**Universidad de Chile**
**Facultad de Ciencias Químicas y Farmacéuticas**
**Departamento de Bioquímica y Biología Molecular**

# Ingeniería Genética en Eucariontes

**Bibliografía :**
**Recombinant DNA, Third Edition: "Genes and Genomes – A short Course". James D. Watson y cols. W. H. Freeman y CSHL Press. 2007.**

## MANIPULACION GENETICA EN EUCARIONTES

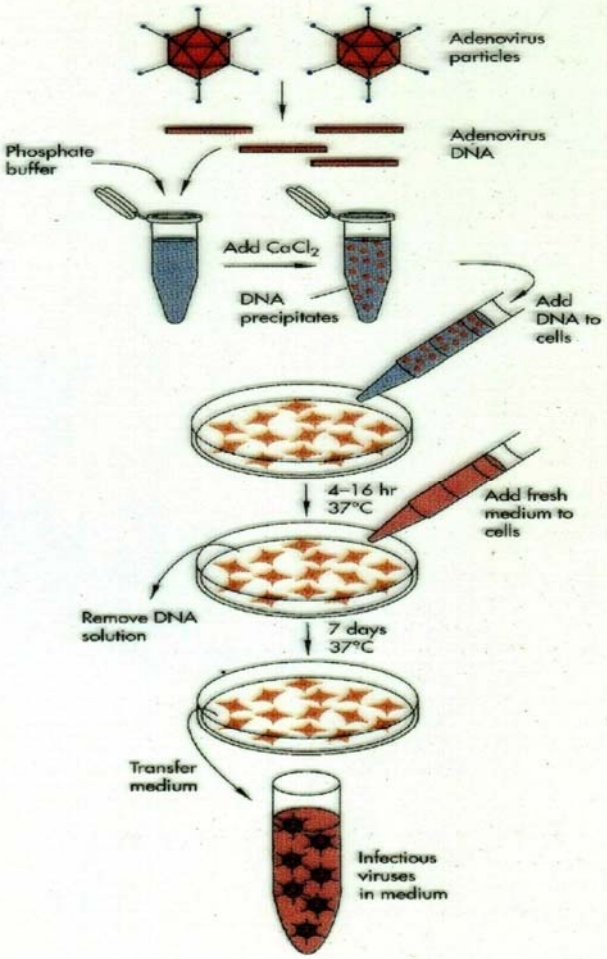
Existen tres herramientas básicas para estudiar la función de los genes de un organismo eucariótico:

1) Aislar el gen mediante clonamiento. ¿¿??
2) Manipular la secuencia del gen *in vitro*
3) Retornar el gen silvestre (wt) o alterado a las células eucarióticas para determinar cómo funciona

El punto 3) es el que ha presentado los mayores obstáculos…….*"el contexto genómico".*

# TRANSFECCIÓN: "Transformación de células eucarióticas en cultivo usando DNA desnudo"

FIGURE 12-1
Transfection of DNA by calcium phosphate coprecipitation. The figure depicts an updated version of the original Graham and van der Eb experiment, which demonstrated that transfer of naked adenovirus DNA yields infectious virus. Many variations of this basic protocol are now in use. Adenovirus particles are purified, and their DNA is extracted. The DNA is mixed with a carefully buffered solution containing phosphate. Addition of calcium chloride forms a fine precipitate of calcium phosphate and DNA (which contains phosphate groups in its backbone). The precipitate is pipetted onto a monolayer of cells growing in a petri dish and left on the cells for several hours, during which time many of the cells take up the precipitated DNA. The precipitate is removed from the cells, which are then incubated in fresh growth medium for several days. Infectious virus particles soon emerge from the cells.

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# Definiciones

La **transfección** consiste en la introducción de algún tipo de material genético externo en células eucariontes mediante plasmidios, vectores virales (en este caso también se habla de *transducción*) u otras herramientas para la transferencia.

El término **transfección** para métodos no-virales se usa en referencia a células de mamíferos, mientras que el término **transformación** se prefiere para describir las transferencias no-virales de material genético en bacterias y células eucarióticas no animales como hongos, algas o plantas.

"**Transfección**" originalmente viene de "**infección mediante transformación**", ya sea mediante la introducción de DNA o RNA de un virus o de un bacteriófago.

"**Infección**" es la colonización de una célula huesped u organismo vivo por células externas o virus.

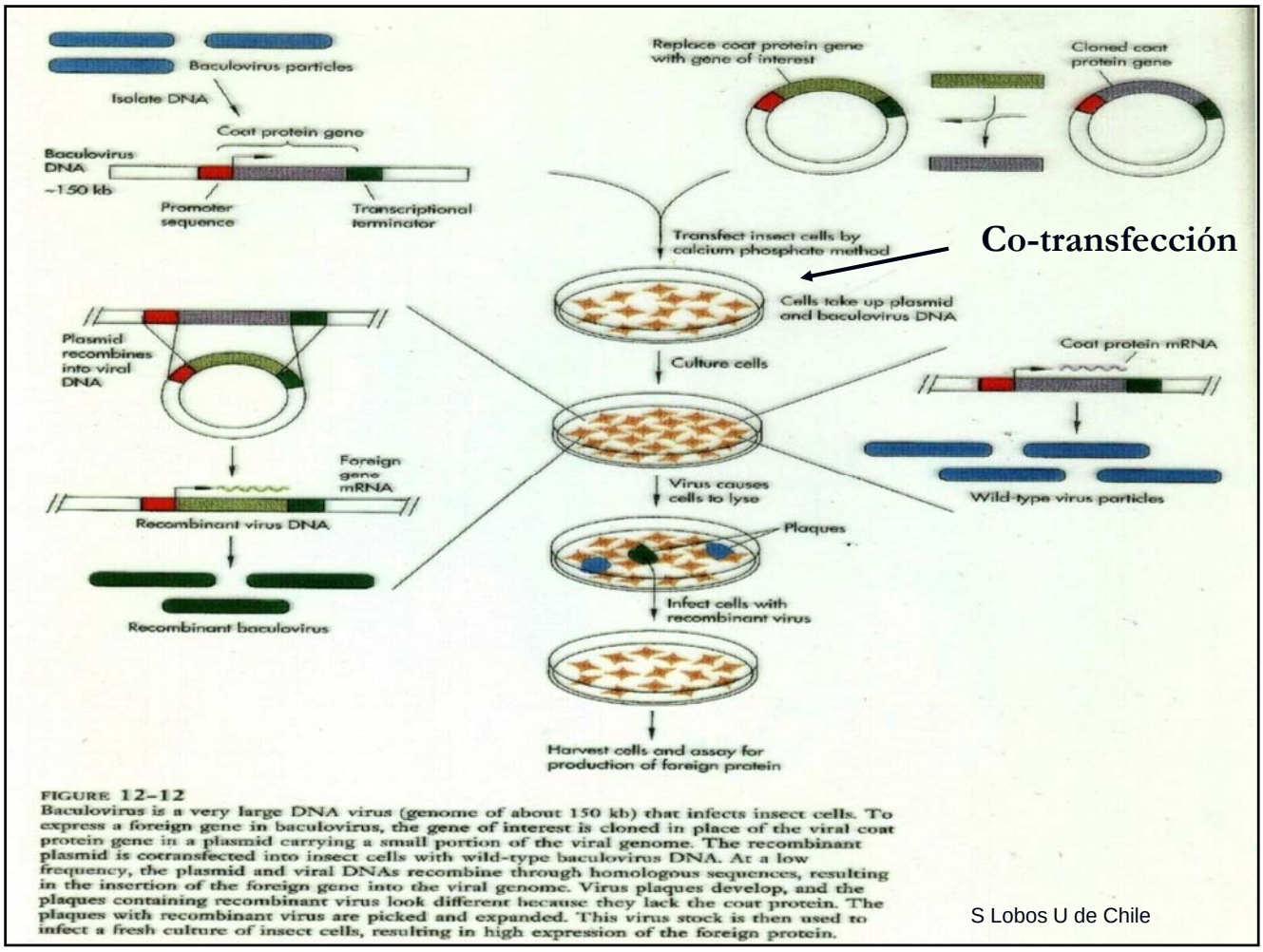


FIGURE 12–12
Baculovirus is a very large DNA virus (genome of about 150 kb) that infects insect cells. To express a foreign gene in baculovirus, the gene of interest is cloned in place of the viral coat protein gene in a plasmid carrying a small portion of the viral genome. The recombinant plasmid is cotransfected into insect cells with wild-type baculovirus DNA. At a low frequency, the plasmid and viral DNAs recombine through homologous sequences, resulting in the insertion of the foreign gene into the viral genome. Virus plaques develop, and the plaques containing recombinant virus look different because they lack the coat protein. The plaques with recombinant virus are picked and expanded. This virus stock is then used to infect a fresh culture of insect cells, resulting in high expression of the foreign protein.

