Lecture 20: DNA Delivery in Host (Part-I)

Introduction: The delivery of DNA into the host is required for generation of genetically modified organism. DNA delivery to host is a 3 stage process, DNA sticking to the host cell, internalization and release into the host cell. As a result, it depends on 2 parameters-

Surface chemistry of host cell-Host cell surface charges either will attract or repell DNA as a result of opposite or similar charges. Presence of cell wall (in the case of bacteria, fungus and plant) causes additional physical barrier to the up-take and entry of DNA.

Charges on DNA-Negative charge on DNA modulates interaction with the host cell especially cell surface.

Modulation of these two properties is achieved in different methods to deliver DNA into the host cell and it is the topic of the discussion of today's lecture.

Transformation- it is the natural process, through which bacterial population transfer the genetic material to acquire phenotypic features. The event of transformation was first time demonstrated by **Frederick Griffith** in 1928. The schematic presentation of the experiment is given in Figure 20.1. Griffith has used two different *Streptococcus pneumonia* strains, virulent (**S**, causes disease and death of mice) and avirulent (**R**, incapable of causing disease or death of mice). In a simple experiment he injected 4 different combination of bacterial mixture, (1) live S, (2) heat killed S, (3) live R, (4) mixture of live R and heat killed S in to the mice. The observation indicates that live S has killed the mice where as mice were healthy with heat killed S or live R. Surprisingly, mice injected with mixture of live R with heat killed S were found dead, and bacteria isolated from these dead mice were virulent. Based on these observations, Griffith hypothesized the existence of a transforming agent (Protein, DNA) being transferred from heat killed virulent strain to the avirulent strain and proposed the concept of transformation. Later, Oswald has proved that the transforming factor is DNA rather than protein.

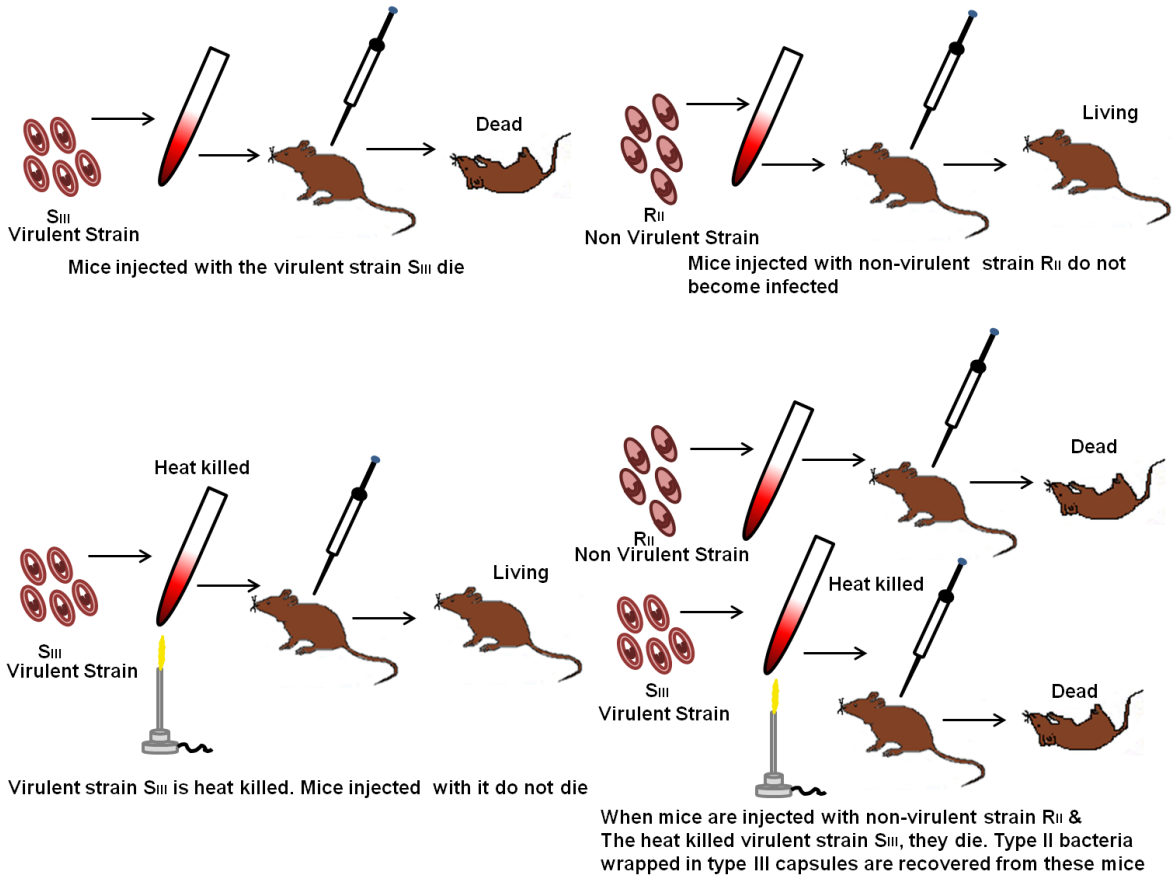

Figure 20.1: Discovery of Transformation

Mechanism of Transformation- Transformation is the process by which cell free DNA is taken up by another bacteria. The principle steps of transformation are given in Figure 20.2.The DNA from donor bacteria binds to the competent recipient cell and DNA enters into the cell. The DNA enters into the recipient cell through a uncharacterized mechanism. The DNA is integrated into the chromosomal DNA through a homologous recombination. Naturally transformation is common between closely related species only.

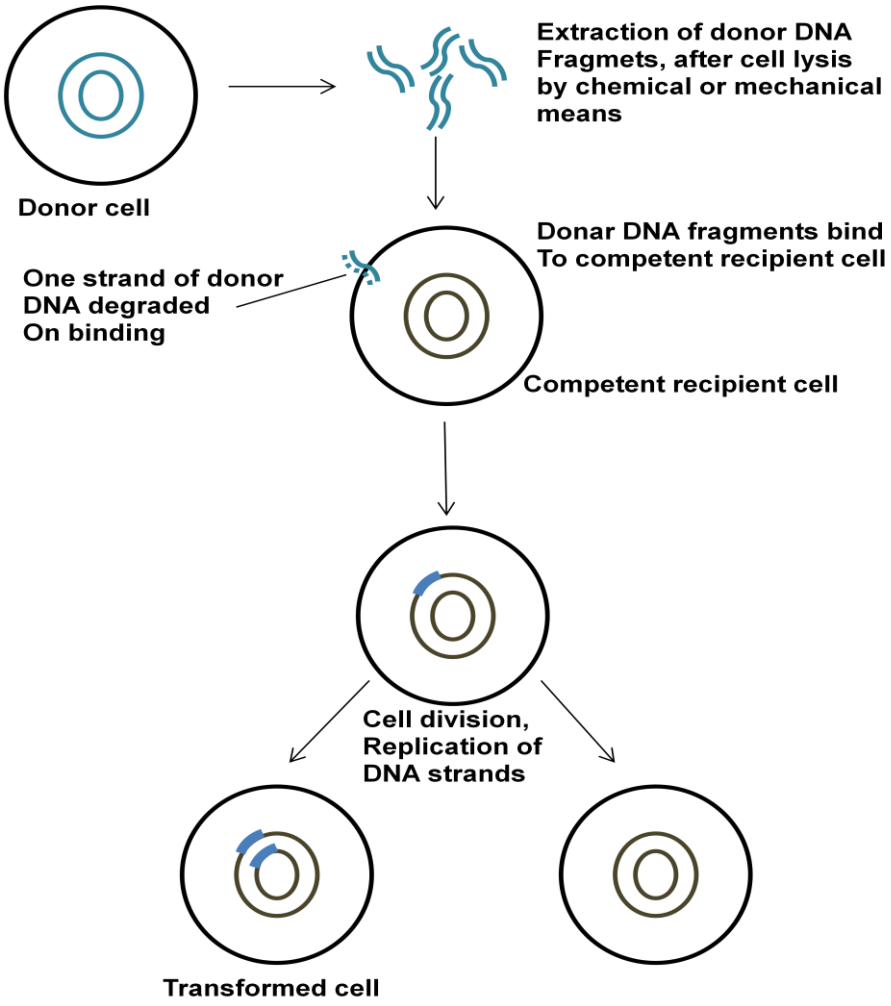

Figure 20.2: Principle steps in transformation.

