Expression in animal cells

Once the DNA encoding the protein of interest is introduced into the cells, expression may be transitory over a period of days or weeks until the DNA is lost from the population. The ability to express the heterologous DNA during a short period of time is called **"transient expression"**. A few transfected cells may incorporate the exogenous DNA into their genome by a recombination mechanism leading to the **"stable expression"** of a gene. Figure 3.3 shows the different phases involving the expression of a heterologous gene.

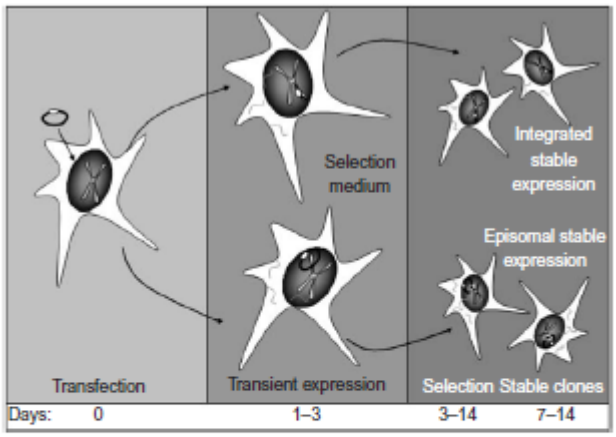


Figure 3.3

Expression of a heterologous gene in mammalian cells. The transfection of host cells with the recombinant vector can be performed by different methods (see Section 3.7). During the initial phase, first to third day upon transfection, the cells express the gene transiently. In the absence of selection agent, the DNA is eventually lost from the cell population. In a small proportion of transfected cells, the exogenous DNA is incorporated, by non-homologous recombination, leading to the generation of cell lines that stably express the gene of interest. If the vector contains episomal replication elements, then a stable episomal expression of the exogenous gene takes place. If the vector integrates into the cell genome, then a process known as integrated stable expression occurs.

The success of transfection, either stable or transient, depends on several factors that must be taken into account and can be summarized as follows:

(i) the transfectability and physiology of the cell line;

(ii) the characteristic of the genetic marker in the expression vector;

(iii) the type of expression desired;

(iv) the size of the expression cassette and the quality of the DNA to be introduced;

(v) the compatibility of the transfection method and/or reagents with the cell line;

(vi) the type of assay to be used for detection of the recombinant product; and

(vii) the presence of fetal bovine serum and/ or antibiotics in the culture medium. Additionally, the selection of an appropriate vector is as important as the selection of the cell line and/or of the transfection conditions.

2. Transient expression

In general, transient expression allows fast production of a desired protein to characterize it or to verify the integrity, functionality, and efficiency of different recombinant vectors. This represents a convenient means of comparing the performance of different vectors and ensuring their functionality before the more laborious procedure of isolating and characterizing stably transfected cell clones. Transient expression obviates the clonal variation commonly observed in stable transfections. The production of large amounts of recombinant protein has recently been reported for transient expression systems on large scales. Different cell lines can be used for transient expression. These include COS, BHK, CHO, and two variants of genetically modified HEK-293 cells: HEK-293 T and HEK-293 EBNA.

Table 3.1 Cell lines commonly used in the biopharmaceutical industry

Cell line	Source	Applications
COS	Monkey	Transient expression Production of recombinant viruses
HEK-293	Human	Transient and stable expression Production of recombinant viruses
BHK-21	Hamster	Transient and stable expression Vaccine production
CHO.K1, CHO dhfr-	Hamster	Transient and stable expression
Hybridomas, NS0, SP2/0	Mouse	Stable expression Monoclonal antibodies production
MDCK	Dog	Stable expression Vaccines production
Per.C6™	Human	Stable expression Production of recombinant viruses and vaccines
Vero	Monkey	Vaccine production
Sf9, Sf21	Insect	Production of recombinant proteins and baculoviruses
Tn-368, High-Five® BTI-TN-5B1-4	Insect	Recombinant protein production