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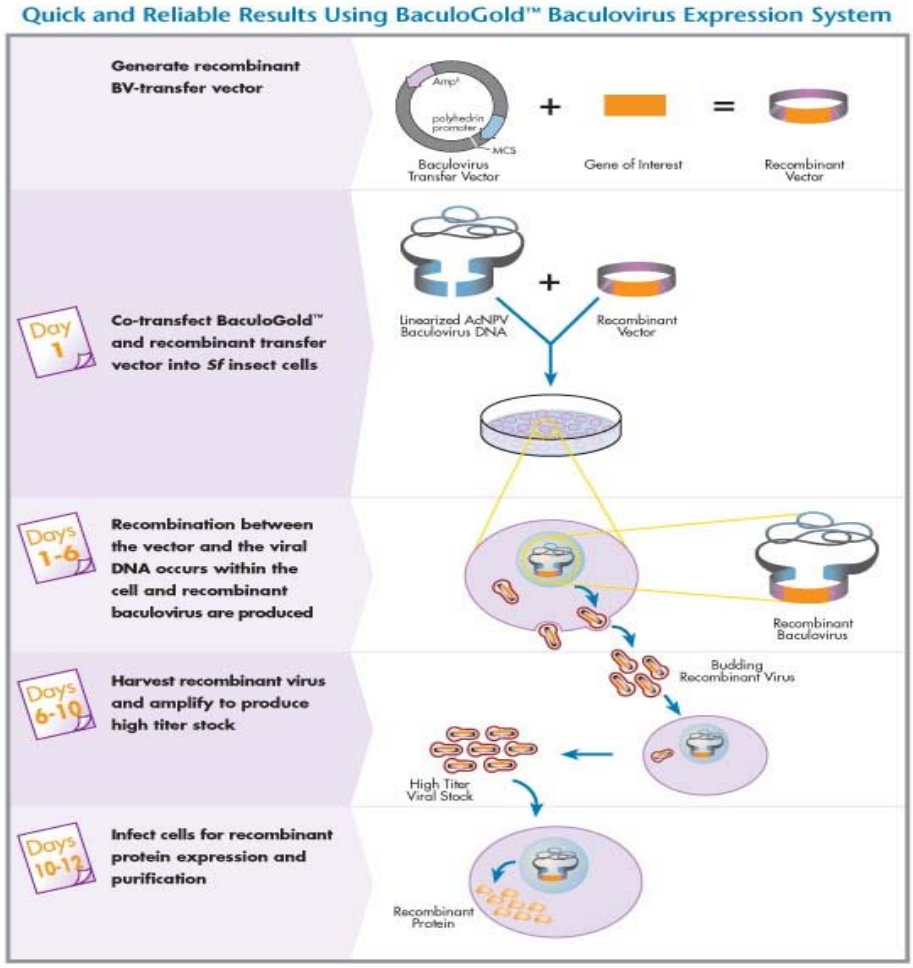
**Infección de células de insectos para expresión de proteínas recombinantes con correcto procesamiento post-traduccional (glicosilación, fosforilación, etc)**

**Baculovirus kit conteniendo linea celular de *Spodoptera frugiperda***

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**Transfección de células eucarióticas con vectores retrovirales.**

**Las lineas celulares utilizadas contienen un provirus integrado que aporta todas las proteínas requeridas para el ensamblaje del virus recombinante. El provirus está desarmado y no puede empaquetar su propio RNA.**

**Las partículas infecciosas recombinantes tienen amplio rango de huesped y su DNA se integra en forma estable expresando el producto génico**

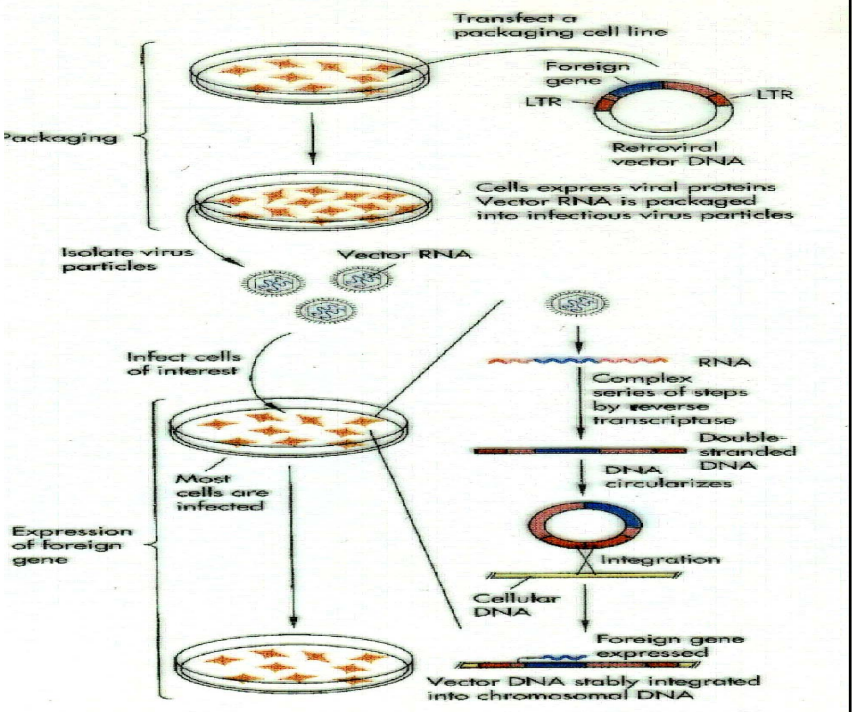
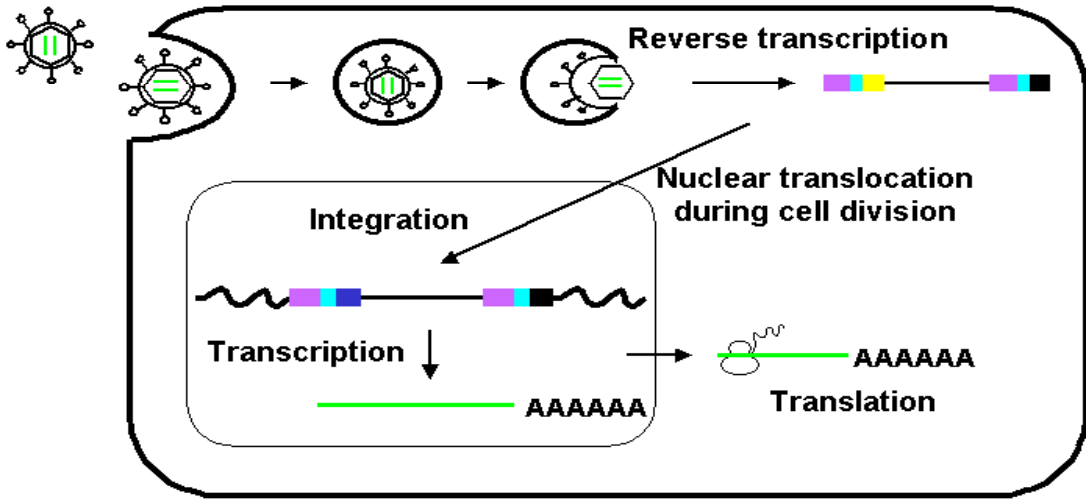



FIGURE 12-13
Use of a retrovirus vector for stable, long-term expression of a foreign gene. The gene is cloned into a retrovirus vector that lacks most viral genes. The gene is usually expressed under the control of the strong viral promoter in the LTR. The recombinant plasmid is transfected into a special packaging cell line that harbors an integrated provirus. The provirus has been crippled so that, although it produces all the proteins required to assemble infectious viruses, its own RNA cannot be packaged into virus. Instead, RNA produced from the recombinant virus is packaged. The virus stock released from the packaging cells thus contains only recombinant virus. The virus can be used to infect virtually any other cell type, resulting in the integration of the viral genome and the stable production of the foreign gene product.

