TECHNIQUES IN GENETIC ENGINEERING

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CHAPTER 7

Cell Culture

“Some cultural phenomena bear a striking resemblance to the cells of cell biology, actively preserving themselves in their social environments, finding the nutrients they need and fending off the causes of their dissolution.”

Daniel Dennett III (1942 -)

Tissue culture refers to the *in vitro* cultivation or "culturing" of an entire tissue, such as epidermal tissue of the skin. Obviously, due to intrinsic features of the cells such as their dividing capacities, some tissues are easier to grow *in vitro* than others.

Cell culture refers to the *in vitro* culturing of dispersed cells derived from either primary tissue (in which case it is called **primary cell culture**), from a cell line, or from a cell strain (we will discuss the difference of these latter two terms later).

Organ culture refers to the 3-dimensional culturing of undisintegrated tissue (thereby preserving some of the features of the organ although it is detached from the body).

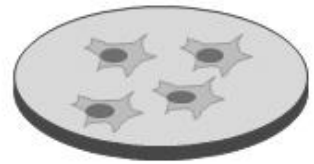
Organotypic culture, on the other hand, refers to the recombination or co-culturing of cells from different lineages *in vitro*, so as to re-create some of the features of the organ. And of course, all of the above terminologies may refer to animal cells, plant cells or insect cells.

Depending on what type of culture one wishes to use in one's studies, there are some important issues to be considered, such as

(a) source of cells;
(b) dispersion methods (not every tissue can be dispersed in the same way, due to differences in matrix material);
(c) defined media (ie antibiotics, nutritional supplements, growth factors, hormones, etc);
(d) temperature;
(e) pH;
(f) incubators; and
(g) method of propagation of cells over passages

Cells can be grown as either adherent or suspension cultures, depending on the source or origin:

for example, fibroblast or epidermal cells require tight attachment or adherence to an extracellular matrix, and they have to be grown as adherent cells.



On the other hand, leukocytes (white blood cells) are cells that normally circulate in the bloodstream without any significant attachment, and are highly mobile, therefore they can be grown as suspension cells.

Genetic manipulation of cells

The method for genetic manipulation cells is largely dependent on the type of cell in question; however one can sub-divide these methods into 5 major categories:

- **Electrical**, such as in electroporation;
- **Mechanical**, such as in microinjection or gene gun;
- **Chemical**, such as with liposomes or calcium phosphate;
- **Viral**, such as with baculoviral, retroviral or lentiviral vectors; and
- **Laser**, such as in phototransfections.