3. Stable expression

In the case of stable transfections, the selection agent is added to the cultures approximately 2 days post-transfection. The selection phase normally lasts days to weeks. Those cells surviving the selection pressure harbor the selection marker, grow as discrete colonies, and can either express or not the protein of interest. These transfectants are soon isolated by means of traditional techniques, which include single cell cloning by cylinders, limiting dilution or soft agar. These methods have nowadays been replaced at the industrial level by fully automated techniques that are faster, less laborious, and enable high-throughput screening, for example, by flow cytometry and using robots. It is important to stress that generally less than 1% of a cell population that transiently expresses a gene will give rise to

stable cell lines. Table 3.2 compares the main advantages and disadvantages of transient and stable expressions.

Table 3.2 Comparison of transient and stable expression

Transient expression	Stable expression
Short time-frame for the generation of the product	Long time-frame for the generation of the product
Simple, does not require selection marker	Selection and/or amplification markers are required
No need to screen for recombinant clones	Tedious and time-consuming screening of the recombinant clones
Expression levels are not influenced by the "positioning effect"	The expression levels are influenced by the "positioning effect" and the gene copy number
Useful to confirm the integrity of expression vectors, convenient for high-throughput screening methods, e.g. to study different mutant genes	Once a stable expressing clone is identified, it constitutes an unlimited source for protein production
Circular DNA is used	The use of linearized or circular DNA depends on the transfection method, but, in general, linearized DNA is advisable

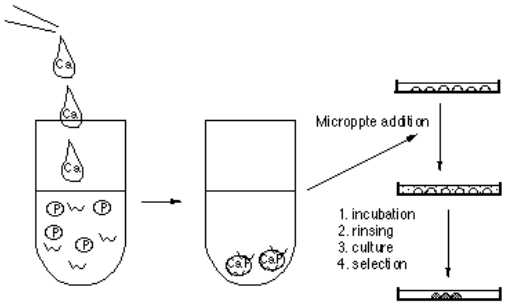
4. Introduction of DNA into mammalian cells

A wide variety of methodologies and reagents are currently employed to introduce different molecules into eukaryotic cells. The incorporation of DNA can be achieved by two different mechanisms: **infection or transfection**. The first consists of a biological process mediated by a virus (the viral infection of cells is mediated by receptors), while the second makes use of physical or biochemical methods to incorporate the DNA into the cell. Although the virus-mediated methods are more efficient, they are more laborious and time-consuming compared with transfection. Additionally, the nature of the infection process requires the presence of virus-specific receptors in the host cell to allow viral penetration, which restricts the spectrum of possible host cells. Another limitation of viral infection as a method for DNA transfer is that, unlike plasmid transfection, it is not possible to simultaneously transfer multiple recombinant viruses into the cell. In the next sections, the transfection methods most commonly used in cell culture laboratories will be described. It is difficult to predict the best method for transfection, so the expression vector, the cell type, and the facilities of each laboratory should be taken into account before deciding which technique to apply.

4.1 Calcium phosphate co-precipitation method

Although this technique was originally used to increase the infectivity of adenoviral DNA, it became more popular after extending its application to plasmid DNA. Although the original method has been frequently modified to increase the transfection efficiency, **it consists**

basically of mixing purified DNA with calcium chloride and phosphate buffer at neutral pH, which results in the formation of a fine, visible precipitate.



The precipitate (DNA–calcium phosphate complex), when added to the cells, is incorporated by phagocytosis. The complexes fuse to the phagosomes and are transported to different cellular organelles, including the nucleus. For many cell lines, the transfection efficiency can be further increased by means of short exposures of the cells to chemical agents like glycerol or dimethyl sulfoxide (DMSO) in concentrations between 10 and 20%. Variables such as pH, precipitate quality, incubation time with the precipitate, DNA concentration, cellular state, temperature, and concentrations of chemical agents, can influence the transfection efficiency. **This method is widely used because of its low cost, the lack of requirements for sophisticated infrastructure, and its applicability to a wide range of different adherent and suspension cell lines.**

On the other hand, cytotoxicity, high mutation rates, and the need for optimization and standardization of the transfection conditions are recognized as the major disadvantages of this method.

