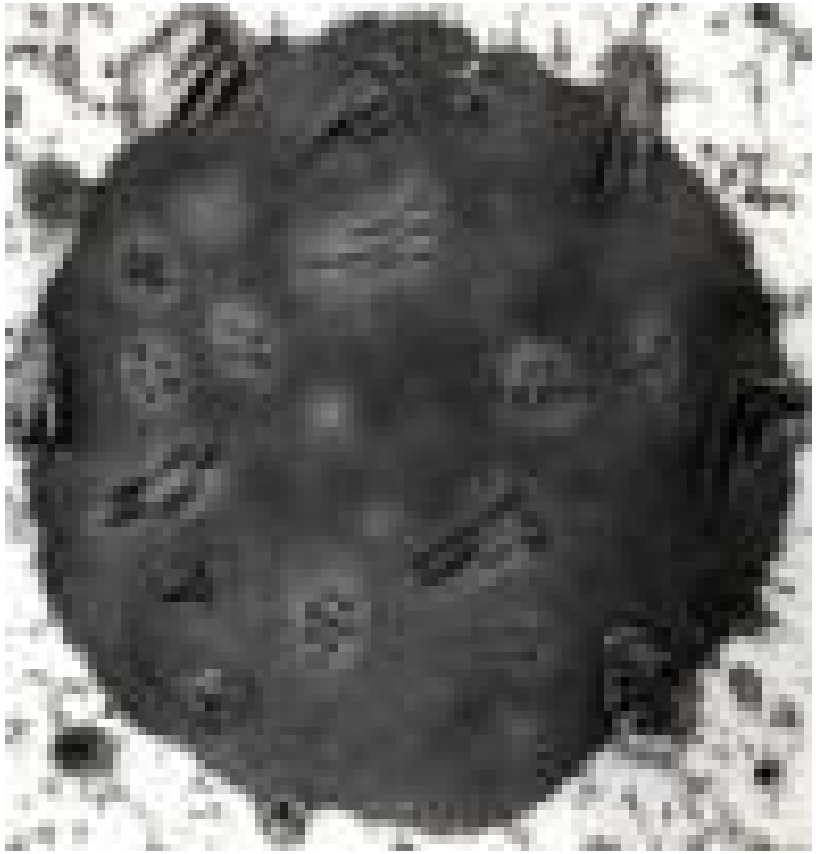
1. Le proteine purificate dai batteri possono contenere composti tossici o pirogeni (endotossine) che le rendono inutilizzabili

2. Proteine eucariotiche sintetizzate dai batteri sono instabili e/o prive di attività biologica a causa della mancanza di modificazioni post-traduzionali:

   -Formazione di ponti disolfuro (disolfuro isomerasi)
   -Taglio proteolitico del precursore
   -Glicosilazione (OH, NH2)
   -Fosforilazione (OH)
   -Acetilazione
   -Carbossilazione
   -Palmitoilazione (acido palmitico)

Baculovirus Expression





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Baculovirus Expression

- *Autographica californica* Multiple Nuclear Polyhedrosis Virus (Baculovirus) AcMNPV
- Virus commonly **infects insects cells** of the alfalfa looper (small beetle) or armyworms (and their larvae)
- Uses **super-strong promoter** **from the Polyhedrin coat protein** to **enhance expression of proteins** while virus resides inside the insect cell

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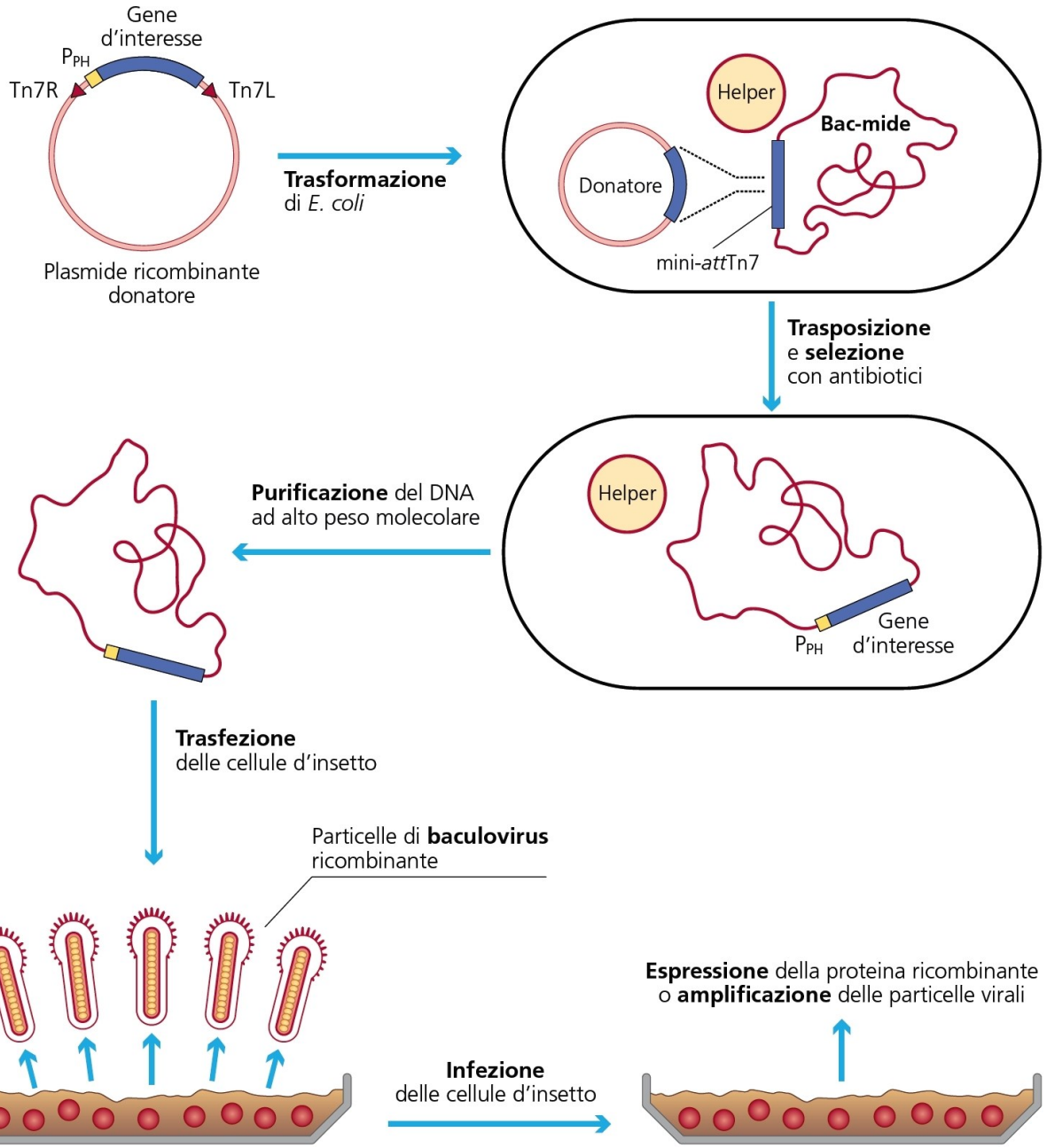
Gene d'interesse
P_{PH}
Tn7R
Tn7L
Plasmide ricombinante donatore
Trasformazione di *E. coli*
Helper
Bac-mide
Donatore
mini-*att*Tn7
Trasposizione e **selezione** con antibiotici
Helper
Gene d'interesse
P_{PH}
Purificazione del DNA ad alto peso molecolare
Trasfezione delle cellule d'insetto
Particelle di **baculovirus** ricombinante
Infezione delle cellule d'insetto
Espressione della proteina ricombinante o **amplificazione** delle particelle virali