**Vectores retrovirales son pantrópicos, es decir, infectan a casi cualquier tipo de células**

## Fate of pantropic retroviral vector

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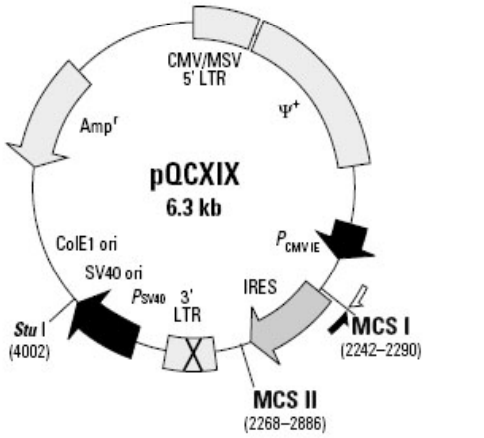
**Típicos vectores retrovirales son comerciales y pueden ser manipulados en bacterias.**
**Vectores de expresión Bicistrónicos**

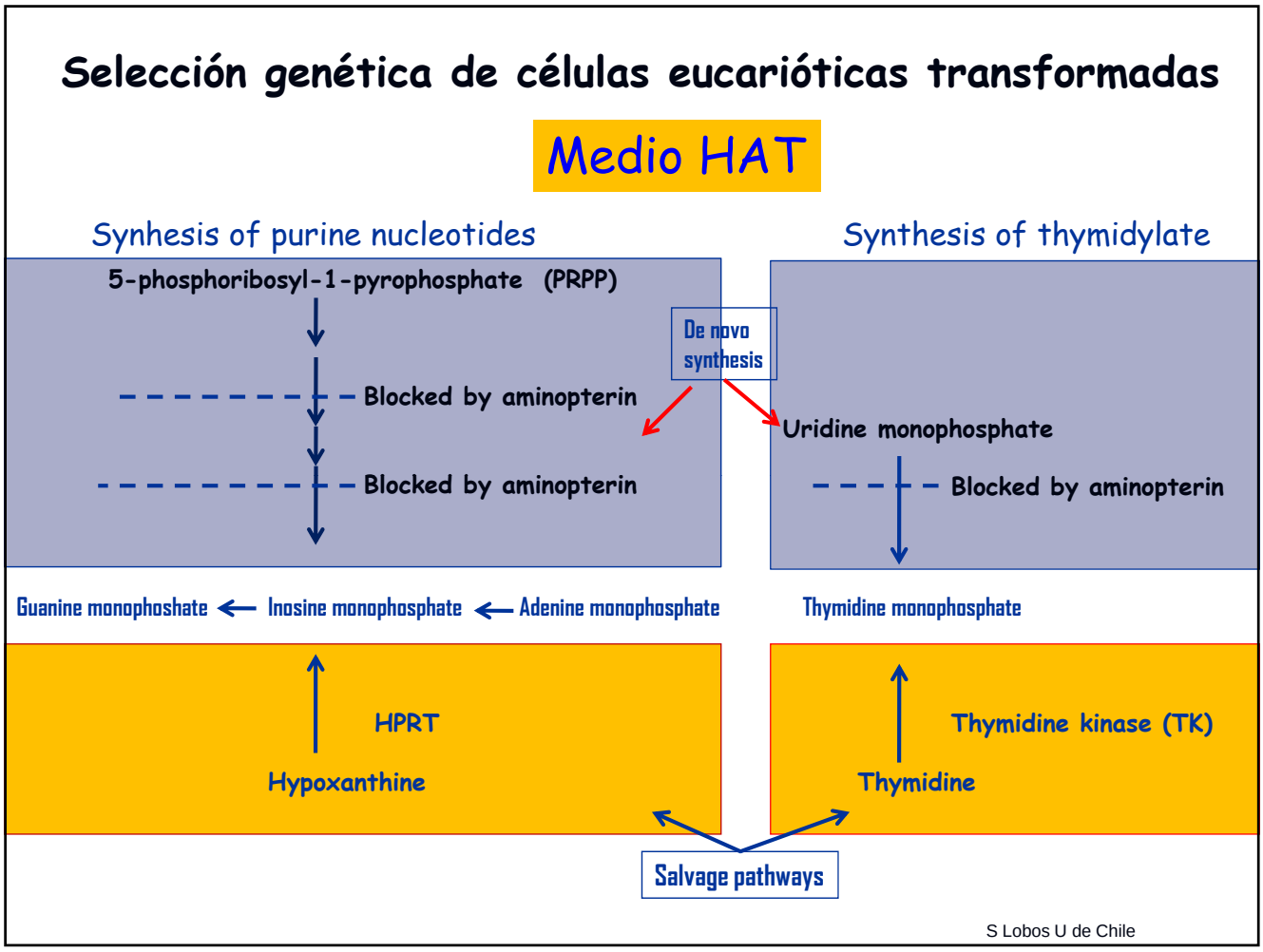
**Vector diseñado para expresar dos genes blanco. Después de transfectar una linea celular, este vector puede expresar temporalmente o integrarse y expresar establemente el primer gen a partir del promotor temprano de CMV y el segundo gene usando las secuencias IRES**




MCS I: CGCGGCCGCACCGGTAGGCCTCGTACGCTTAATTAACGGATCCGGAATTCC
Not I Age I Bsi WI Pac I BamH I EcoR I

MCS II: AAGCTTGATCACGCGTCTCGAGATATCAG
Bcl I Mlu I Xho I EcoR V

Restriction Map and Multiple Cloning Sites (MCS I and MCS II ) of pQCXIX Retroviral Vector. Unique restriction sites are in bold.


Selección genética de células eucarióticas transformadas
Medio HAT
Synhesis of purine nucleotides
Synthesis of thymidylate
5-phosphoribosyl-1-pyrophosphate (PRPP)
De novo synthesis
Blocked by aminopterin
Blocked by aminopterin
Uridine monophosphate
Blocked by aminopterin
Guanine monophoshate
Inosine monophosphate
Adenine monophosphate
Thymidine monophosphate
HPRT
Hypoxanthine
Thymidine kinase (TK)
Thymidine
Salvage pathways
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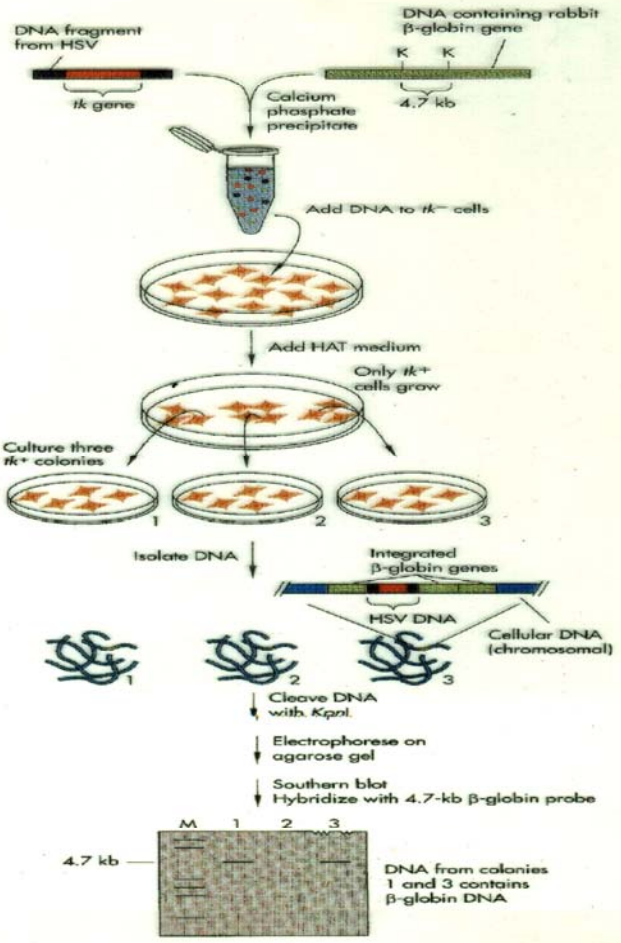
## CO-TRANSFECCIÓN
## Gen marcador + gen de interés no-seleccionable