Procedure: Natural ability of a bacterium to take up cell free DNA present in extracellular environment is low and only 1% bacteria are capable to take DNA. Hence, a number of (chemical/physical) treatments make the cell competent to take DNA through transformation. A list of agent used for different organism is given in Table 20.1.

TABLE 20.1: LIST OF SELECTED AGENT AS POTENTIAL TO MAKE CELL COMPETENT

Bacterial Strains	Competent agents
Streptococcus pneumoniae	mitomycin C, fluoroquinolone
In *B. subtilis*	UV light
Helicobacter pylori	ciprofloxacin
Legionella pneumophila	mitomycin C, norfloxacin, ofloxacin, nalidixic acid, bicyclomycin, hydroxyurea, UV light
E.Coli	Calcium chloride, Rubidium Chloride

The most popular reagent for making E.coli competent cell is calcium chloride. The complete procedure of transformation is given in Figure 20.3 and it has following steps:

1. Bacterial Culture- The growth stage of the bacteria has a significant impact for its ability to take up foreign DNA. The bacterium at log phase is more active and efficient to perform DNA damage and repair than stationery phase. As a result, it is preferred to use a bacteria of log phase for making competent cells for transformation.

2. Preparation of Competent Cell-Bacteria is incubated with divalent cation (Calcium chloride,Manganese chrloride or Rubidium chloride) for 30mins at 4^0C. During this process, cell wall of treated bacteria is swell and it gather factors required for intake of DNA docked on the plasma membrane.

3. On the day of transformation, competent cells are incubated with DNA or circular plasmid containing appropriate resistance gene such as ampicillin resistance gene for 30mins on ice.

4. Heat Shock-Competent cells are given a brief heat shock (42^0C for 90 sec) to relax the cell wall and high temperature stress causes upregulation of the factor responsible for DNA recombination and repair.

5. A chilled media is added for faster recovery of transformed cells.

6. it is plated on the solid media with appropriate antibiotics such as ampicillin and allowed to grow for another 18-24 hrs.

7. Transformed cells with appropriate resistance will grow and give colony.

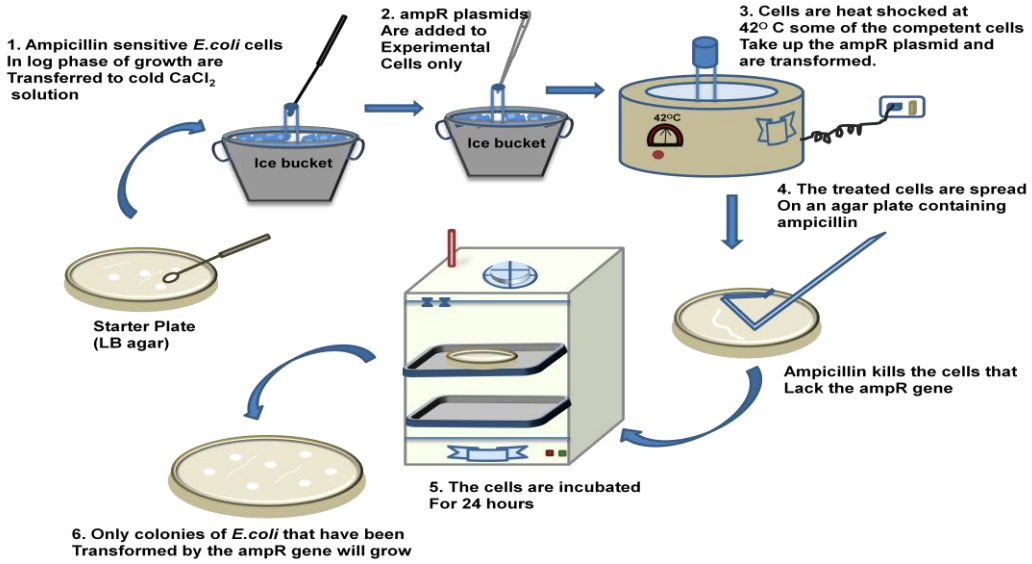

Figure 20.3: Steps in bacterial transformation by $CaCl_2$ method.

TRANSFORMATION IN YEAST

1. Lithium Acetate/ssDNA/PEG Method: In this method, yeast cells are incubated with a transformation mixture of lithium acetate, PEG 3500, single stranded carrier DNA and foreign plasmid at 42^0C for 40mins. The purpose of adding carrier DNA is to block the non-specific sites on cell wall and made plasmid available for uptake. Post-transformation, cells are pelleted to remove transformation mixture and re-suspended in 1ml water. It is plated on a solid media with an appropriate selection pressure such as antibiotics.

2. Spheroplast Transformation Method: In this method, yeast cell wall is removed partially to produce spheroplast. Spheroplasts are very fragile for osmotic shock but are competent to takes up free DNA at high rate. In addition, polyethyl glycol (PEG) is used to facilitate deposition of plasmid and carrier DNA on cell wall for easier uptake. The mechanism of DNA uptake in yeast is not very clear. A schematic of spheroplast method is given in Figure 20.4. (1) In the spheroplast method, yeast cells are incubated with zymolyase to partially remove cell wall to produce spheroplast. (2) They are collected by centrifugation and incubated with carrier DNA and plasmid DNA for 10mins at room temperature. (3) It is now treated with PEG and calcium for 10mins with gentle shaking. (4) Transformed spheroplast are plated on selective solid media and incubated on 30^0C for 4 days.

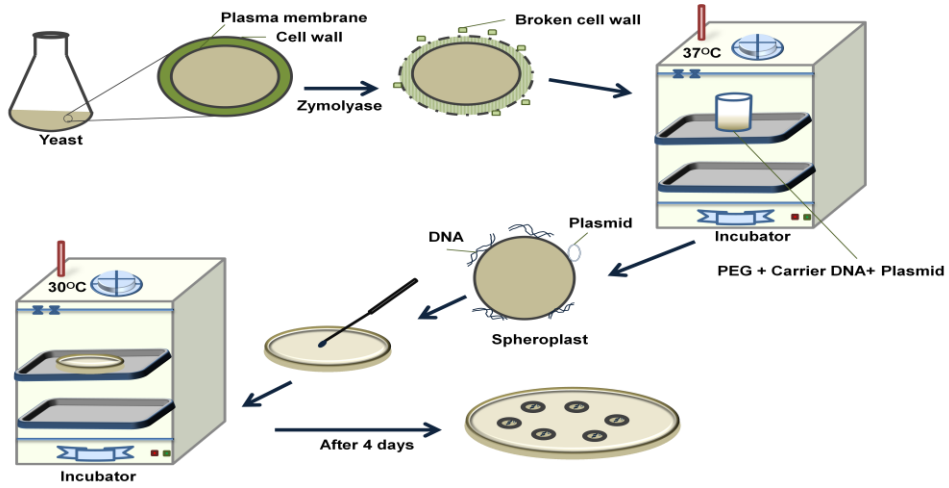

Figure 20.4: Steps in yeast transformation by sphereplast method.