Costruzione di un **BACMIDE** (4)

- Controllo dell'integrità del gene clonato tramite PCR
- Transfezione del BACMIDE ricombinante in cellule di insetto e espressione della proteina

Esistono anche protocolli per effettuare le manipolazioni genetiche di AcMNPV in cellule di lievito, utilizzando vettori navetta lievito-cellula di insetto

Baculovirus Systems

Disadvantages

- Grow very slowly (10-12 days for set-up)
- Cell culture is only sustainable for 4-5 days
- Set-up is time consuming, not as simple as yeast

Advantages

- Can express large proteins (>50 kD)
- Correct glycosylation & signal peptide removal
- Has chaperonins to help fold "tough" prtns
- Very high yields, cheap

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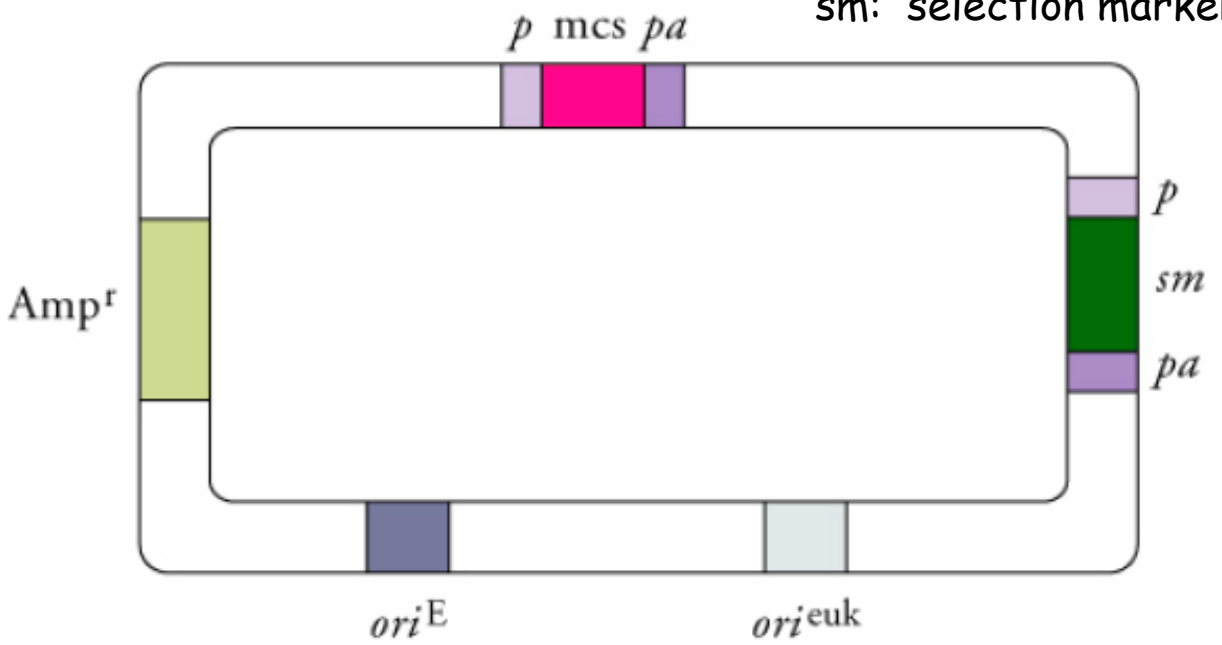
Vettori di espressione per cellule di mammifero

Funzioni:

1. Studio della funzione e regolazione dei geni di mammifero
2. Produzione di proteine ricombinanti "autentiche" utilizzabili in applicazioni terapeutiche

Sono simili agli altri vettori eucariotici

mcs: multiple cloning site
sm: selection marker



Vettori di espressione eucarioti

- **Origine di replicazione**
  - Generalmente derivata da virus animali (SV40)
- **Promotori/sequenze di arresto**
  - Generalmente derivati da virus animali o da geni di mammifero altamente espressi

    SV40, cytomegalovirus (CMV), herpes simplex virus (HSV)

    actina β, timidina chinasi, ormone della crescita bovino

Mammalian Cell-line Expression

- Sometimes required for difficult-to-express proteins or for "complete authenticity" (matching glycosylation and sequence)
- Cells are typically derived from the Chinese Hamster Ovary (CHO) cell line
- Usually use:
  - Vectors SV-40 virus,
  - CMV or vaccinia virus promoters
  - DHFR (dihydrofolate reductase) as the selectable marker gene