FIGURE 12–3
Transfer of a nonselectable gene by cotransfection with a marker gene. A herpes simplex virus DNA fragment carrying the *tk* gene was mixed with a fragment carrying the rabbit β-globin gene. The two fragments were applied to dishes of mouse *tk*⁻ cells as a calcium phosphate precipitate. The precipitate was removed, and the cells were incubated in HAT medium, which supports only the growth of *tk*⁺ cells (see Figure 12–2). After 2 to 3 weeks, colonies of *tk*⁺ cells grew up on the dishes. The colonies were picked and grown up. Chromosomal DNA was isolated, digested with the restriction enzyme *Kpn*I, (which cleaves at the sites indicated by "K" in the β-globin gene), and analyzed by Southern blotting, using a radioactive probe that detected the rabbit β-globin sequence in the fragment generated by cleavage with *Kpn*I. Most of the *tk*⁺ colonies had also acquired the rabbit-β-globin gene. Detailed analysis of the structure of the transfected DNA in these clones showed that it integrated as large arrays carrying multiple copies of both transfected fragments, usually at one or a few chromosomal sites. M, molecular size markers.

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# Otros genes marcadores seleccionables

TABLE 12-1
Dominant Selectable Markers Used in Transfection Experiments

| ENZYME (abbreviation) | DRUG FOR SELECTION | SELECTION MECHANISM |
|---|---|---|
| Aminoglycoside phosphotransferase (APH) | G418 (inhibits protein synthesis) | APH inactivates G418 |
| Dihydrofolate reductase (DHFR): Mtx-resistant variant | Methotrexate (Mtx; inhibits DHFR) | Variant DHFR resistant to Mtx |
| Hygromycin-B-phosphotransferase (HPH) | Hygromycin-B (inhibits protein synthesis) | HPH inactivates hygromycin-B |
| Thymidine kinase (TK) | Aminopterin (inhibits de novo purine and thymidylate synthesis) | TK synthesizes thymidylate |
| Xanthine-guanine phosphoribosyltransferase (XGPRT) | Mycophenolic acid (inhibits de novo GMP synthesis) | XGPRT synthesizes GMP from xanthine |
| Adenosine deaminase (ADA) | 9-β-D-xylofuranosyl adenine (Xyl-A; damages DNA) | ADA inactivates Xyl-A |

**Transfección con otros tipos de vectores es ineficiente e inestable y solo pocas de las células seleccionadas integran el DNA transfectado en el cromosoma. Estas pocas células deben ser crecidas por varias generaciones.**

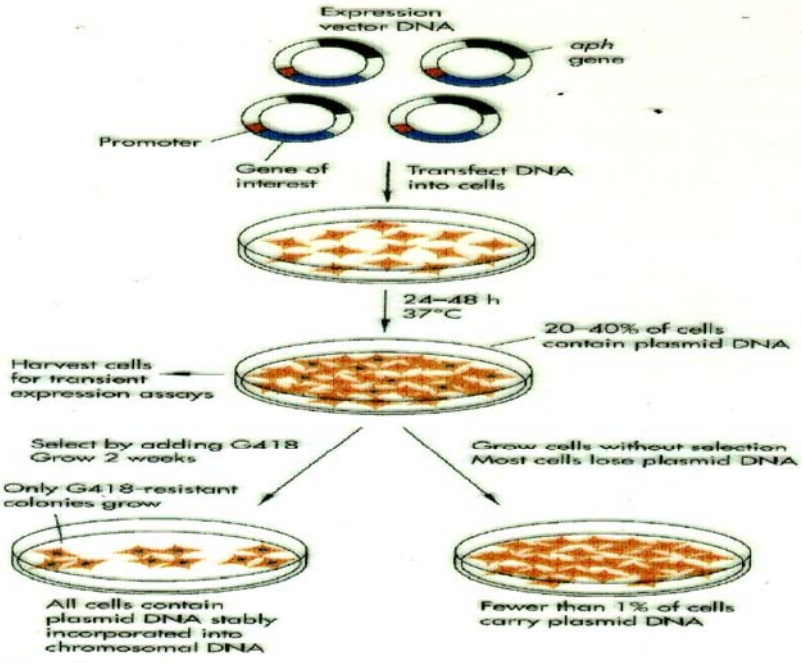


FIGURE 12–4
Transfected DNA is maintained transiently in many cells, but stably integrates in only a few. During the first 48 hours following transfection, as many as 50 percent of the cells in the culture carry the transfected DNA. This is termed the *transient phase*, and for many kinds of experiments, the cultures are used at this point. As the cells continue to incubate, the transfected DNA is progressively lost from most of them (owing to a combination of degradation and dilution, because unintegrated DNA is not usually replicated along with the host cell chromosomes). In only a few cells on the dish does the DNA become stably integrated into a chromosome. Isolating those cells and their descendants requires the application of a *selection* for a transfected marker gene (left). The selection kills off all the cells that failed to integrate transfected DNA, allowing the rare integrants to grow up into large isolated colonies (clones), which can be recovered for further analysis.

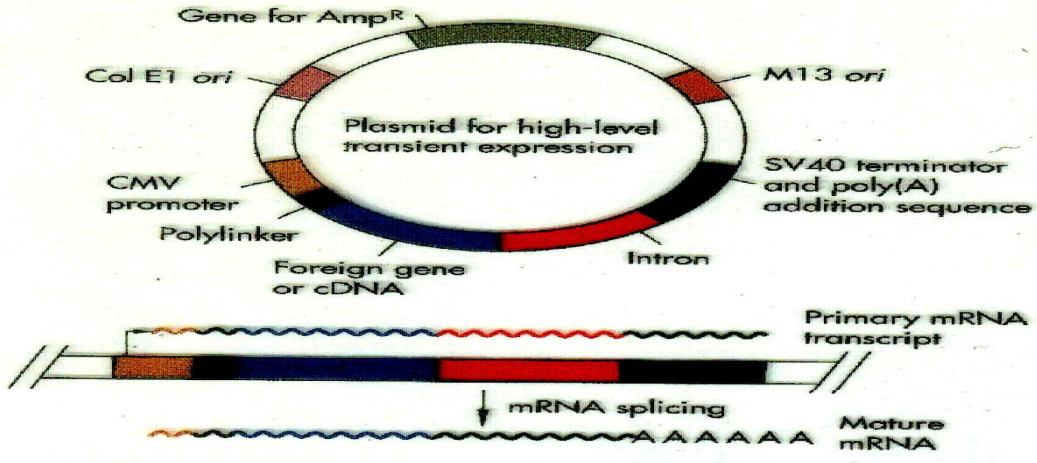


FIGURE 12–5 *(Above)*
A typical mammalian expression vector for high-level protein expression. Modern expression vectors are stitched together from several distinct units that provide the different components of a functional gene. The vector shown here carries a potent promoter from cytomegalovirus (CMV), an intron from a globin gene, and the poly(A) addition signal from SV40 virus. Such plasmids usually contain several restriction sites (collectively called a polylinker) for inserting the foreign gene to be expressed. The plasmid backbone carries the usual sequences for replication (Col E1 *ori*) and drug-resistance (Amp$^R$) in *E. coli*, including in this case an M13 origin of replication (*ori*), allowing this vector to be recovered from bacteria as single-stranded phages for easy mutagenesis.