Electroporation- Plasma membrane is composed of lipid and protein. These macromolecules give a partial conductance to the cell membrane. When a high electric pulse is given to the cell, the charge run across the membrane and partially disturbs the arrangement of lipid molecule. As a result, it makes formation of pore and allow easy passage of macromolecule especially charged molecule like DNA into the cell. After the electroporation, cell is allowed to recover from the damage and it forms colony on the selective solid media.

Lecture 21: DNA Delivery in Host (Part-II)

DNA Delivery in mammalian cells- mammalian cell membrane surface chemistry, intracellular comparatmentization and uptake mechanism is different from the prokaryotic cells or yeast. Hence specialized methods have been developed to suit mammalian cells. There are 4 major strategies to deliver the DNA in mammalian cells:

1. Chemical transfection techniques-The principle behind the chemical transfection technique is to coat or complex the DNA with a polymeric compound to a reasonable size precipitate (Figure 21.1). It facilitates the interaction of the precipitate with the plasma membrane and uptake through endocytosis. There are multiple chemical compounds have been discovered which can be able to make complex and deliver DNA into the mammalian cell.

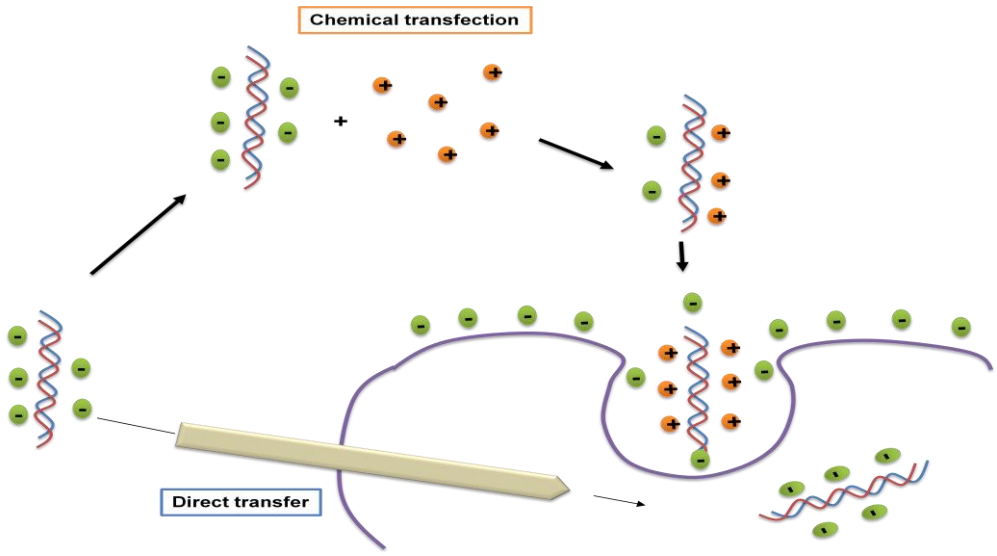

Figure 21.1: Mechanism of chemical method mediated DNA delivery in animal cells.

Calcium Phosphate method-In this method, DNA is mixed with calcium chloride in phosphate buffer and incubated for 20mins. Afterwards, transfection mixture is added to the plate in dropwise fashion. DNA-calcium phosphate complex forms a precipitate and deposit on the cells as a uniform layer. The particulate matter is taken up by endocytosis into the internal storage of the cell. The DNA is then escapes from the precipitate and reach to nucleus through a unknown mechanism. This method suited to the cell growing in monolayer or in suspension but not for cells growing in clumps. But the technique is inconsistent and the successful transfection depends on DNA-phosphate complex particle size and which is very difficult to control.

Polyplexes method- The disadvantage of calcium phosphate method is severe physical damage to the cellular integrity due to particulate matter settling on the cell. It results in reduced cellular viability and cyto-toxicity to the cell. An alternate method was evolved where DNA was complexed with chemical agent to form soluble precipitate (polyplexes) through electrostatic interaction with DNA (Figure 21.2). A number of polycationic carbohydrate (DEAE-Dextran), positively charged cationic lipids (transfectin), polyamines (polyethylenimines) etc are used for this purpose. The soluble aggregates of DNA with polycationic complex is readily been taken up by the cell and it reaches to the nucleus for expression (Figure 21.3).

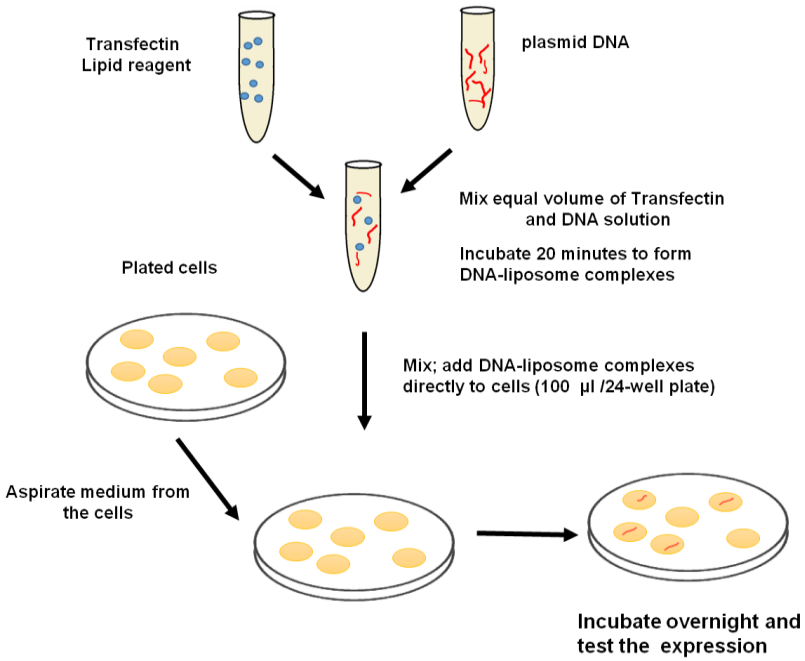

Figure 21.2: Transfection of animal cell with tranfectin (polyplexes)