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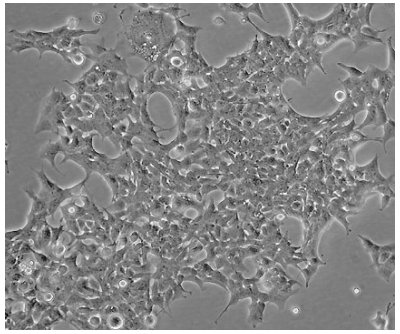
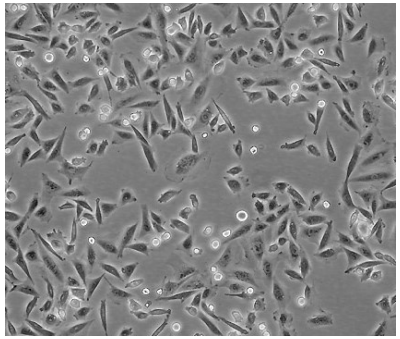
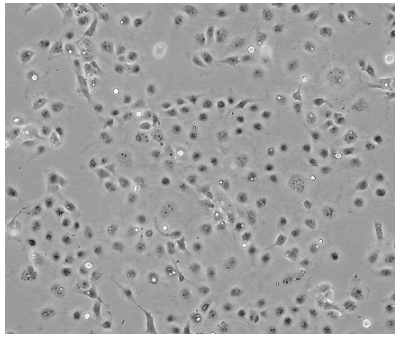
USED MAMMALIAN CELLS



HEK 293: Human embryonic kidney cells



CHO: Chinese Hamster Ovary cells



COS: Simian fibroblasts

TRANSFECTION OF MAMMALIAN CELLS

- Electroporation
- Calcium phosphate transfection
- Liposome based transfection reagents
- Micro-Inj
- Gun
- Virus-lentivirus

TYPES OF TRANSFECTION

- Transient
- Stable
- Episomal

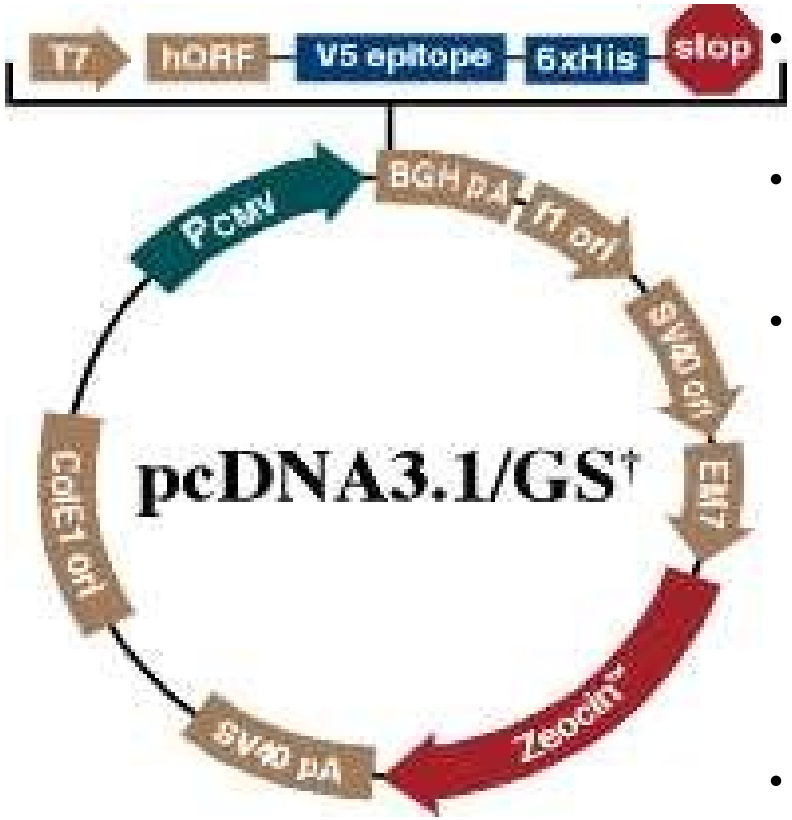
TRANSIENT TRANSFECTION

- Gene to protein in days
- Testing expression
- Functional studies
- Low yield
- Used in high-throughput structural studies (293 cells)

STABLE TRANSFECTION

- Gene to protein in ≥2 months
- Complex process
- Gene of interest integrates into genome of host cell
- High yields (from 1 to 5 mg/l and higher)
- Stock of cells expressing desired recombinant protein