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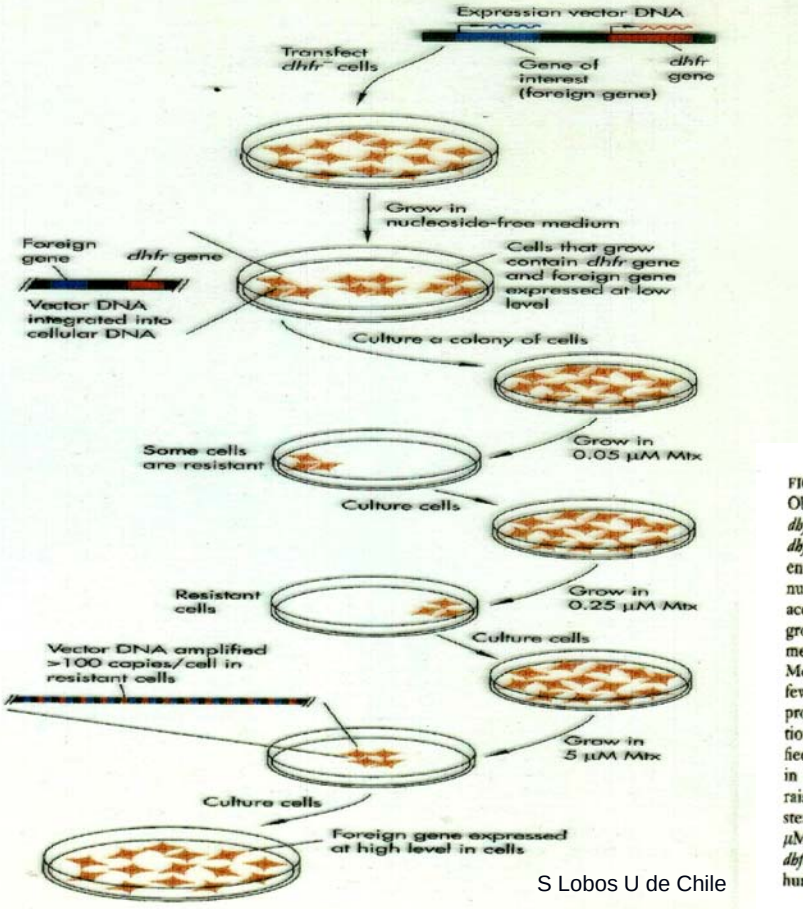


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## Co-amplificación

FIGURE 12-6 *(Right)*
Obtaining high-level expression by coamplification with *dhfr*. The gene to be expressed is transfected together with a *dhfr* gene into a cell line that lacks endogenous DHFR enzyme activity. The cells are grown in a special nucleoside-free medium in which only cells that have acquired a *dhfr* gene can grow. Clonal colonies are picked, grown up, and shifted to medium containing 0.05 μM methotrexate (Mtx), an inhibitor of the DHFR enzyme. Most cells fail to grow at this concentration of Mtx. But a few will have amplified their *dhfr* genes, allowing them to produce sufficient amounts of the enzyme to escape inhibition by Mtx. After a few weeks of growth, cells with amplified *dhfr* genes will have multiplied enough to predominate in the culture. At this point, the concentration of MTX is raised, and the cycle is repeated. The Mtx concentration is steadily raised in small increments until it reaches about 50 μM. Cells growing at this concentration have amplified their *dhfr* genes along with the linked foreign gene several hundredfold.

## Otros método de transferencia genética:

- **Electroporación**
- **Lipofección**

## Tipos de células

| Mammalian Cells | Bacterial Cells | Fungal Cells |
|---|---|---|
| *CHO* | *E. coli* | *S. cerevisiae* |
| *COS7* | *A. tumefaciens* | *P. pastoris* |
| *3T3* | *P. aeruginosa* | *C. albicans* |
| *293* | *S. aureus* | *S. pombe* |
| *HeLa* | *B. cereus* | *D. discoideum* |
| *BHK21* | *S. pyrogenes* | |
| *A549* | *L. plantarum* | |
| *CV1* | | |
| *K562* | | |
| *HL60* | | |
| *Jurkat* | | |
| *HuT78* | | |

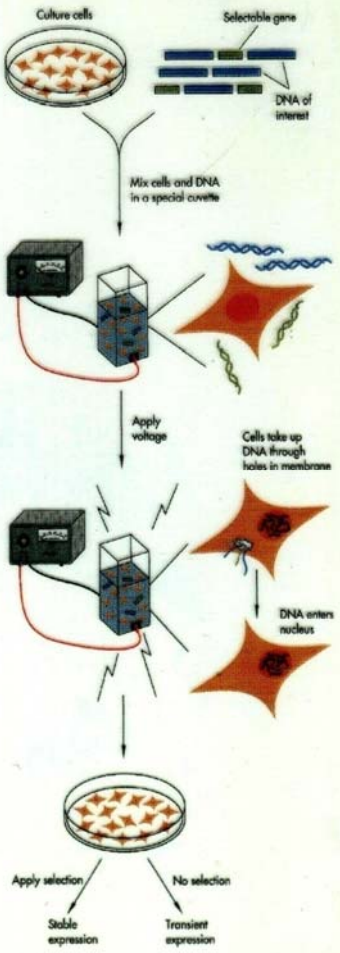
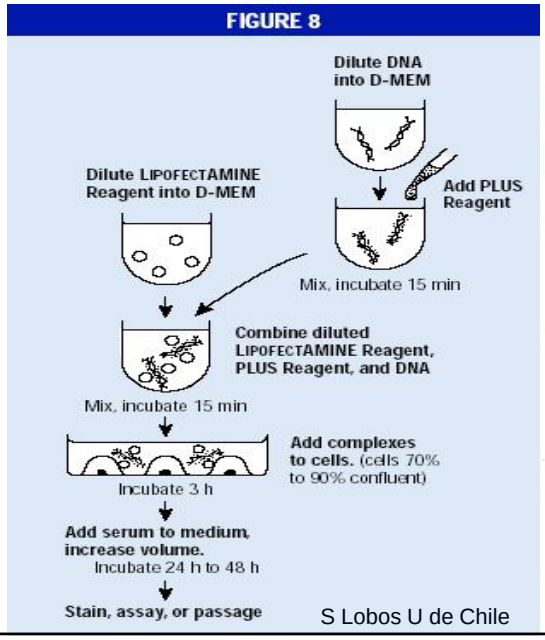



FIGURE 12-7
Gene transfer by electroporation. Cells are concentrated, mixed with the DNA to be transfected, and placed in a small chamber with electrodes connected to a specialized power supply. A brief electric pulse is discharged across the electrodes, transiently opening holes in cell membranes. DNA enters the cells, which are removed and plated in fresh medium. The cultures can be harvested during the transient expression phase, or selection can be applied to isolate stably transfected clones.

**Lipofección:**
**Utiliza liposomas con el DNA de interés encapsulado.**

**Los liposomas son fabricados con reactivos lipídicos catiónicos o lipofectaminas.**

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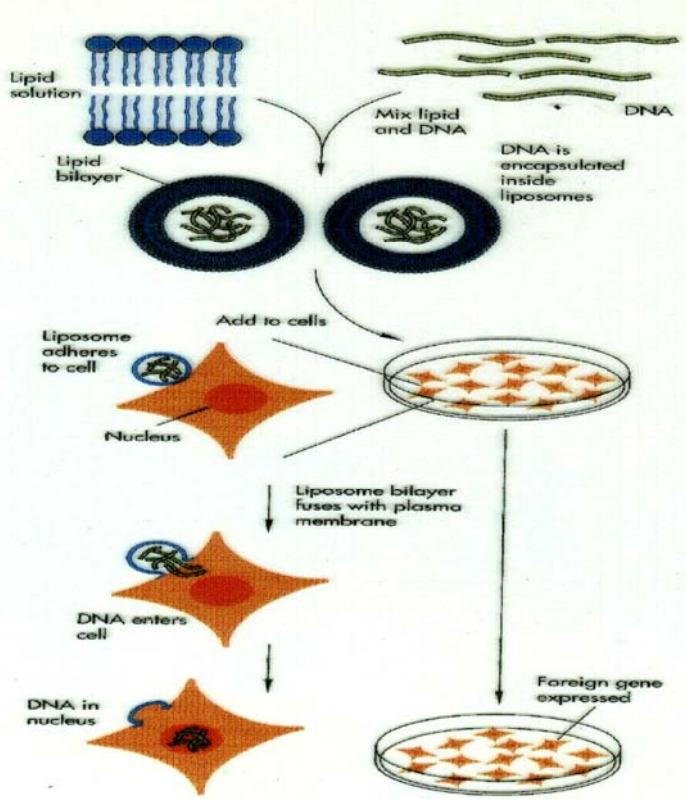


FIGURE 12-8
Liposome-mediated gene transfer (*lipofection*). A number of methods have been developed to encapsulate DNA in synthetic lipid bilayers resembling cell membranes. These *liposomes* are essentially spheres of synthetic membrane filled with DNA. They fuse spontaneously to cell membranes, disgorging their contents into the cytoplasm. Making liposomes from scratch is complicated, but commercial reagents are available that considerably simplify the procedure.