4.2 Cationic polymers

Several polycations with a good buffering capacity below physiological pH, such as lipopolyamines and polyamidoamine polymers, have proved to be efficient transfection agents. Two of the most popular methods based on polycations are discussed below: **diethylaminoethyl- dextran (DEAE-dextran)** and **polyethylenimine (PEI).**

DEAE-dextran. Like the calcium phosphate co-precipitation method, the DEAE-dextran technique was originally developed to increase the viral infectivity of animal cells, and its application was later extended to transfection processes. Although it is simple, efficient, and appropriate for transient expression, its use for stable transfections has not given satisfactory results. The DNA is incorporated by endocytosis, and thus exposed to extreme pH levels and cellular nucleases, which may explain, to a certain extent, the high frequency of mutations

observed when transfecting by this method. This transfection technique can be applied to both adherent and suspension cell lines.

PEI. Boussif et al. (1995) reported for the first time the use of PEI as a vehicle for gene delivery into cells and, since then, there has been a growing interest in the in vitro and in vivo applications of this method to animal cells**. PEI is an organic polymer with a high density of amino groups that can be protonated**. At physiological pH, the polycation presents a high affinity for binding DNA and can mediate the transfection of eukaryotic cells. Although the precise mechanism mediating gene transfer by PEI remains unknown, it has been postulated that the buffer capacity of the polycation at the lysosomal level would protect the DNA:PEI particles from nuclease degradation. The critical parameters for PEI- mediated transfections are the amount of DNA, the PEI/DNA ratio, the cell density at the time of transfection, and the order of addition of reagents. The cost of the PEI-based method enables its use in transient transfections on a large scale.

4.3 Lipid-mediated gene transfer (lipofection)

Since the first work of Felgner et al. (1987), describing the use of a synthetic cationic lipid for transfection, more than a dozen cationic liposomes have been developed. Under optimal conditions, the cationic lipids form small unilamellar liposomes when formulated in water. **The surface of these liposomes is positively charged and interacts with the phosphate backbone of the DNA, forming complexes that present high affinity for the negatively charged surface of the cell membrane.** The uptake and delivery of lipid–DNA complexes into the intracellular compartment is mediated **by endocytosis**. Normally, cationic liposomes contain an amphiphilic cationic lipid (DOSPA, DOTMA, etc.) and a neutral helper lipid, generally DOPE. This innovative technology stands out as being easy to accomplish, and its diverse applications offer reproducible results associated with very good yields. The large variety of formulations available has conferred to **lipofection** a great versatility of applications: stable cell lines and primary cultures, transient and stable transfections, adherent or suspension cells. Different formulations presenting high transfection efficiencies are commercialized by several companies. Unfortunately, the **high cost** of these products precludes their use in large-scale processes. Multivalent cationic lipids present better transfection efficiencies than those composed of monovalent cationic lipids. For example, LipofectamineTM (composed of DOSPA:DOPE, i.e. a multivalent cationic lipid associated with a helper lipid) turned out to be more effective than

Lipofectin (DOTMA:DOPE, a monovalent cationic lipid associated with a helper lipid). Both these formulations are commercialized by Invitrogen.

4.4 Electroporation

In this method, the delivery of DNA molecules to the cells is mediated by short strong electrical pulses. The exposure of the cells to an electrical field induces a potential difference across the cellular membranes that generates **temporary pores through which the DNA reaches the cytoplasm**. Compared with other methods, such as calcium phosphate co-precipitation and DEAE-dextran, **electroporation presents lower mutation frequencies**, perhaps because the DNA remains free in the cytoplasm and nucleoplasm. Several cell types resistant to transfection by other procedures, including lymphocytes, hematopoietic stem cells and murine embryonic stem cells, have been successfully electroporated. Furthermore, this technique renders higher efficiencies when linearized DNA is used. The advantages of this technique include its simplicity, reproducibility, applicability for transient and stable transfections, for adherent and suspension cells, and the possibility to control gene copy number in transfectants. On the other hand, the pulse time and the intensity of the electrical field raise concerns and must be determined empirically for each cell type, since succesful transfection can be achieved in a very limited range of voltage.