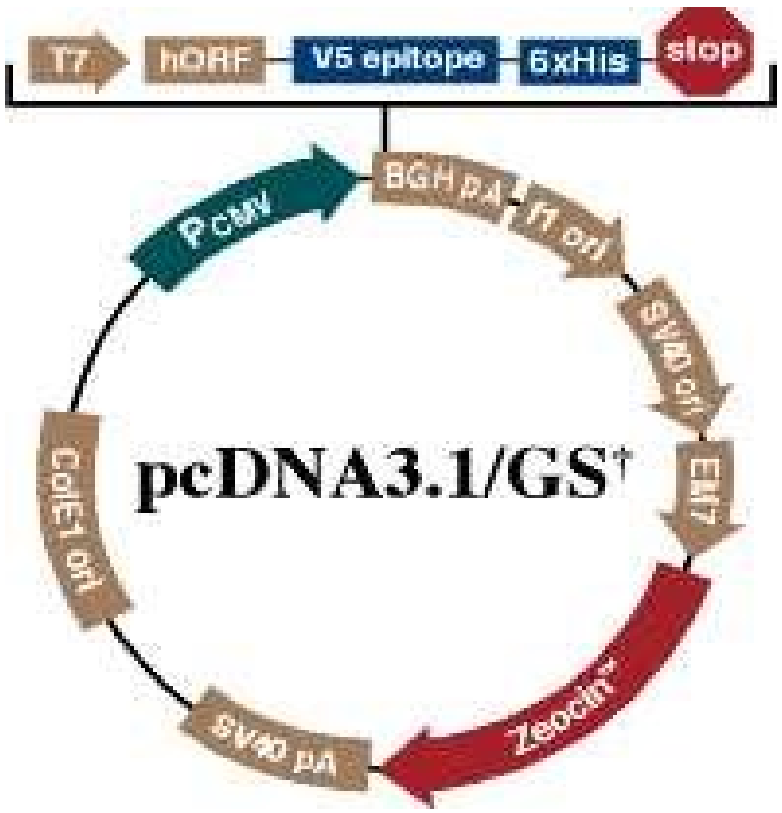
Example: Expression Vector



For eukaryotic expression, this vector (from Invitrogen) has

- a cauliflower mosaic virus promoter (P_{CMV}),
- a bovine growth hormone polyadenlyation site (BGHpA).
- The DNA inserted at "hORF" gets fused to a short peptide called an epitope, for which very specific anitbodies exist. It also gets fused to 6 histidines, which allow easy purification on a column that has nickel ions bound to it (an affinity tag).
- For growth in mammalian cells, it has an SV40 viral origin of replication (SV40ori),

Example: Expression Vector



For eukaryotic expression, this vector (from Invitrogen) has …….

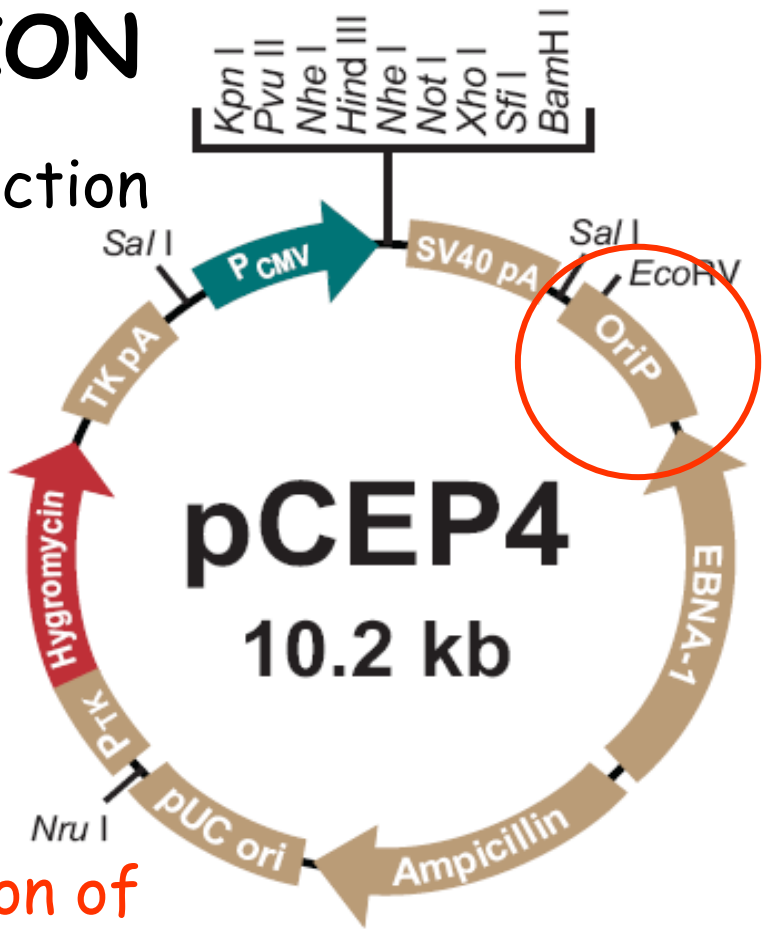
- a zeocin resistance gene (Zeocin, with SV40 promoter/enhancer and SV40 poly A site).
- For growth in E. coli it has the ColE1 replicon.
  Zeocin works as a selectable marker in bacteria as well as in eukaryotic cells.

There is also a

- T7 promoter for making RNA from the inserted gene, and an
- f1 origin of replication for making single stranded DNA (useful for sequencing).

EPISOMAL TRANSFECTION

- Gene to large scale protein production in ~ 4 weeks
- Straightforward process



- HEK EBNA cells (293 stably transfected with EBNA-1 gene)
- EBNA-1 driven episomal replication of Ori-P containing vectors
- Very high yields (5 to 20 mg/l and higher)

Mammalian Expression

- Gene initially cloned and plasmid propagated in bacterial cells

- Mammalian cells transformed by electroporation (with linear plasmid) and gene integrates (1 or more times) into random locations within different CHO chromosomes

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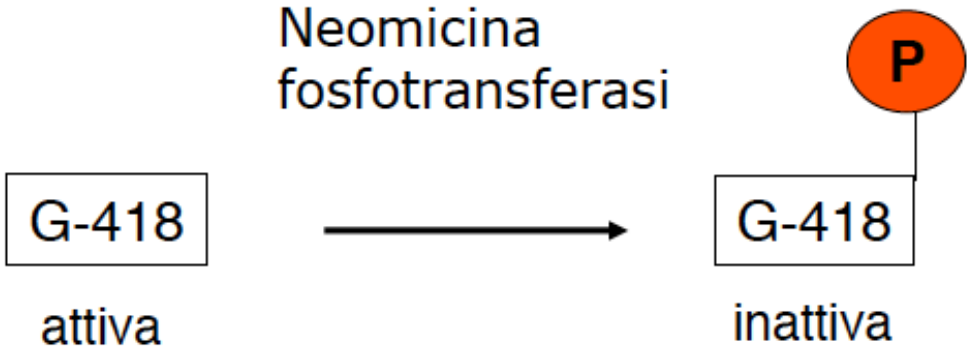
Marcatori selezionabili (1)