## Otros métodos:

Micro-inyección

Biobalística

**A pesar de todos estos métodos siguen habiendo tipos celulares recalcitrantes.**

**Muchos investigadores retornan a los virus y vectores derivados. Adenovirus, SV40, virus Vaccinia, etc., especialmente por sus diseños para expresión de proteínas y sobre-expresión.**

Co-transfección

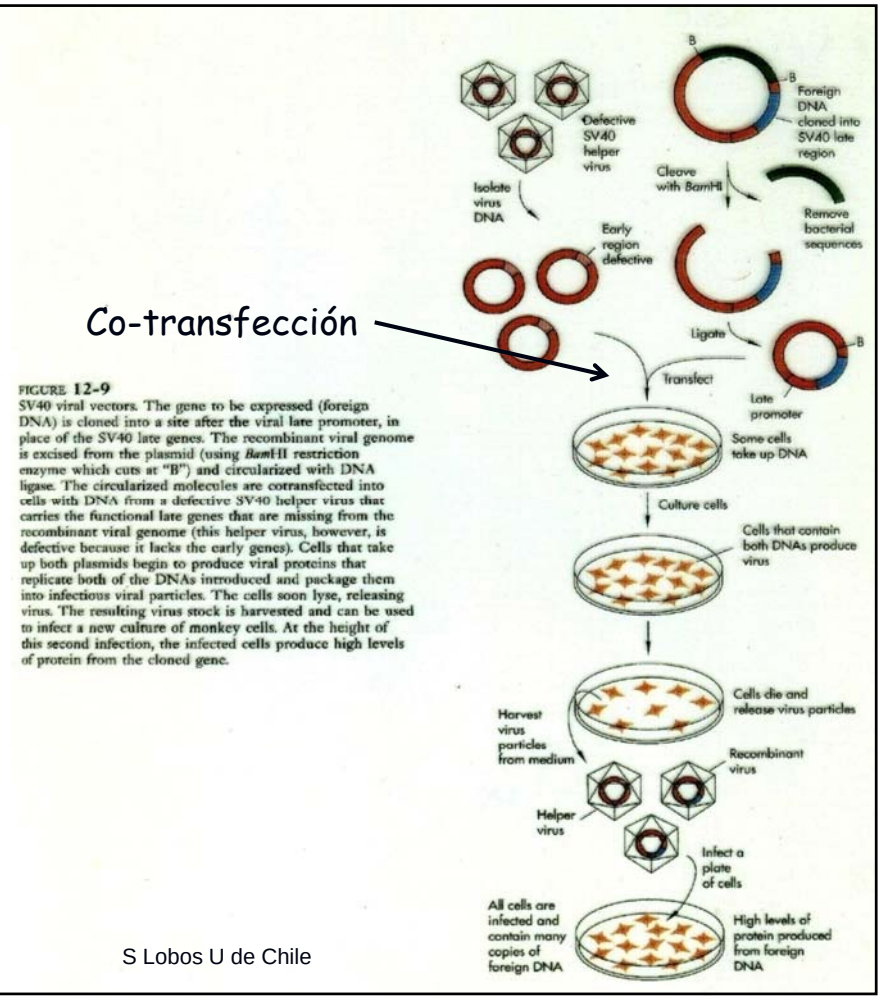


FIGURE 12-9
SV40 viral vectors. The gene to be expressed (foreign DNA) is cloned into a site after the viral late promoter, in place of the SV40 late genes. The recombinant viral genome is excised from the plasmid (using *BamH*I restriction enzyme which cuts at "B") and circularized with DNA ligase. The circularized molecules are cotransfected into cells with DNA from a defective SV40 helper virus that carries the functional late genes that are missing from the recombinant viral genome (this helper virus, however, is defective because it lacks the early genes). Cells that take up both plasmids begin to produce viral proteins that replicate both of the DNAs introduced and package them into infectious viral particles. The cells soon lyse, releasing virus. The resulting virus stock is harvested and can be used to infect a new culture of monkey cells. At the height of this second infection, the infected cells produce high levels of protein from the cloned gene.

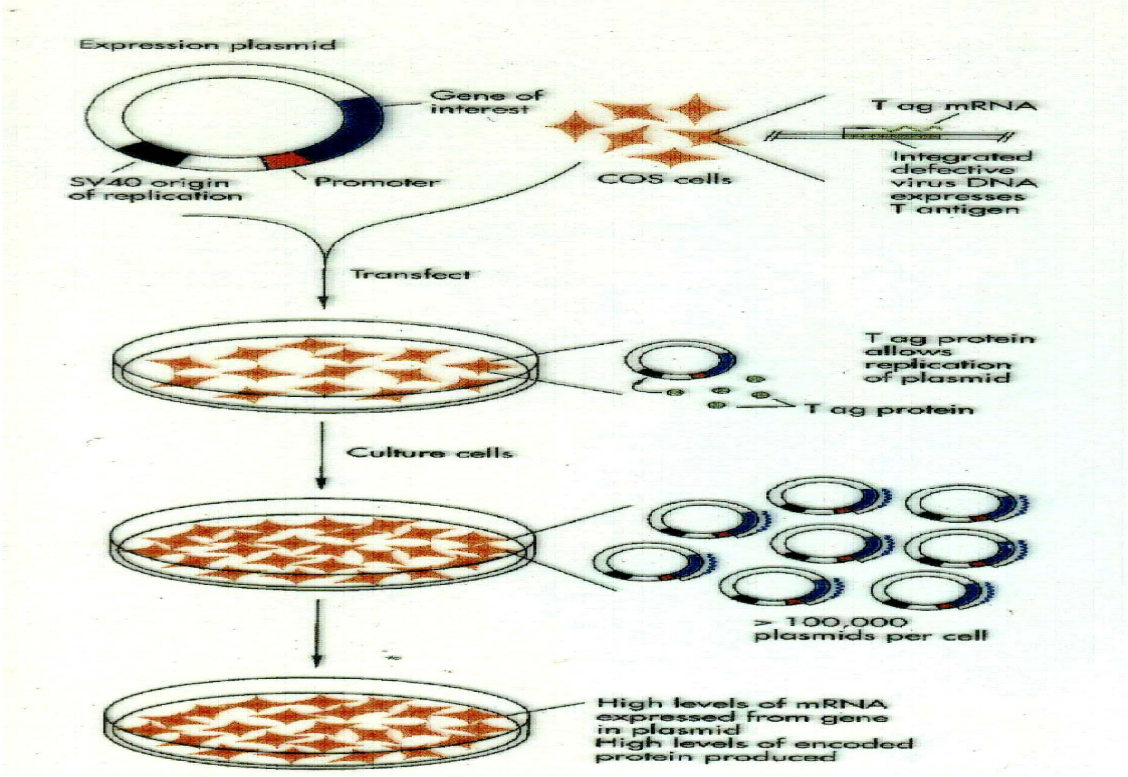


FIGURE 12–10
Use of COS cells for high-level protein expression. The gene to be expressed is cloned into a plasmid that carries a small piece of SV40 DNA that includes the viral origin of replication. The plasmid is transfected into a special cell line, COS, that contains an integrated defective SV40 genome that expresses the viral T antigen (T ag), required for replication of viral DNA. The plasmid, because it contains the viral origin of replication, is recognized by T ag, causing the plasmid to be massively replicated. Before the transfected cells finally die a few days later, protein expressed from the gene on the plasmid accumulates to high levels.