A number of electroporation devices are commercially available, which are safe, easy to handle, and allow the control of different electroporation parameters. In addition to the physical properties of the electrical pulse, the type of buffer solution, and the quality and concentration of cells (exponentially growing cells, between 106 and 107 cells/ml) have to be considered.

5. Selection markers

Upon transfection, there are cells that incorporate the plasmid (**transfected**) and others that do not (wild-type). A selection system allows the separation of these two cell populations. In this regard, the detection of morphologic changes or, more commonly, the use of some toxic metabolite for which the vector confers resistance (biochemical markers) have become the methods of choice to select positive transfectants.

5.1 Morphological changes

In general, cells transfected with DNA that contains transforming genes, derived from oncogenic viruses, lose the growth feature called ‘‘contact inhibition,’’ which limits their

growth and proliferation. These transfectants can be visualized under the microscope and isolated as colonies.

5.2 Biochemical markers

Many genes that encode proteins causing biochemical tranformations in the cells have been isolated, characterized, and employed in eukaryotic expression systems. Among these biochemical markers, those conferring resistance to cytotoxic drugs are among the most commonly used. They allow the selection of stably transfected cells from a heterogeneous population cultured in the presence of toxic metabolites. Plasmids employed for stable expression contain a selection cassette with a gene that will provide a new property to the transfected cell. Both the sequence encoding the product and the selection gene can be contained in the same plasmid or in individual vectors, and this has no influence on the expression levels of the recombinant protein.

The resistance to a drug can be **recessive** or **dominant.** Those genes providing recessive resistance need a particular host, which must be deficient in the activity by which it will be selected, whereas the genes confering dominant resistance can be used independently of the biochemical background of the cell. Use of the TK gene is one example of selection employing a recessive biochemical marker. Mammalian cells display an absolute dependency on the TK enzyme when they are cultivated in the presence of aminopterin, which blocks the de novo synthesis of thymidine. The selection medium HAT, containing hypoxanthine, aminopterin, and thymidine, is used to rescue cells that express TK. Obviously, this selection system requires cells that lack TK, also identified as TK–. In this respect, TK– cell lines are available from human and murine origin.

The selection systems based on a **dominant marker** are based on **resistance to antibiotics**. A large number of drugs are used for this purpose: neomycin, hygromycin, puromycin, bleomycin, and zeocin, among others. However, two aminoglycoside phosphotransferases are most routinely used, namely **neomycin-phosphotransferase** and **hygromicin-B-phosphotransferase**. Both confer resistance by phosphorylation-mediated inactivation of the corresponding antibiotics. The toxic metabolites added to the culture medium are **G418** (analog to gentamicin and also known as geneticin) and **hygromycin B**, both of which inhibit protein synthesis.

5.3 Reporter markers

The advent of reporter markers has greatly contributed to a better understanding of gene expression in eukaryotic cells. A reporter gene encodes for a protein that can be monitored by simple and sensitive methods. Reporter genes are commonly used to:
(i) optimize and monitor DNA delivery methods;
(ii) study a variety of promoters and regulatory elements, since the expression levels of the reporter protein will reflect the transcription activity;
(iii) analyze the traffic and/or the cellular compartmentalization of the studied protein;
(iv) characterize transcription factors and associated proteins, such as co-activators;
(v) identify protein–protein interactions; and
(vi) develop high-throughput screening of compounds with potential therapeutic effects.

An ideal reporter gene should meet the following characteristics:
(i) to be absent or expressed in very low levels in the cell;
(ii) to be detected by a simple, sensitive, and reliable method;
(iii) not to induce pleiotropic effects in the host cell.