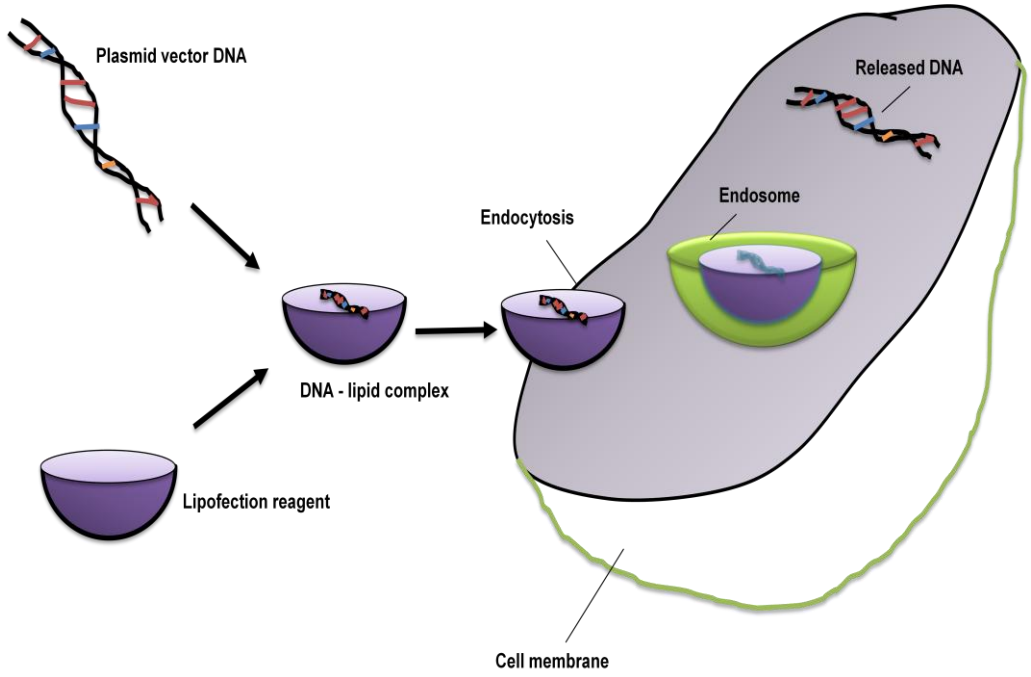

Figure 21.3: Proposed mechanism of DNA-lipid complexes in mammalian cells

Liposome and lipoplex method-Another approach of DNA transfection in animal cell is to pack the DNA in a lipid vesicle or liposome. In this approach, DNA containing vesicle will be fused with the cell membrane and deliver the DNA to the target cell. Preparation of liposome and encapsulating DNA was a crucial step to achieve good transfection efficiency. Liposome prepared with the cationic or neutral lipid facilitates DNA binding to form complex (lipoplex) and allow uptake of these complexes by endocytosis. The lipoplex method was applicable to a wide variety of cells, and found to transfect large size DNA as well. Another advantage of liposome/lipoplexes is that with the addition of ligand in the lipid bilayer, it can be used to target specific organ in the animal or a site within an organ.

2. Bactofection-This mode of gene transfer is very popular in plant where agrobacterium tumefaciens is used. In animal cell, bacteria is actively been taken up by the host cell through phagocytosis and entrapped in a membraneous vesicle known as phagosome. Then bacteria get escape from phagosome and get lysed to release the DNA into the cytosol. In alternate mechanism, bacteria get lysed inside the phagosome and DNA is released into the cytosol. The bacterial species used in this methods are salmonella, shigella etc. Most of the strain used to deliver the DNA are attenuated so that they should not harm host cell (Figure 21.4).

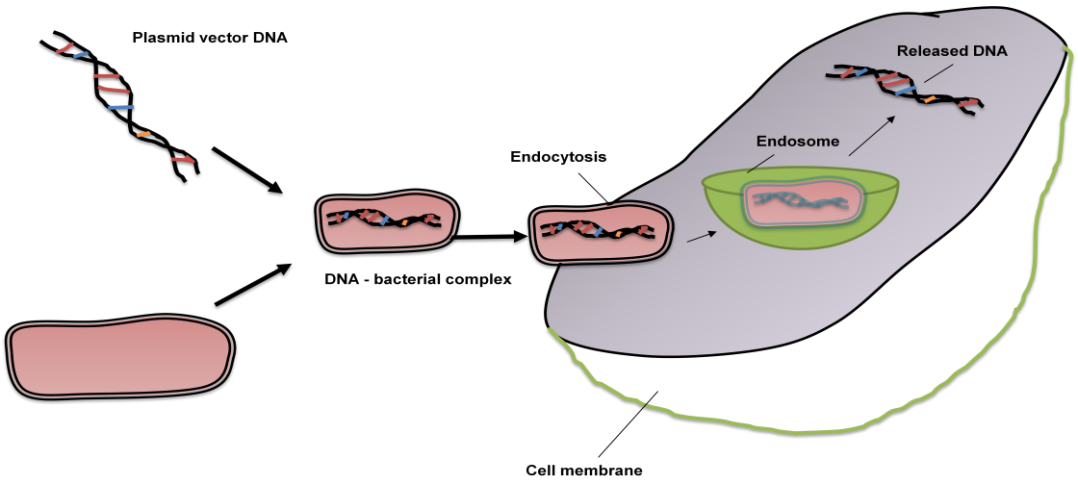

Figure 21.4: Proposed mechanism of transfection of mammalian cell with bacteria.

3. Transduction (Virus mediated)- Viral particle has a natural tendency to attack and deliver the DNA into the eukaryotic cells. As discussed previously, cloning gene of interest in to the viral vectors is a innovative way to deliver the DNA into the host cell. If the recombination sequences are available, the delivered DNA is integrated into the host and replicate. Virus has essential components for expression of proteins required for DNA replication, RNA polymerase and other ligand for attachment onto the host cell. In addition, it has additional structural components to regulate infection cycle. The virus vector contains cassettes to perform all these functions then it is fully sufficient to propagate independently. Few virus strains may cause disease if their propagation will be uncontrolled. A mechanism has been devised to keep a check on the uncontrolled propagation of virus in cell. Few crucial structural blocks are placed on another helper plasmid, in this case virus propagate only if helper plasmid has been supplied along with

the viral vector. This particular arrangement is made with the virus strains which can cause disease after integrating into the genome such as lentivirus.

Quiz

Q1: What is the role of heat shock in the transformation of bacteria in $CaCl_2$ method?

Q2: Why generation of spheroplast gives better transformation efficiency in yeast?

Q3: What are the draw back of calcium-phosphate method of DNA delivery in mammalian cells?

Q4: The process through which DNA-lipid complex is taken up by the mammalian cells?

Q5: What is the significance of a helper plasmid in virus mediated DNA delivery in mammalian cells?

Lecture 22: Screening of recombinant clone (Part-I)

Introduction- The different vectors are used in cloning techniques to produce recombinant DNA or clone. Transformation of recombinant DNA into the suitable host gives colonies and screening of the clone containing desired gene fragment is required for down-stream applications.

Chromogenic Substrate- The use of chromogenic substrate to detect a particular enzymatic activity is the basis to screen the desired clone. The most popular system to exploit this feature is **"Blue white screening"** where a coloroless substrate is processed to a colored compound. The colorless compound X-gal or 5-bromo-4-chloro-3-indolyl-β-D-galactoside used in this screening method is a substrate for β-galactosidase (Figure 22.1). The enzyme β-galactosidase is the product of lacZ gene of the lac operon. It is a tetrameric protein and an initial N-terminal region (11-41) of the protein is important for activity of the protein. In this system, host contains lacZ gene without the initial region where as vector contains α-peptide to complement the defect to form active enzyme. As a result, if a vector containing α-peptide will be transformed into the host containing remaining lacz, the two fragment will reconstitute to form active enzyme. In addition, the α-peptide region in vector contains multiple cloning site and as a result of insertion of gene fragment, consequently α-peptide will not be synthesized to give fully active β-galactosidase. The enzyme β-galactosidase oxidizes x-gal to form 5-bromo-4-chloro-indoxyl and galactose. The indoxyl derivative is oxidized in air to give a blue colored dibromo-dichloro derivative (Figure 22.2). Hence, blue colored colonies indicate the presence of an active enzyme or absence of insert where as colorless colonies indicate presence of an insert.