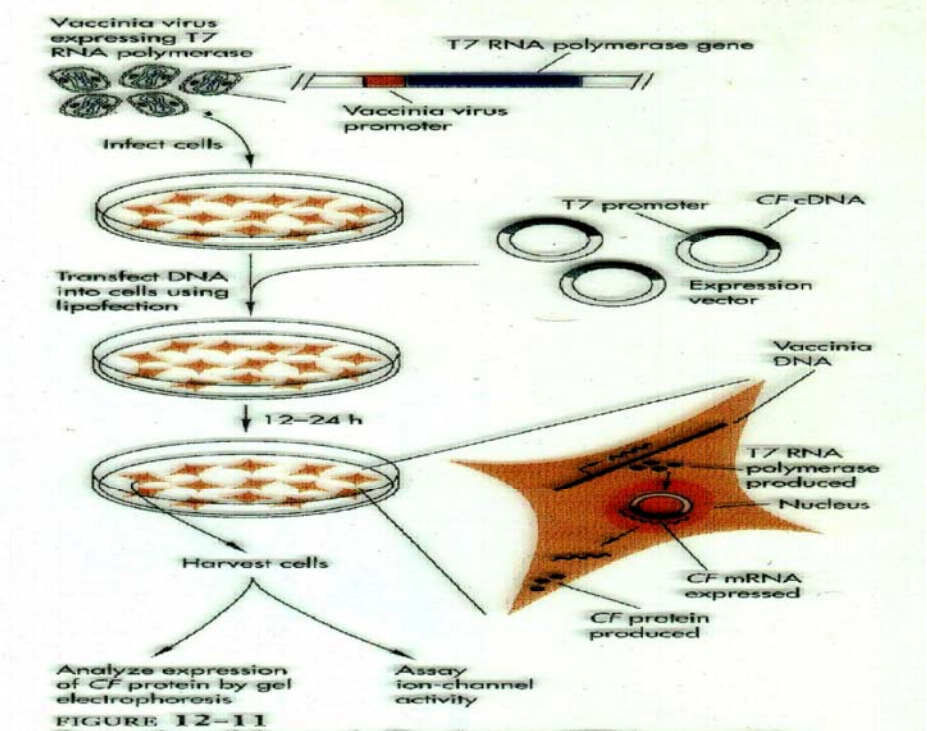


FIGURE 12–11
Expression of the cystic fibrosis gene (*CF*) in a vaccinia virus vector. To prove that a wild-type *CF* gene could correct the ion-transport defect in cells from a cystic fibrosis patient, the wild-type gene was expressed in these cells using a two-component vaccinia virus expression system. First, the cells were infected with a specially engineered vaccinia virus that carries the gene encoding bacteriophage T7 RNA polymerase. The infected cells began to produce the RNA polymerase. Next, the cells were transfected with a plasmid carrying the wild-type *CF* gene downstream of a bacteriophage T7 promoter. The T7 RNA polymerase efficiently transcribed the *CF* gene into mRNA, and the mRNA was translated into protein. The resulting cells were analyzed for their ion-transport activity and found to have normal activity, proving that the cloned *CF* gene could correct the defect in the patient's cells.

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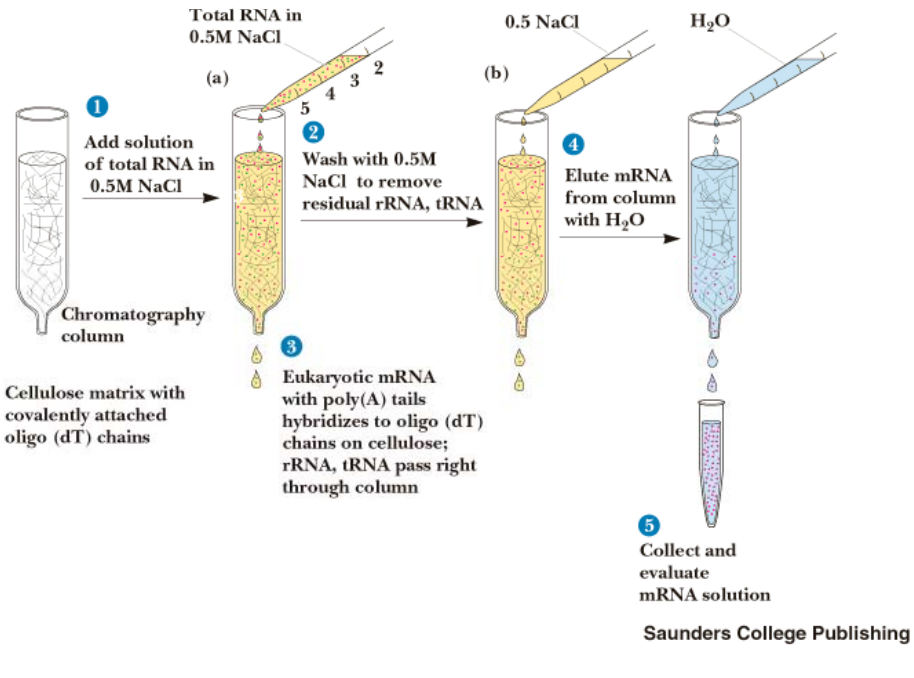
# Construcción de una genoteca de cDNA (cDNA library)

## A partir de *mRNA-poli(A+)*

Garrett & Grisham: Biochemistry, 2/e
Figure 13.13

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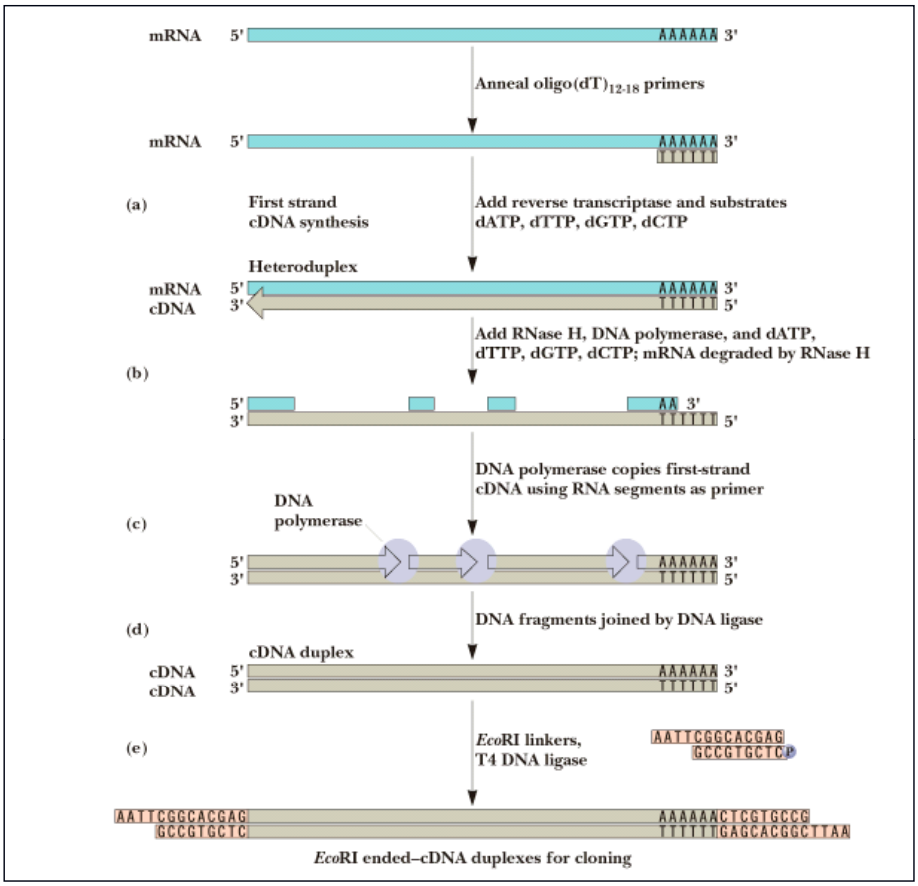