Neo^r: gene batterico che codifica neomicina fosfotransferasi

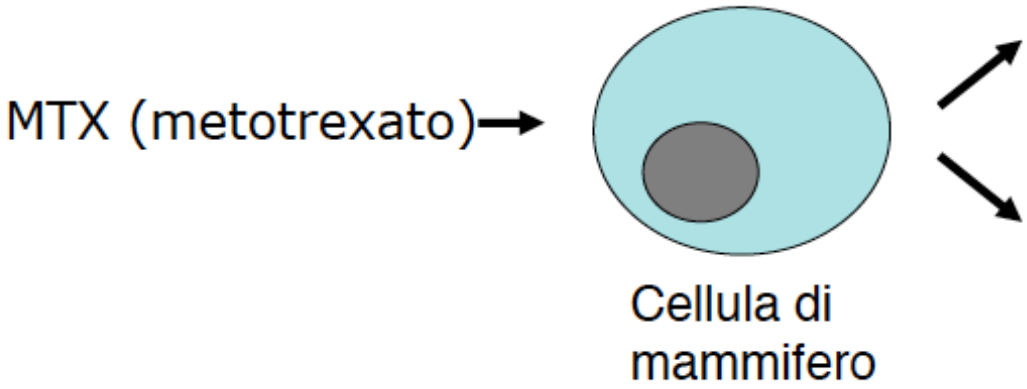
G-418 (geneticina): agente che blocca la traduzione e uccide la cellula



Marcatori selezionabili (2)

DHFR: gene che codifica diidrofolato reduttasi

Utilizzabile con cellule DHFR$^-$



-DHFR : inibizione della crescita

+DHFR : proliferazione normale

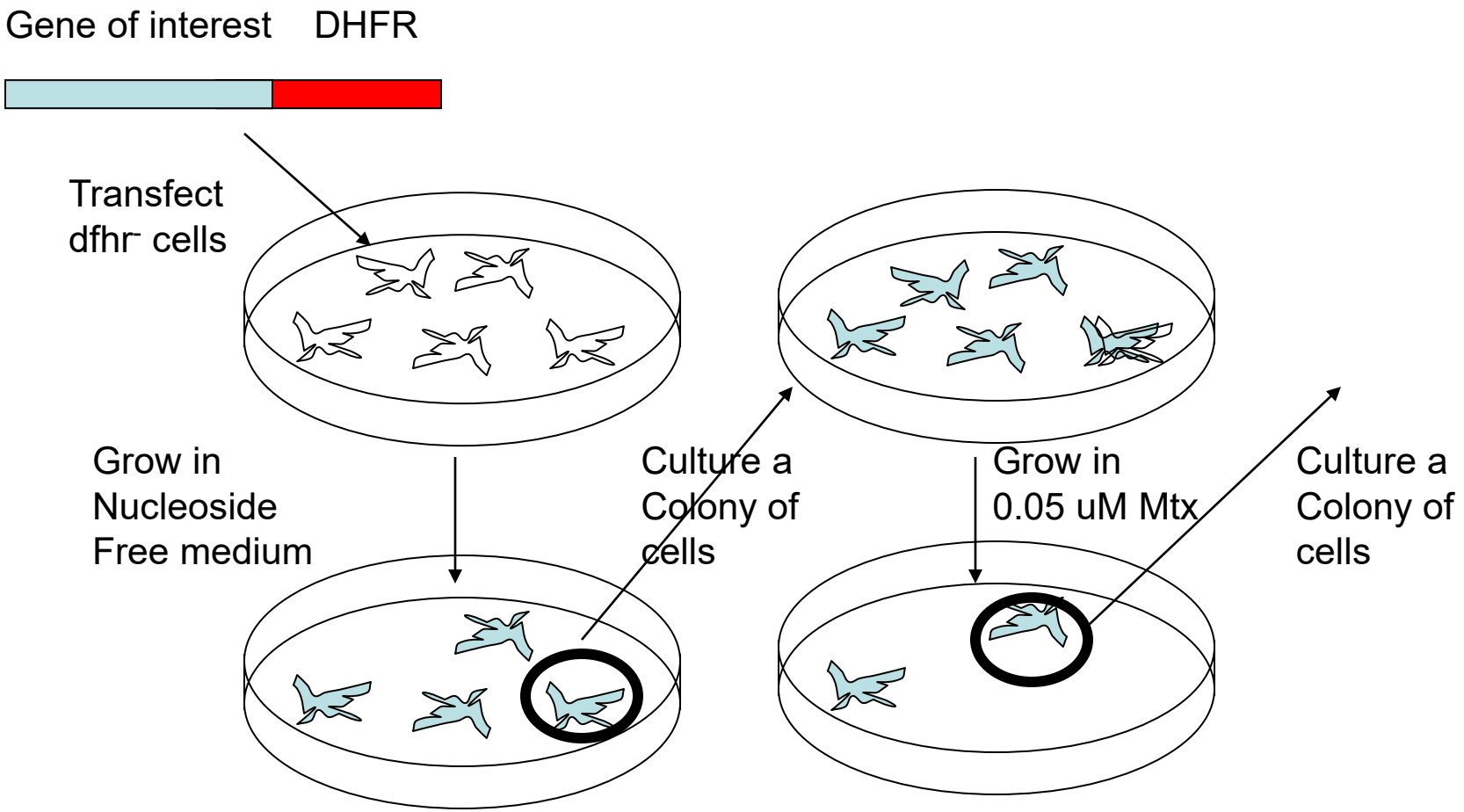
Aumento di [MTX] e selezione di cellule contenenti più copie del vettore

Molteplici cicli di crescita e selezione → utilizzando metotrexato per selezionare le cellule con la più alta espressione e integrazione di DHFR e del gene di interesse

Mammalian Expression (2)

- MTX si lega a DHFR, inibendo così la produzione di tetraidrofolato.
- Con livelli insufficienti di DHFR, le cellule vengono private dei precursori nucleosidici (ipoxantina e timidina) e muoiono.
- La tecnica utilizzata:
  - Trasformazione cellule con DNA ricombinante costituito dal gene di interesse strettamente legato al gene per DHFR.
- Durante l'amplificazione genica le cellule vengono coltivate a livelli sempre più alti di MTX.
- Vengono selezionate quelle cellule CHO che hanno un numero maggiore di copie del gene DHFR, e quindi livelli più elevati dell'enzima