Viral methods

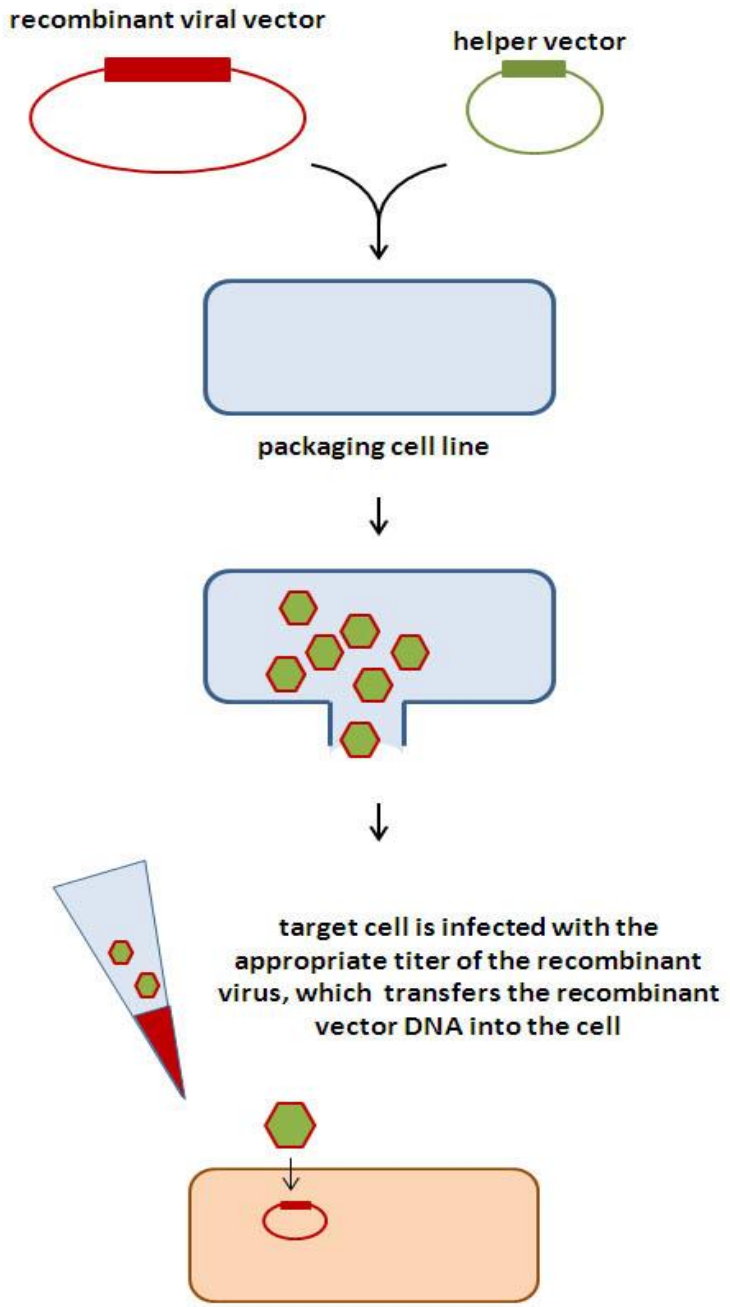


Figure 7.1. A simplified scheme of how viral vectors are typically used. The viral vector that is constructed to carry the insert DNA is co-transfected to a packaging cell line along with a helper vector. The recombinant DNA gets packaged into viral particles; after calculation of viral titer (Fig.7.2), target cells are infected with the recombinant virus, allowing for the transfer of recombinant DNA