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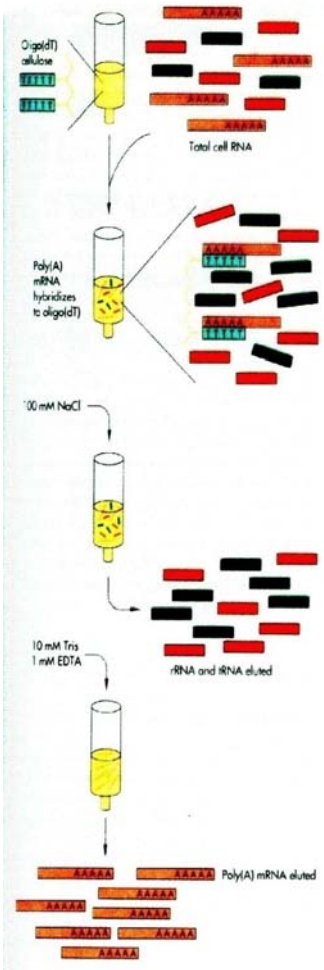


FIGURE 7-2
Isolation of poly(A) RNA. Most eukaryotic mRNAs carry a poly(A) tail, which can be used to purify the mRNA fraction from the bulk of cellular RNA. Cellular RNA is passed over a column consisting of an inert material, often cellulose or agarose, to which oligonucleotides consisting entirely of deoxythymidine (dT) residues have been attached. The poly(A) tails hybridize to this oligo(dT), causing the mRNA to stick to the column, while the rest of the RNA runs through. After extensive washing of the column to remove the last traces of contaminating material, the column is washed with a buffer of low ionic strength. Under these conditions the poly(A)·oligo(dT) hybrids dissociate, and the purified mRNA washes off the column.

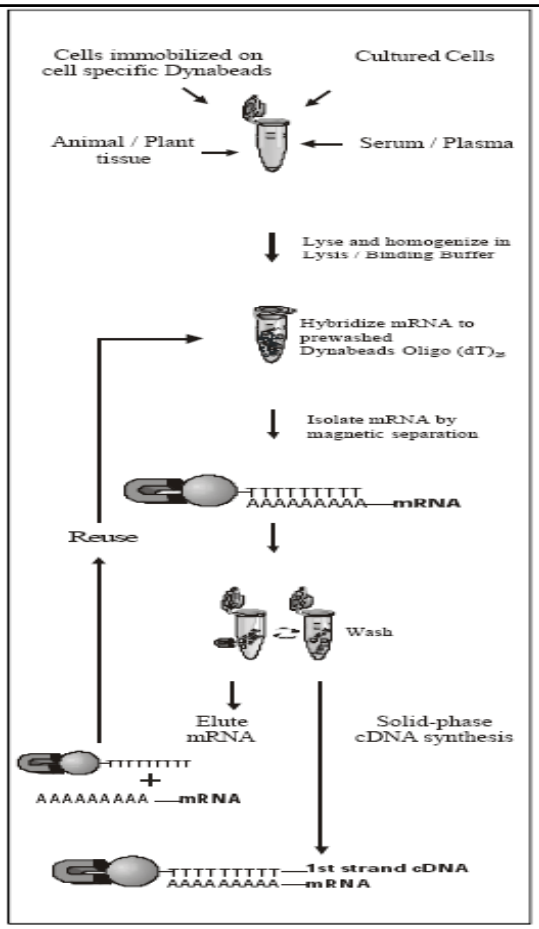


*Fig. 1: Schematic flow-chart of mRNA isolation with the Dynabeads mRNA DIRECT ™ Kit*

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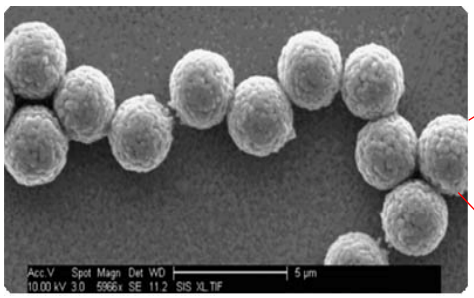
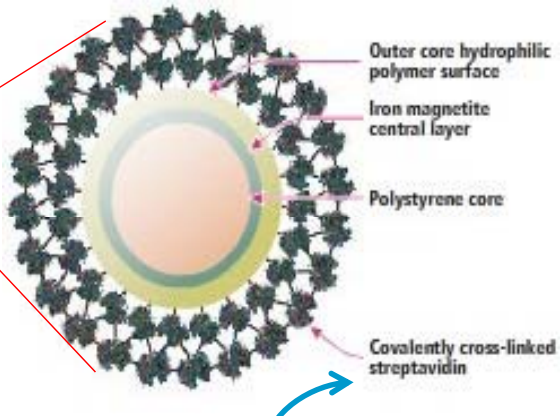
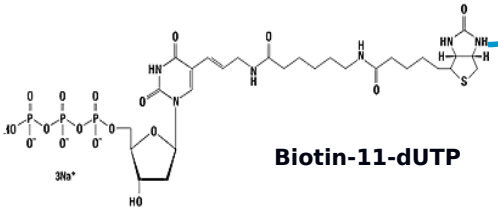
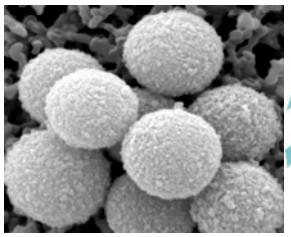



*OligodT*$_{12\text{-}25}$




**Biotin-11-dUTP**



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**Una genoteca de cDNA es representativa del conjunto de mRNAs poli-A de un tipo celular determinado, creciendo bajo condiciones específicas y a una edad o tiempo de cultivo determinado.**

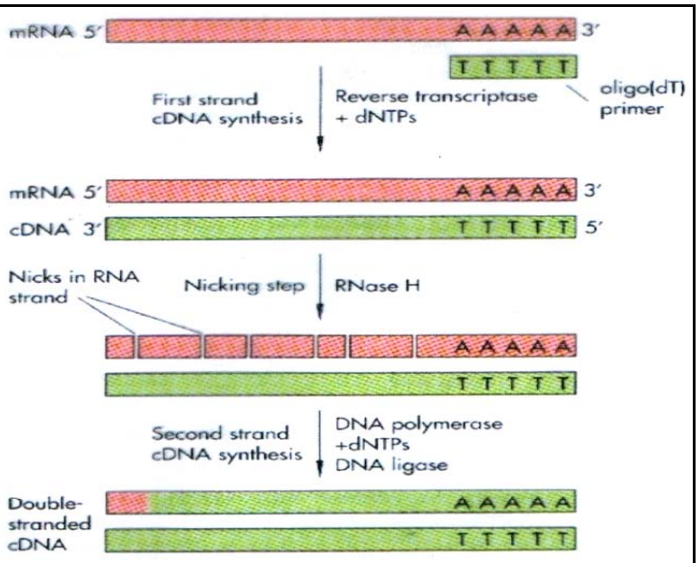


FIGURE 7-3
One method of converting mRNA to double-stranded cDNA. Poly(A) RNA is incubated with short deoxythymidine-containing oligonucleotides [oligo(dT)], which hybridize to the poly(A) tails, forming primed templates for the enzyme reverse transcriptase. The result of this reaction is a collection of RNA–DNA hybrids. To clone the cDNA molecules, the RNA strands must be destroyed and replaced with DNA. One way this is done is to use an enzyme called *RNase H*, which nicks the RNA–DNA hybrids. These nicks then serve as initiation sites for DNA synthesis by *E. coli* DNA polymerase. Eventually, most of the RNA fragments are replaced by DNA. The double-stranded DNA molecules are finally treated with DNA ligase, which seals up any remaining nicks in the new DNA strand. To prepare cDNA enriched for a specific sequence, cDNA synthesis can be primed with a specific oligonucleotide primer. This method is often used when the goal is to obtain the missing 5′ end of a partial cDNA clone.