Insertional inactivation-In this approach a foreign DNA is cloned within the coding gene responsible for a phenotype. As a result of insertion, the gene product is not available to modulate the phenotype of the host. This approach is known as insertional inactivation, and it can be used with a suitable genetic system.

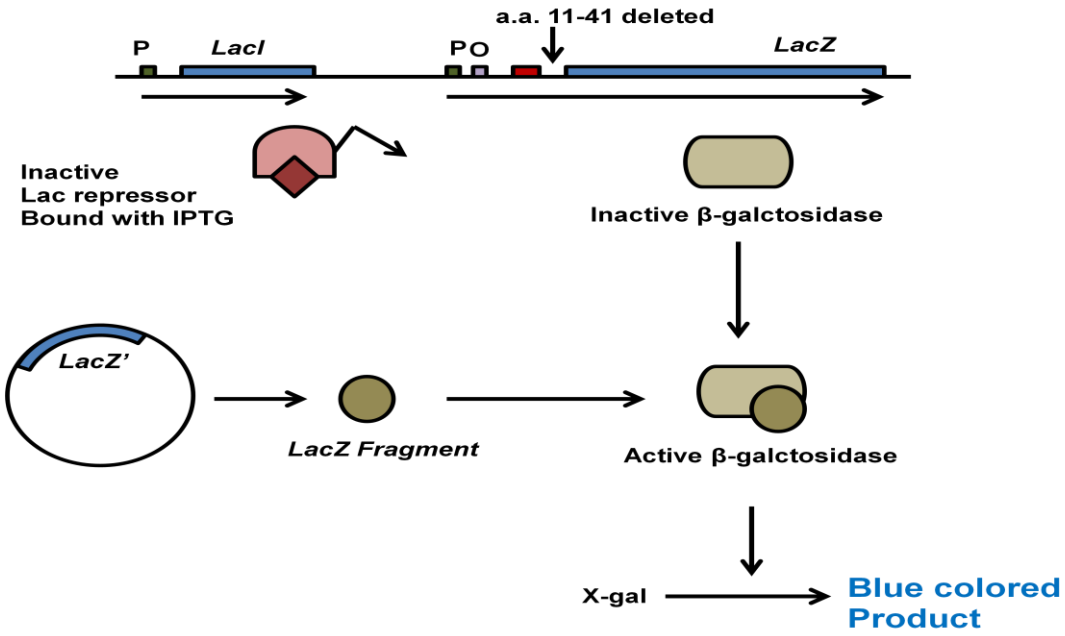

Figure 22.1: Molecular Mechanism of blue-white screening.

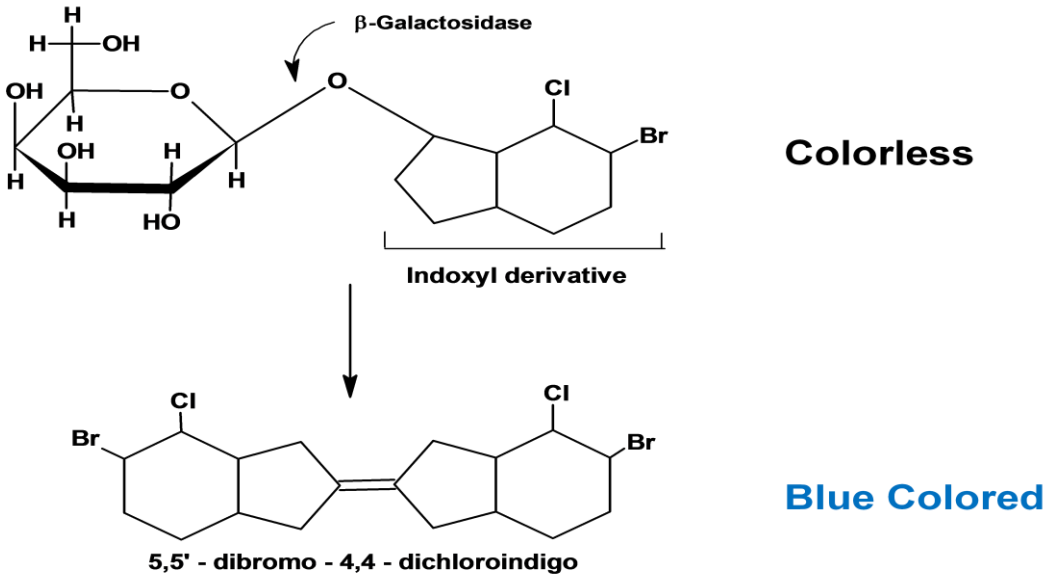

Figure 22.2: Chemical conversion of X-gal during blue-white screening.

(1) Insertional Inactivation of antibiotic resistance gene- As discussed in an earlier lecture, bacterial plasmid PBR322 has two antibiotic resistance gene, Ap^r and Tc^r. If a gene fragment will be cloned in ScaI, it will disrupt the Ap^r gene. As a result, the clone will be ampicillin sensitive and Tc^r. where as the original plasmid will be Ap^r and Tc^r. To select the clone, first the transformed e.coli is plated on tetracycline containing media. Subsequently, a replica plate will be made on ampicillin containing media to identify the clone growing on tetracycline media but not on ampicillin media. This approach is schematically depicted in Figure 22.3.

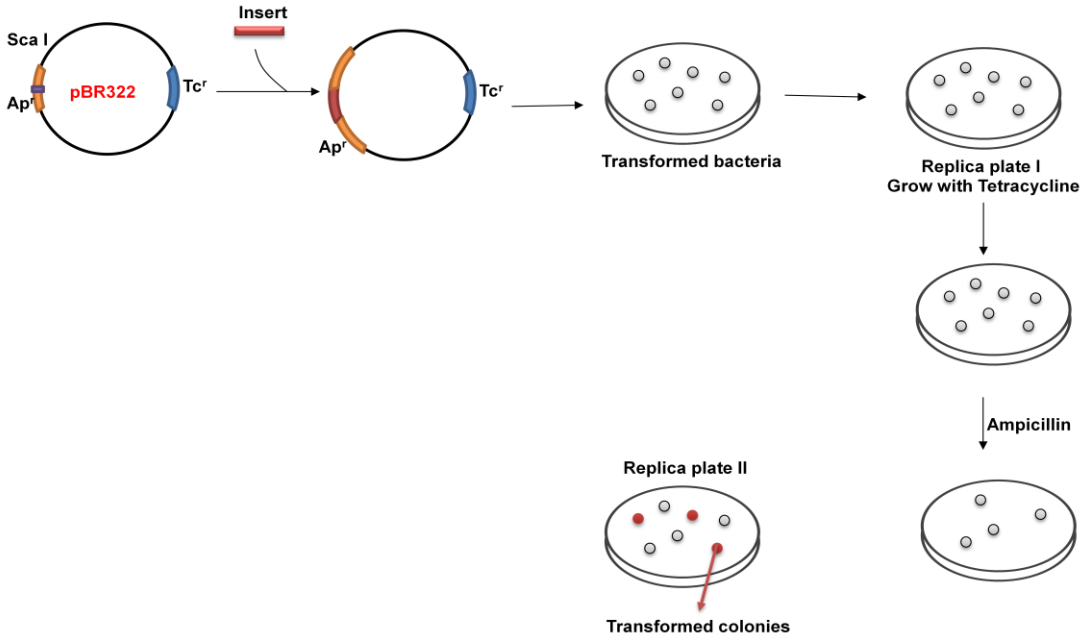

Figure 22.3: Insertional Inactivation of antibiotic resistance gene in pBR322 to screen recombinant clone.