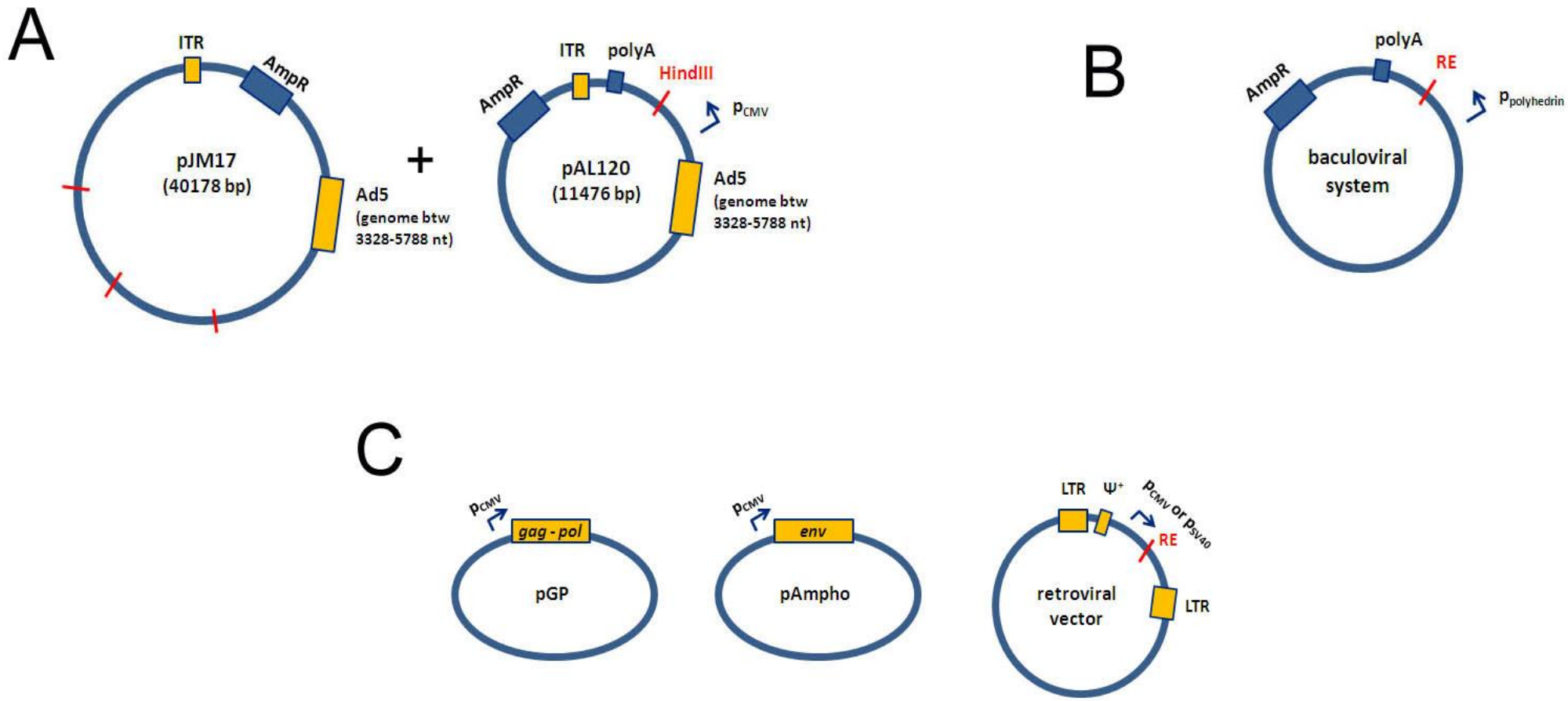


Figure 7.3. Schematic representations of some common viral vectors. **(a)** Adenoviral vector pAL120 and helper vector pJM17, based on Adenovirus type 5. ITRs are inverted terminal repeats; pJM17 vector contains the replication-defective portion of the Ad5 genome (note that there are various HindIII sites within the plasmid, shown with red lines). The pAL120 vector is used to clone the gene of interest into the HindIII site, under pCMV promoter. Upon co-infection, homologous replication will transfer the gene of interest to the Ad5 genome in pJM17, in place of E1 gene, and allow for adenoviral particle production. **(b)** Baculoviral vectors are used typically to express genes under the polyhedron promoter of baculoviruses. **(c)** Retroviral vectors (rightmost) are usually used to clone genes under the constitutive CMV or SV40 promoters, and include the psi-packaging elements (Ψ+), however require the presence of other helper vectors that express gag, pol and env proteins (see text for details). LTR: long terminal repeats.

Reporter plasmids and genes

The plasmids used for transfection and analysis in (animal) cells broadly fall into 4 different catergories based on the promoters used:

- Plasmids containing minimal promoters, such as the HSV *tk* (Herpes Simplex Virus *thymidine kinase*) promoter, are essentially used to study enhancer elements or activating regulatory motifs;

- Plasmids containing constitutive promoters, such as CMV (cytomegalovirus) or SV40 (simian virus 40) promoters;

- Plasmids with cell type-specific promoters;

- Plasmids with regulatable promoters.

Reporter plasmids and genes

The genes commonly used for reporter activity include

- lacZ
- luciferase
- GFP or other fluorescent proteins

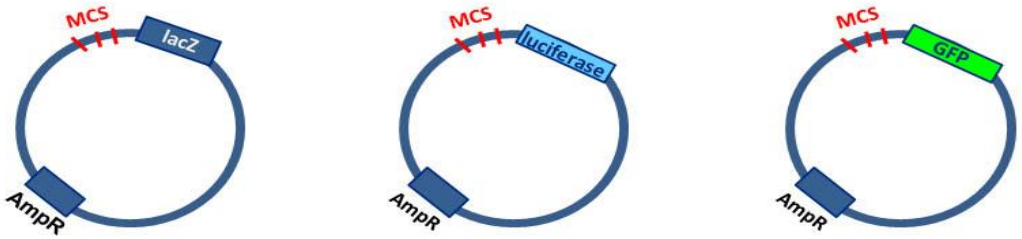


Figure 7.5. Simplified diagrams of typical reporter vector backbones. Hypothetical LacZ, luciferase and GFP reporter vectors are shown (left to right).