Methotrexate (MTX) Selection (2)



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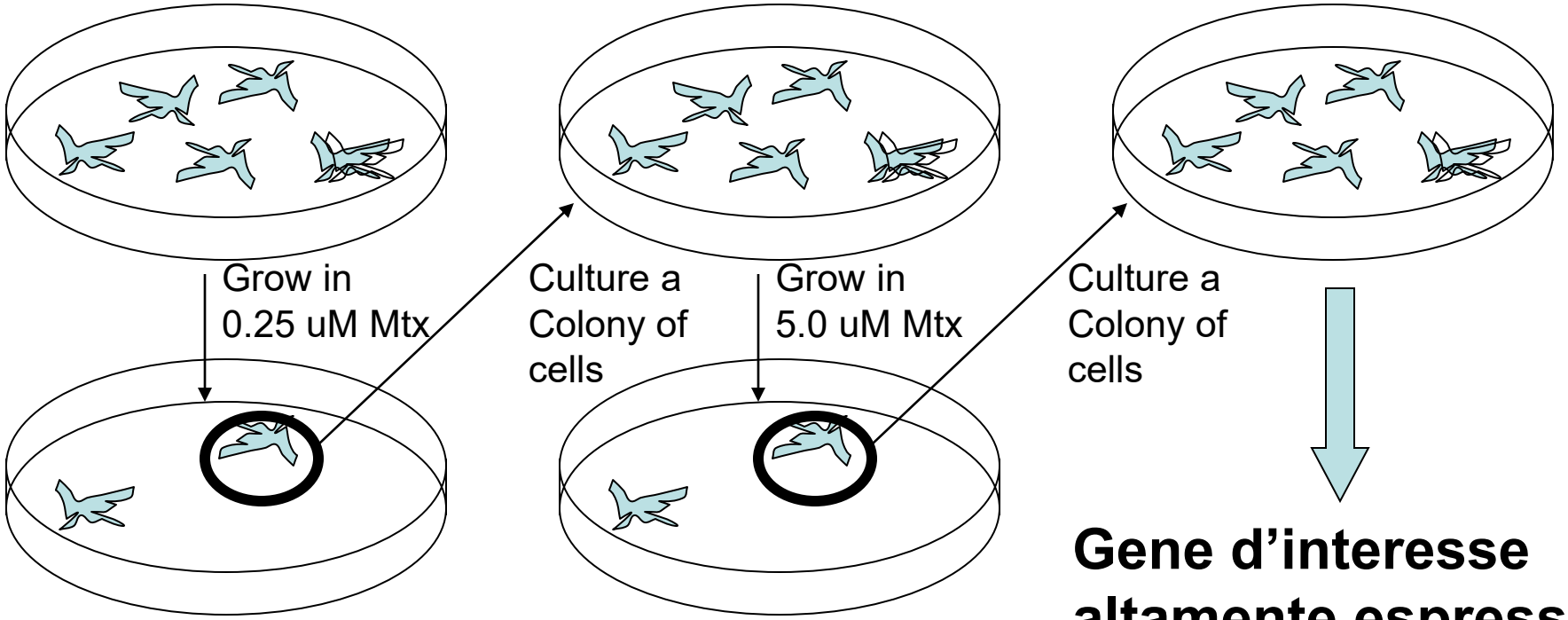
Methotrexate (MTX) Selection (2)



Gene d'interesse altamente espresso in Cellule CHO

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Marcatori selezionabili (3)

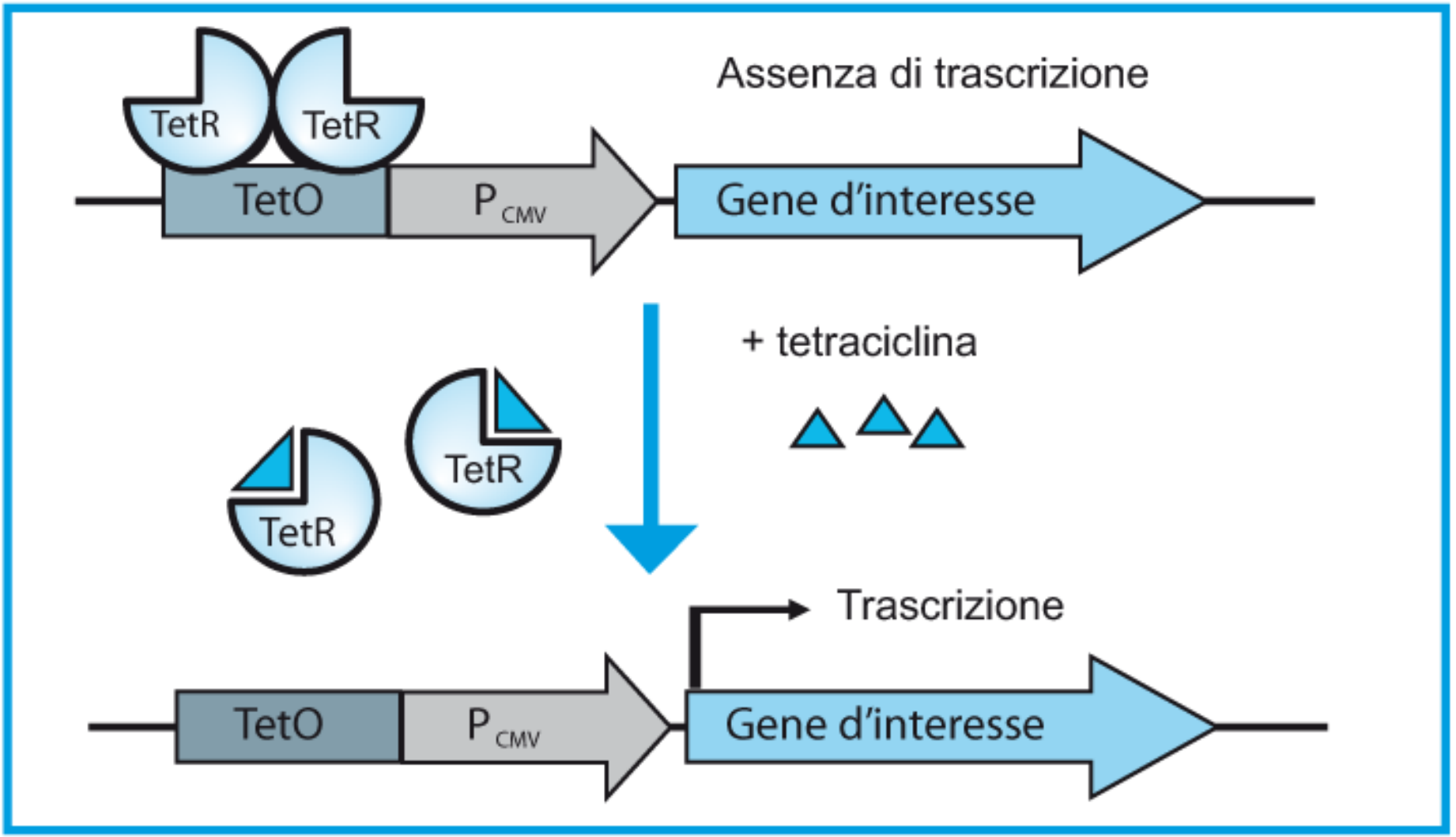
GS: gene che codifica glutammina sintetasi