(2) Insertional Inactivation of LacZ gene- LacZ is a part of lac operon and responsible for synthesis of β-galactosidase. As discussed earlier, X-gal system can be used to detect the insertional inactivation of LacZ gene to screen the cloned fragment. If the gene is inserted into the lacz, the clone will not be able to produce a functional β-galactosidase. Hence, blue colored colonies indicate the presence of an active enzyme or absence of insert where as colorless colonies indicate presence of an insert. This approach is schematically depicted in Figure 22.4.

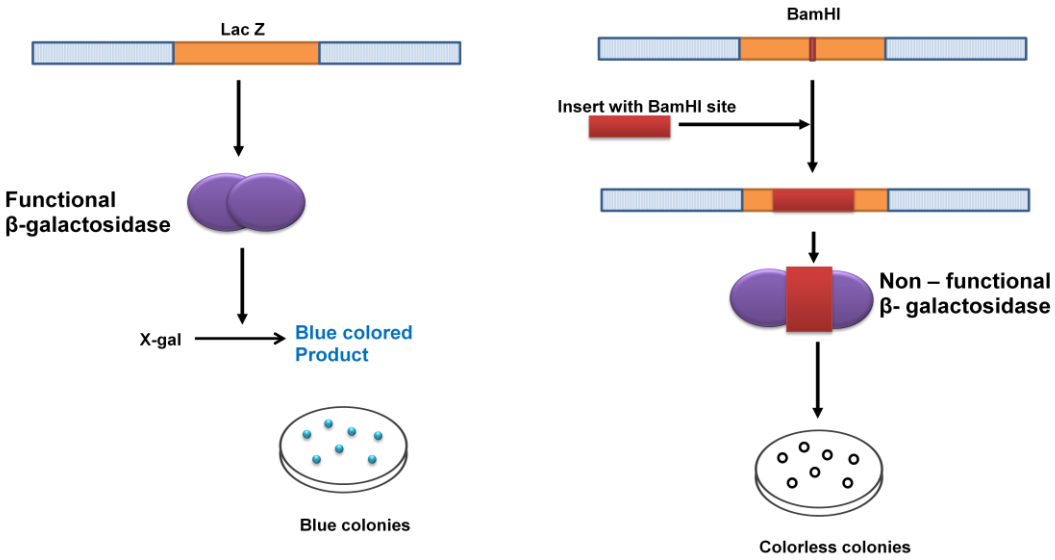

Figure 22.4: Insertional Inactivation of LacZ gene to screen recombinant clone.

(3) Insertional Inactivation of cI gene-During an infection cycle, virus undergoes a lytic and lysogenic stages. The lytic phase is responsible for lysis of host to release the virus particle where as lysogenic stage allow the replication of virus without lysis of host. The cI gene encodes for cI repressor and which is responsible for the formation of lysogens. In the presence of functional cI, the plaques contains unlysed host cells and has a turbid appearance where as in the absence it will clear. This feature can be use to screen the clone to detect functional cI (absence of clone) or absence of cI (presence of insert). This approach is schematically depicted in Figure 22.5.

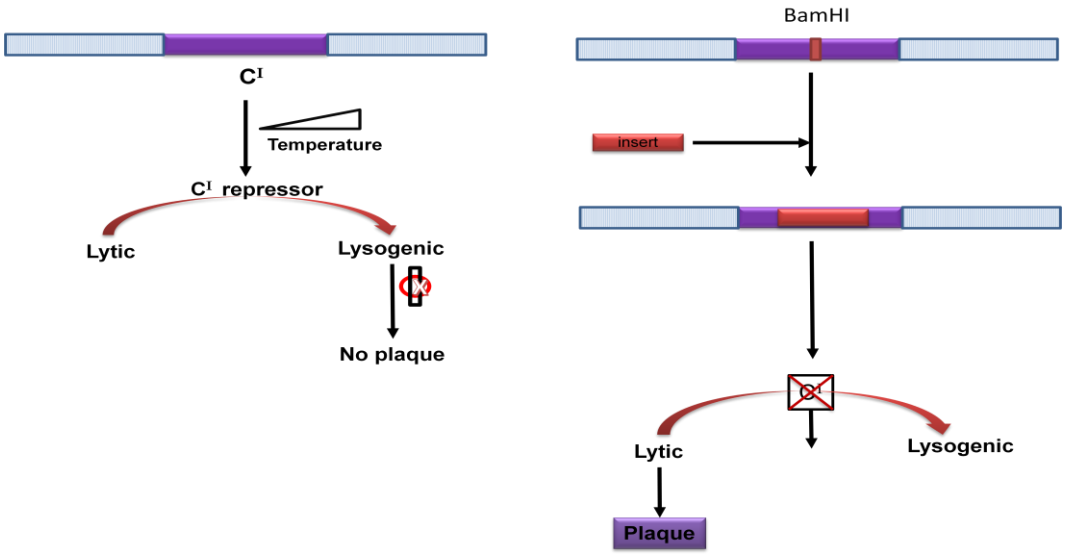

Figure 22.5: Insertional Inactivation of cI gene to screen recombinant clone.

Antibiotic sensitivity- Vector carries a functional selection marker such as antibiotic resistance gene to be use to select the clone. The antibiotic resistance gene product has multiple mechanism to provide resistance in host cell (Table 22.1). In this approach, a circular plasmid containing antibiotic resistance can be able to replicate into the host cell plated on a antibiotic containing media. In the cloning of a fragment into this plasmid, the plasmid is cut with restriction enzymes and a fragment in ligated to give circular plasmid with insert. The transformation of both DNA species; cut plasmid and circularized clone into the host and plated onto the antibiotic containing solid media. Only circularized clone will give colonies where as cut plasmid will not grow as it has lost antibiotic resistance gene. This approach is schematically depicted in Figure 22.6.

TABLE 22.1: ANTIBIOTICS RESISTANCE GENE AND THEIR MODE OF MECHANISM.