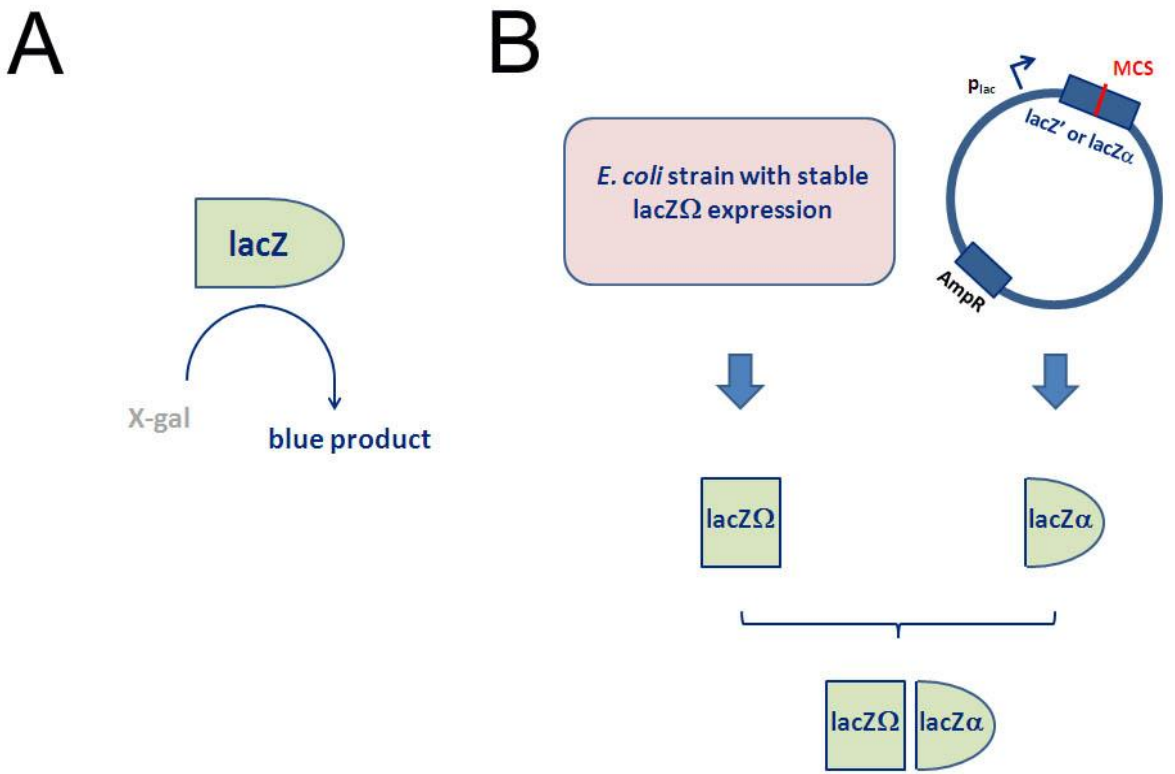


Figure 7.4. Schematic representation of how lacZ is used as a reporter. **(a)** a simplified scheme of the catalytic reporter activity of lacZ, whereby a colorless substrate X-gal is cleaved to generate a blue-colored product (see text for details). **(b)** LacZ complementation is based on the principle that the enzyme can be split into two polypeptides that individually cannot function, but only exhibit catalytic activity when expressed in the same cell at the same time

Types of transfection

Transient transfection

the gene transferred to the cell is not integrated into the genome of the cell

the transfected gene gets diluted in the daughter cells with each cell division, in the absence of selection pressure