Conferisce resistenza al composto citotossico metionina-sulfossimmina (MSX)

✓ Non è necessaria una linea cellulare GS^- perché solo copie multiple del gene GS conferiscono resistenza a MSX

=> potenziale vantaggio rispetto al sistema DHFR-MTX

UTILIZZO del SISTEMA Tet-ON



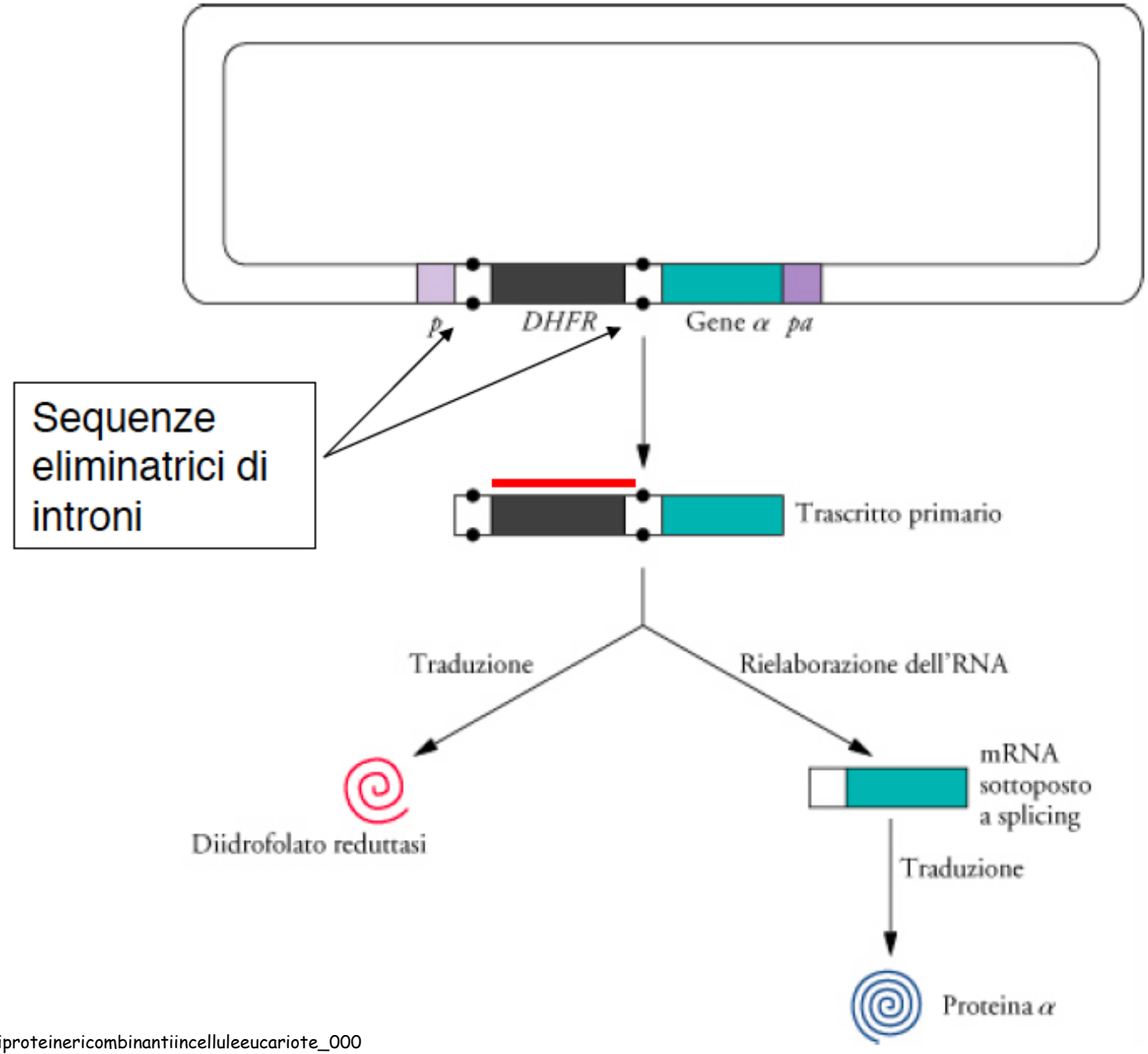
Presenza degli introni

La resa della proteina ricombinante può essere migliorata inserendo un introne tra il promotore e il gene clonato

Possibile spiegazione:

Gene clonato può contenere siti di splicing criptici e la presenza dell'introne rende meno probabile lo splicing in questi siti

Espressione coordinata di un marcatore selezionabile e del gene clonato



Espressione di 2 geni in una stessa cellula

- Necessaria quando la forma attiva di una proteina è costituita da 2 proteine diverse (eterodimero)

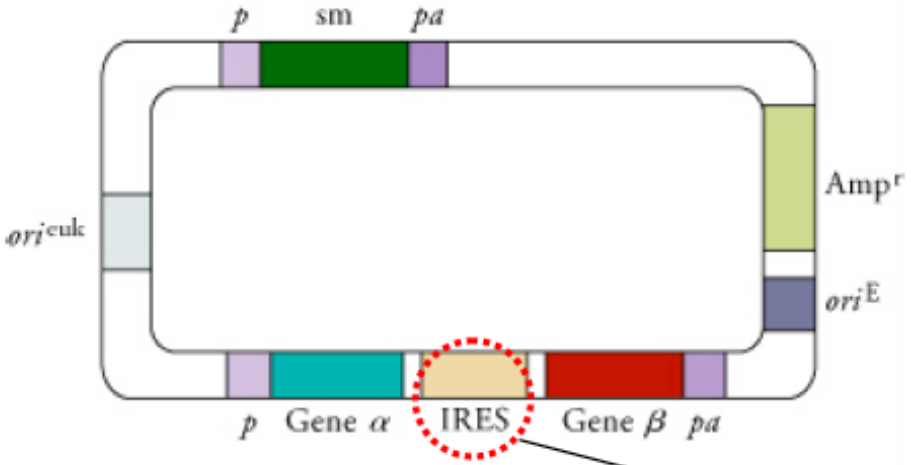
  Ormone stimolatore della tiroide ($\alpha\beta$)

  Emoglobina ($\alpha 2 \beta 2$)

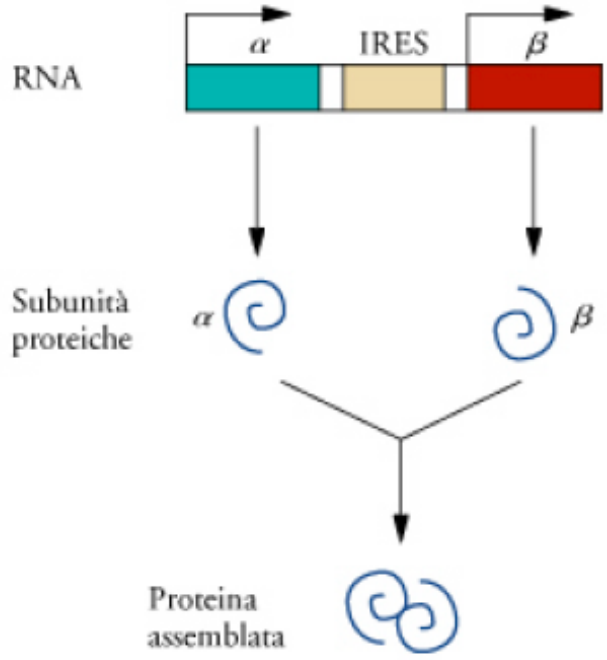
- Preferibile all'assemblaggio *in vitro*

- Necessità di esprimere le subunità contemporaneamente e stechiometricamente

Vettori dicistronici



Sito di accesso ribosomiale interno di origine virale



La seq virale
IRES (internal ribosome entry site) permette che le proteine siano tradotte comteporaneamente

Advantages of Eukaryotic Systems

- Rapid growth (doubling in 90 minutes)
- Simple manipulation with the cells
- Posttranslation modification
- Number of strong constitutive promoters have been used to drive target gene expression
- Number of expression vectors and mutated host strains have been prepared
- Possibility to direct proteins to secretion (signal sequence for secretion)
- Different codon usage (96% of amino acids is encoded only by 25 codons from 61 available combinations)
- Lower yield of expressed proteins

Disadvantages of animal cells (compared to *E. coli*)

- Complex nutritional requirements
- Slower growth
- More susceptible to damage
- Increased costs

Mammalian Systems

Disadvantages

- Selection takes time (weeks for set-up)
- Cell culture is only sustainable for limited period of time
- Set-up is very time consuming, costly, modest yields

Advantages

- Can express large proteins (>50 kD)
- Correct glycosylation & signal peptide removal, generates authentic proteins
- Has chaperonins to help fold "tough" prtns

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Expression in animal cells

- Major advantage- correct PT modifications
- Naturally glycosylated proteins produced in:
  - CHO - Chinese hamster ovary
  - BHK - baby hamster kidney
  - HEK – human embryonic kidney

4.1 Proteins as Biotechnology Products

TABLE 4.2 SOME PROTEIN-BASED PHARMACEUTICAL PRODUCTS (MOST PRODUCED AS RECOMBINANT PROTEINS)

Protein	Application
Erythropoietins	Treatment of anemia
Interleukins 1, 2, 3, 4	Treatment of cancer, AIDS; radiation- or drug-induced bone marrow suppression
Monoclonal antibodies	Treatment of cancer, rheumatoid arthritis; used for diagnostic purposes
Interferons (α, β, γ, including consensus)	Treatment of cancer, allergies, asthma, arthritis, and infectious disease
Colony-stimulating factors	Treatment of cancer, low blood cell count; adjuvant chemotherapy; AIDS therapy
Blood clotting factors	Treatment of hemophilia and related clotting disorders
Human growth factor	Treatment of growth deficiency in children
Epidermal growth factor	Treatment of wounds, skin ulcers, cancer
Insulin	Treatment of types 1 and 2 diabetes mellitus
Insulin-like growth factor	Treatment of type 1 diabetes mellitus
Tissue plasminogen factor	Treatment after heart attack, stroke
Tumor necrosis factor	Cancer treatment
Vaccines	Vaccination against hepatitis B, malaria, herpes

- **Review this table and discuss other proteins that you would expect to see purified via recombinant means.**