Antibiotic	Gene product	Mechanism
Ampicillin	β-lactamase	Degradation of ampicillin
Kanamycin	Neomycin phosphotransferase II	Covalent modification of kanamycin
Tetracycline	Ribosomal protection proteins	Efflux of tetracycline outside of the bacteria
Chloramphenicol	Chloramphenicol acetyl transferase	Chloramphenicol to acetyl Chloramphenicol

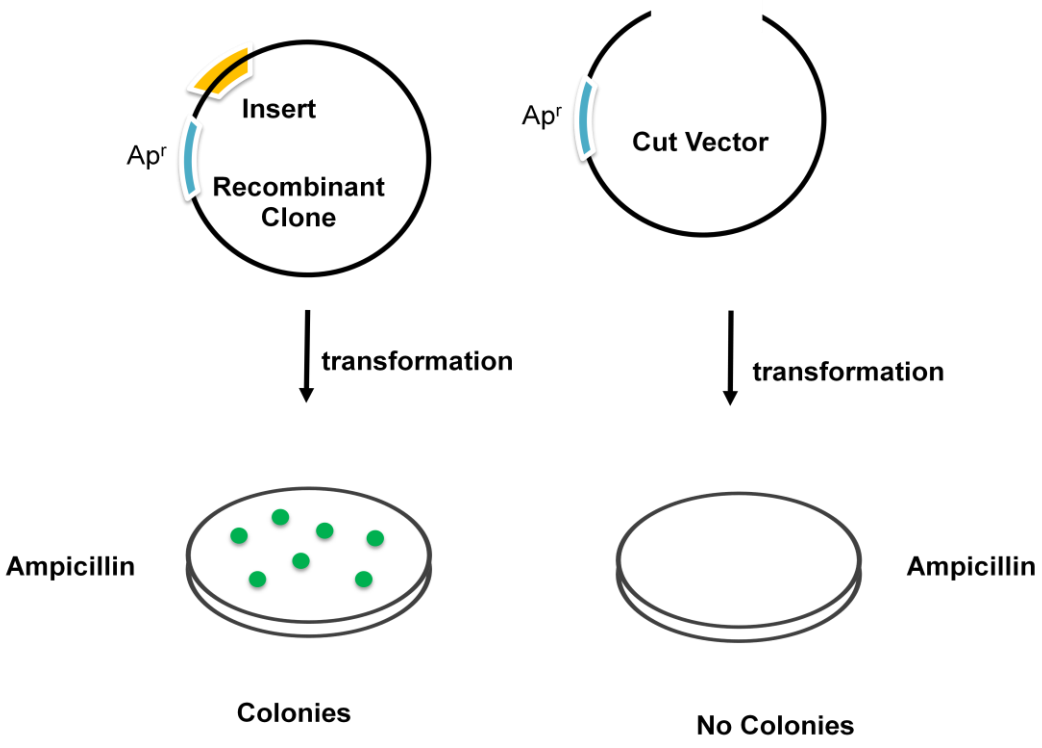

Figure 22.6: Screening of recombinant clones using antibiotic sensitivity.

Complementation of mutation- In this approach, a mutant host strain can be used to screen the plasmid containing the missing gene and the transformant will grow only if the gene product from clone will complement the function. In general genes taking part in metabolic pathway or biosynthetic pathway are routinely been used for this purpose.

There are 3 important requirement for this approach to work-

1. Host strain deficient in a particular gene. If the gene belongs to the biosynthetic pathway, the mutant host in this case are called as auxotroph as host dependent on the gene product or final product of the biosynthetic pathway as a supplement in media for growth.

2. A defined media with missing nutrient.

3. A vector containing the gene to supply the gene product to complement.

Lecture 23: Screening of recombinant clone (Part II)

In continuation of earlier lecture, an auxotroph yeast strain can be use to screen recombinant clone. Yeast vector discussed in previous lecture has 4 different gene *His3, Leu2, Trp1 and Ura3* as selectable marker. Yeast host with a mutation in these gene are available and can be use with the yeast vector to screen the recombinant clone. *Ura3* and *Lys2* marker offer both positive and negative selection.

Positive selection- In the positive selection, host strain doesn't grow on the media lacking the functional gene but the host transformed with the recombinant clone can be able to supply the gene product required to grow in the media.

Negative selection-In the negative selection, a chemical compound is added to the media which will be converted to the cyto-toxic agent in the presenc of gene product, and as a result it doesn't allow the growth of the wild type cells. But host strain transformed with the recombinant clone has non-functional gene product and grows in the presence of the compound in the media. *Ura3* codes for orotine-5'-monophosphate (OMP) decarboxylase and an active enzyme process the 5-fluoro-orotic acid to the toxic fluorodeoxyuridine. Generation of this cyto-toxic agent kills the cells carrying functional *Ura3* gene product.

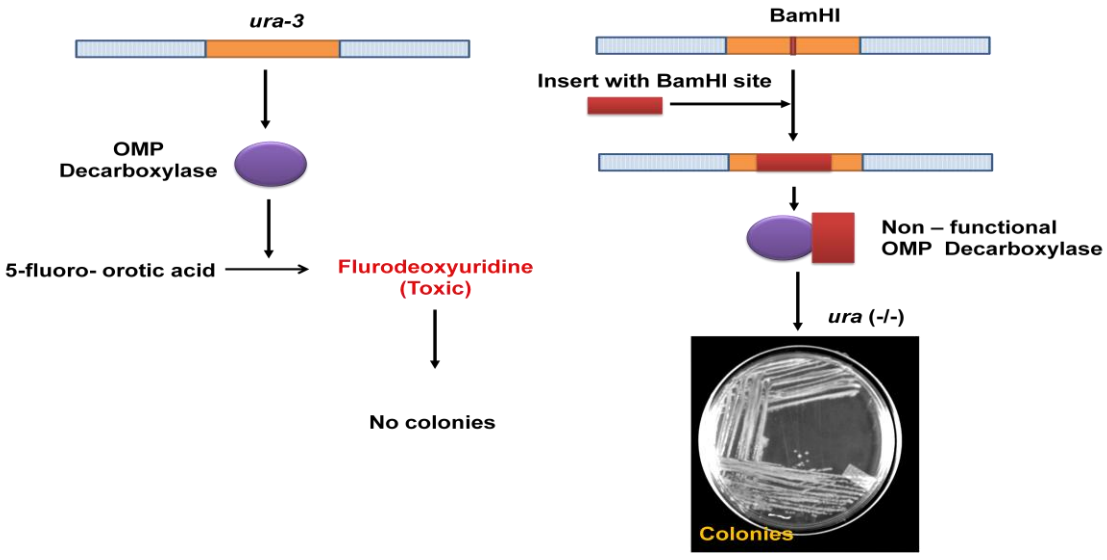

Figure 23.1: Negative selection of yeast cells transformed with the recombinant clone.

Screening of transfected mammalian cells- mammalian cells containing a recombinant clone can be screened using multiple approaches. In the current we will discuss few of these approaches-

Reporter Gene Assay- In the reporter gene assay system, a chimeric construct is produced with an enzyme gene is cloned in front of the promoter of gene of interest. A schematic design of reporter gene construct is given in Figure 23.2. The general reporter gene construct contains a eukaryotic promoter and a enzyme for easy read out. The reporter gene construct is transfected to the mammalian cells with a suitable transfection reagent as discussed in previous lecture. Afterwards, the cells are stimulated with the agents to stimulate the production to transcription factor to binds promoter and drive the expression of the reporter gene. A suitable substrate is added to measure the activity of the reporter enzyme. Different enzymes used for this purpose is given in Table 23.1.