Usually high copy number vectors that contain strong promoters are used for high level expression of proteins, markers or reporters

high level of expression from the transfected gene only lasts for a few days after transfection,

the plasmid DNA is present in large numbers per cell, and thus creates a discrepancy with the rest of the genome that is diploid

Stable transfection

the transfected DNA gets integrated into the genome, albeit with varying probability (the efficiency of integration depends on cell type, method used, or selection pressure applied)

the results of stable transfections are more reliable since there is only one copy of the transfected gene, rather than tens or hundreds, and is therefore more physiological

stable integration ensures transmission to daughter cells over many generations

viral vectors are usually preferred for stable cell line generation, since they have the capacity to integrate into the genome of their target cells

Recombination and integration into genome

Homologous recombination

Homologous recombination is a process in which DNA molecules with overall *similarity* (not *identity!*) exchange corresponding parts of their sequences.

It is mostly studied in crossing over during meiosis, and in post-replicative recombination repair.

Homologous recombination can occur between any two of the recombination hot spots distributed almost randomly throughout the sequence in each chromosome.

Site-specific recombination

Site-specific recombination, in principle, relies on very similar mechanisms to homologous recombination;

however recombination occurs at a specific site of around 30 – 200 bp, outside which no homology is required.

These sites are typically asymmetric in nature, enabling the recombinase enzyme to recognize the left and right “halves” of this motif.

The recombinases that are mostly used in genetic modification are the Cre and Flp recombinases