A significant number of reporter genes are suitable for use in mammalian cells, whose expression is evidenced by colorimetric, fluorescence, or chemiluminescence methods. Some examples are described below.

Chloramphenicol acetyl transferase (CAT). This bacterial enzyme was the first reporter protein used for studying the transcriptional activity of eukaryotic regulatory sequences. CAT inactivates chloramphenicol, an inhibitor of prokaryotic protein synthesis, by converting it to the mono- or di-acetylated species. Measurement of CAT activity requires a 14C-radiolabeled chloramphenicol or acetyl-CoA and, therefore, an additional step is neccessary to separate the radio-labeled reactant from the product. Novel detection methods based on fluorescent substrates or ELISA assays, which do not use radiolabeled reagents, have been described more recently.

Beta-galactosidase (β-gal). The β-gal enzyme from E. coli is the most frequently used reporter marker in science, because it is functionally active and well tolerated in a variety of cell lines. β-gal activity can be monitored both qualitatively, by in situ staining, and quantitatively, in cell lysates, without the need for sophisticated laboratory equipment. The enzyme hydrolyzes many natural or synthetic β -D-galactopyranosides, which allows the use of different detection methods. Because mammalian cells have endogenous β gal activity, this

reporter gene should be used only in cells displaying a low endogenous activity. In situ detection of β -gal requires cell fixation and addition of the colorless substrate 5-bromo-4-chloro-3-indolyl-_-D-galactoside (X-Gal), which is converted into a **blue precipitate** by the enzyme action. On the other hand, determination of β-gal activity in cellular extracts is performed by a simple and fast assay based on the conversion of the colorless orthonitrophenyl- β -D-galactopyranoside (ONPG) into galactose and ortho- β -gal activity can be assessed in vivo using the substrate FdG (fluorescein di-_-D-galactopyranoside). Hydrolysis of this compound releases fluorescein, which is retained intracellularly and can be measured by fluorescent activated cell sorting or FACS.

Luciferase. Luciferase and luciferin are a non-specific enzyme and its substrate, respectively, that upon reaction in the presence of ATP, Mg2þ, and O2 generate **bioluminiscence**. Different genes or cDNAs encoding luciferases have been isolated from different organisms, such as unicellular seaweed, marine bacteria, and fish. Due to the specificity of the reaction, many luciferases have been used as reporter proteins either in prokaryotic or eukaryotic cells. **Firefly luciferase (LUC)** is one of the most widely used. The oxidation reaction produces **oxyluciferin, CO2, and a photon.** The intensity of light emitted during the reaction is measured with a **luminometer.** Luciferase-based techniques are sensitive, simple, rapid, inexpensive, safe (organic solvent or radiolabeled compounds are not required for the test), and applicable to a wide range of cell lines. One of the most recent advances constitutes the development of highly sensitive techniques that make the detection of luciferase activity in living cells and organisms feasible.

Green fluorescent protein (GFP). GFP was discovered during the investigation of marine bioluminescent organisms. In the 1960s, it was demonstrated that Aequoria jellyfish emitted light after the addition of Ca2þ, with no need for other cofactors. The gene encoding the Aequoria GFP was cloned in 1991, and is used as a reporter in cells, where upon excitation at 395 nm a green fluorescence is emitted at 509 nm. The GFP is exceptionally stable with respect to pH, temperature, and proteolysis, and can be detected in living cells and organisms. Due to the prolonged time required between the post-translational maturation and the appearence of fluorescence (3–5 hours) in the wild-type GFP, different mutants exhibiting an increased fluorescence intensity have been generated. The enhanced GFP (EGFP) is a variant with two amino acid substitutions, whose fluorescence is shifted to the red spectrum. The advantages that EGFP presents over the wild-type species are:

(i) an increased fluorescence intensity;
(ii) a shorter time period between protein synthesis and fluorescence; and
(iii) higher expression levels in several animal cells.
Currently, there are also yellow (EYFP) and cyan (ECFP) variants, which are excited at 514 and 434 nm, with a maximal emission at 477 and 527 nm, respectively.