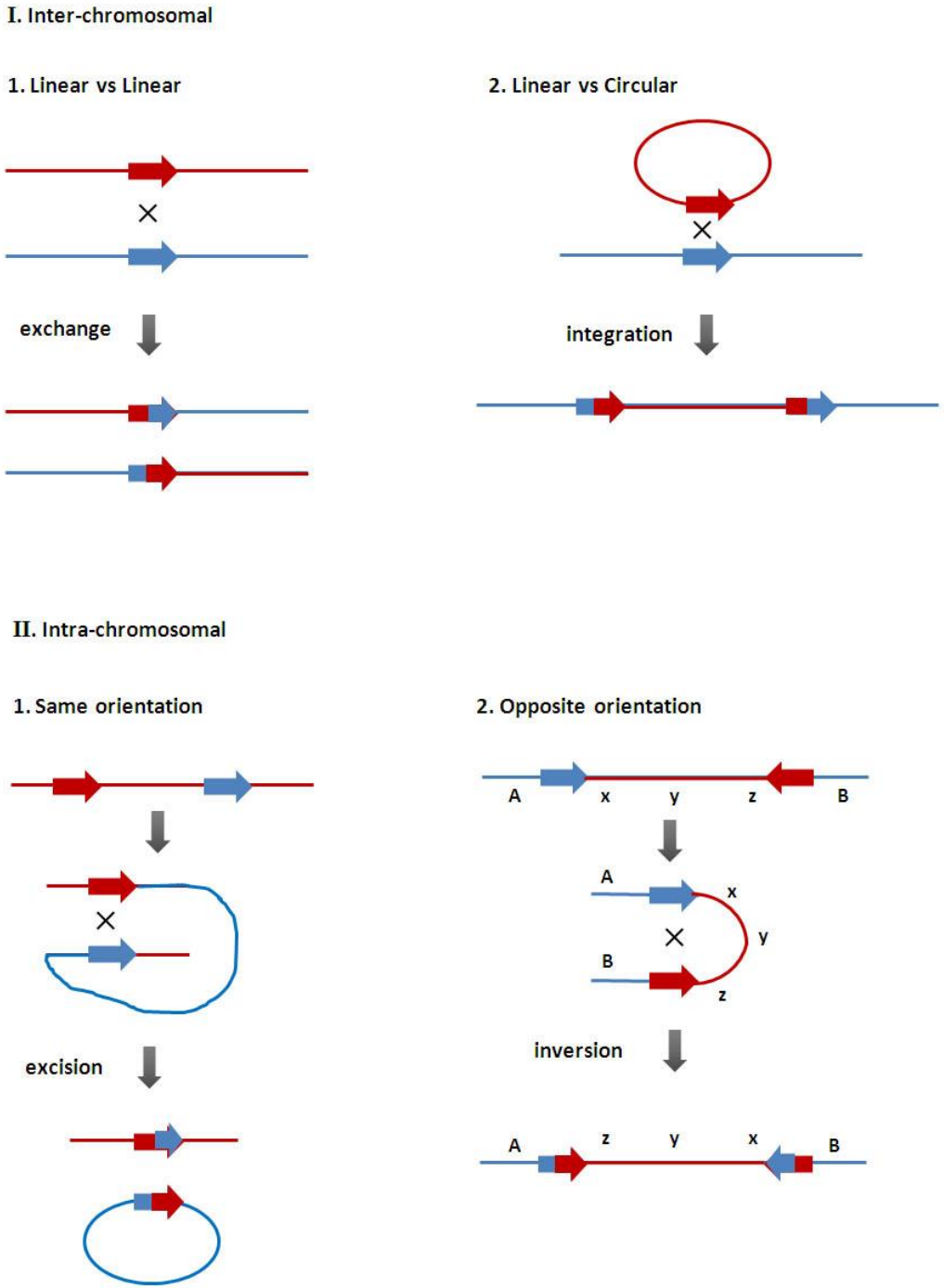


Figure 7.6. Summary of possible products of site-specific recombination.

I. Inter-chromosomal exchange (or inter-molecular exchange) can occur between either

(1) two linear DNA molecules, which results in strand exchange from specific recombination motif (shown with two boxes and a cross), or

(2) one linear and one circular DNA molecule, which results in integration of the circular DNA to the linear one.

II. Intra-chromosomal (or intra-molecular) exchange occurs within the same DNA molecule; however in this case the recombination motif can either

(1) be in the same orientation, which results in excision of the region in the middle as circular DNA, or

(2) in the opposite orientation, which results simply in the inversion of the middle region.

Level of expression

Constitutive expression

Constitutive expression refers to the cases where the gene that is transferred into cells will be expressed at all times, under all conditions. Many mammalian expression vectors such as pCMV series (Fig.2.21) use strong promoters that are always active. This is particularly preferred if high amounts of protein expression is required for preparative purposes.

Inducible expression

Inducible expression in cells is achieved by selecting a promoter that is regulated by a variety of stimuli, which in turn regulate the activity of transcription factors. Some promoters are activated by heat stimulus (eg. Heat shock promoters); some are activated by chemicals (eg. lactose-, lactose analog IPTG-, or tetracycline-inducible promoters); some others are regulated by presence of absence of oxygen (such as hypoxia-inducible promoters).

Tetracycline is perhaps one of the most common inducing agents used in mammalian inducible expression systems, therefore here we choose to explain the basic principles of tet-ON and tet-OFF systems as examples. Essentially, in **tet-ON** systems the transfected gene is turned ON in the presence of tetracycline, or its analog doxycline, in a dose-dependent manner (ie, the higher the tetracycline, the higher the expression level); whereas in **tet-OFF** systems the transfected gene is turned OFF in the presence of tetracycline or doxycline

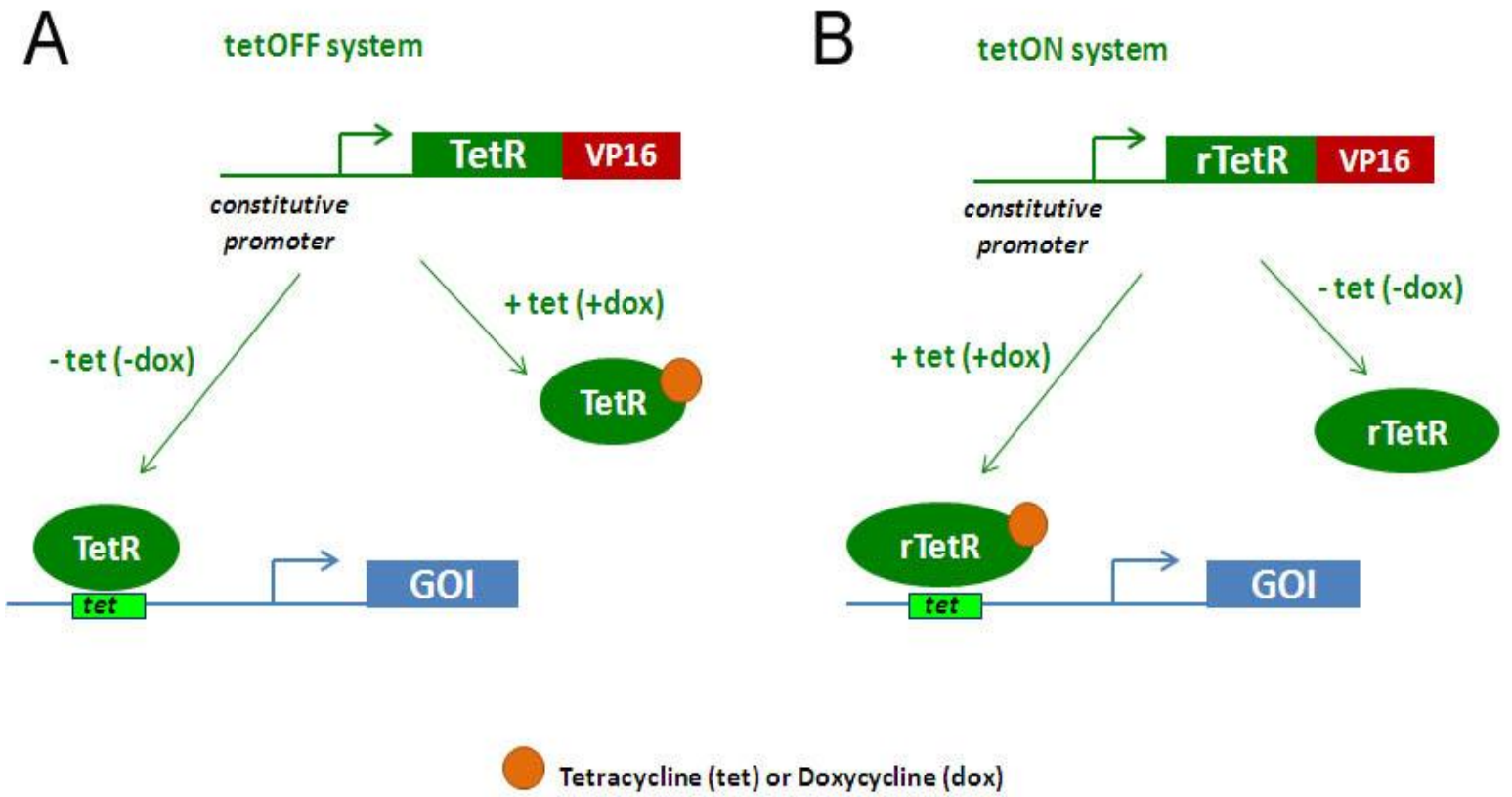


Figure 7.7. Schematic diagram of tetracycline-inducible system. (a) Tet-OFF system relies on the binding of the tetracycline repressor (TetR) to the *tet* motif on target promoters. In this system, TetR is fused to the acidic activation domain of the VP16 transcription factor, and positively regulates transcription from the target promoter in the absence of inducer, upregulating the expression of Gene of Interest (GOI). TetR itself is released from the *tet* motif upon binding of tetracycline (tet) or its analog doxycycline (dox) to the TetR, thereby releasing the TetR-VP16 fusion from the inducible promoter, hence "tet-OFF". (b) In the tet-ON system, TetR is engineered such that its DNA binding properties are reversed: rTetR binds to the promoter in the presence of tet (or dox), and is released from the promoter in the absence of the inducer. Therefore, the GOI is turned "ON" in the presence of tet (or dox)

“Research is what I’m doing when I don’t know what I’m doing.”
Wernher von Braun