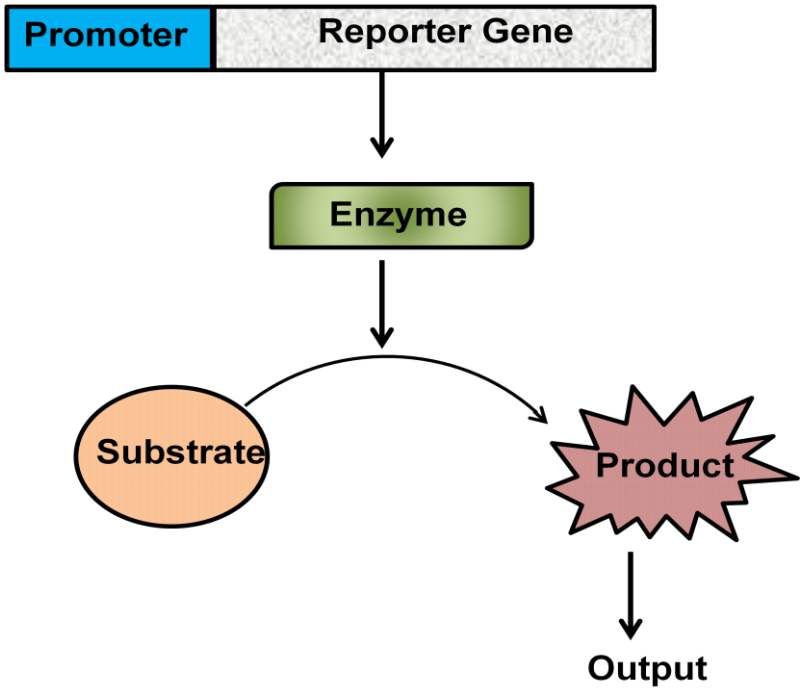

Figure 23.2: an over-view of the reporter gene assay.

Table 23.1: Reporter gene construct used for screening mammalian clones.

Gene	Gene Product	Reaction Catalyzed
CAT	Chloramphenicol acetyl transferase	Chloramphenicol to acetyl Chloramphenicol
lacZ	β-galactosidase	o-nitrophenol-β-galactoside to o-nitrophenol and galacose.
Luc	Luciferase	Luciferin to oxyluciferin
phoA	Alkaline Phosphatase	Release of inorganic phosphate
GFP	Green Fluorescent Protein	Fluorescence

Luciferase reporter gene system-Luciferase is an enzyme present in the abdomen of firefly *photinus pyralis*. The enzyme utilizes D-luciferin as a substrate to form oxyluciferin. In the presence of ATP, Mg2+, luciferin is converted into the luciferinadenylate involving pyrophosphate cleavage and transfer of AMP to luciferin. Luciferin adenylate undergoes oxidative decarboxylation to oxyluciferin with simultaneous emission of light (Figure 23.3). The assay setup is shown in Figure 23.4. The reporter gene construct containing luciferase is transfected to the mammalian cells. The cells are washed with PBS and lysed with a lysis buffer. Take the lysate into the lumiometer cuvette or plate and luciferin substrate is injected to start the reaction and measured immigately in a luminometer.

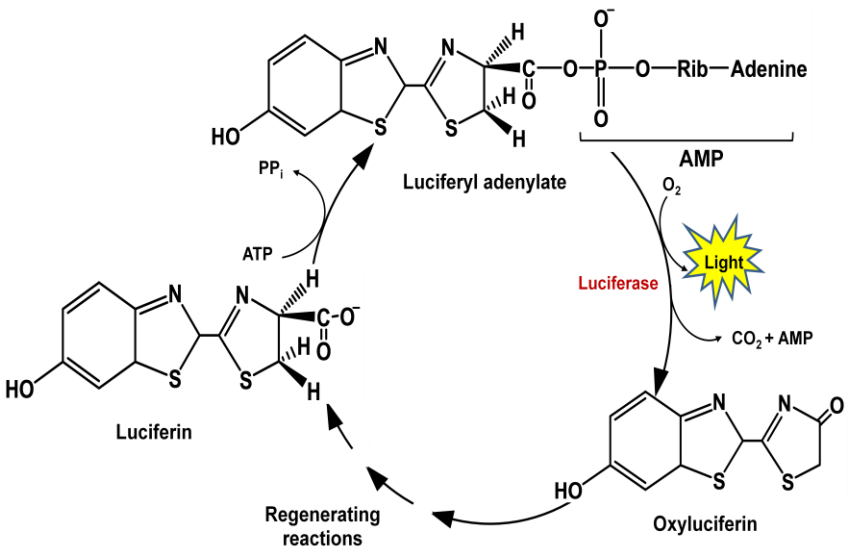

Figure 23.3: Luciferase reporter assay measures the formation of oxyluciferin formation.

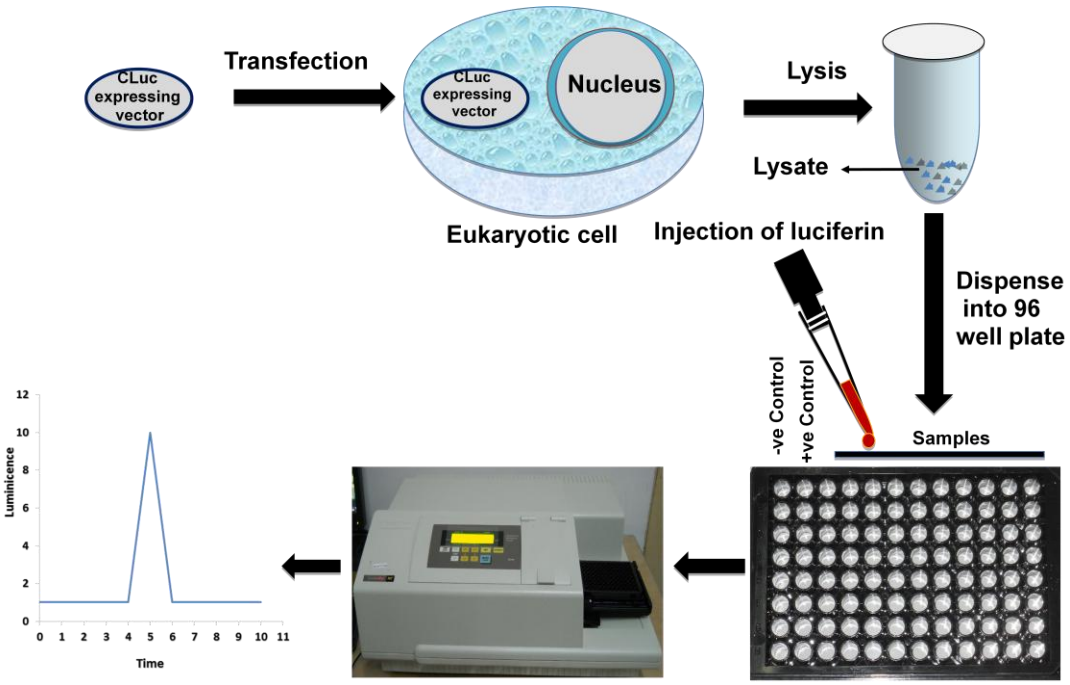

Figure 23.4: Different steps of luciferase reporter assay setup.

Chimeric Construct with green fluorescent protein (GFP) - In the live cell, green fluorescent protein is a good choice as reporter gene to screen cells containing recombinant protein fluorescently tagged with the GFP at their c-terminus (Figure 23.5, A). The cell receiving recombinant DNA will give green fluorescence and it can be visualized with an inverted fluorescence microscope (Figure 23.5,B) and it can be analyzed in flow cytometer to separate the GFP containing cells from the untransfected cells. Flow cytometer analysis the cell based on its shape, size and fluorescence level. A non-fluorescent cell is giving separate peak as compare to the fluorescently labeled cells (Figure 23.5, C) and with the help of flow cytometer, both of these peaks can be collected in separate tubes. Besides, GFP, red fluorescent protein, yellow fluorescent protein, cyan fluorescent protein are also popular to use to label the protein.

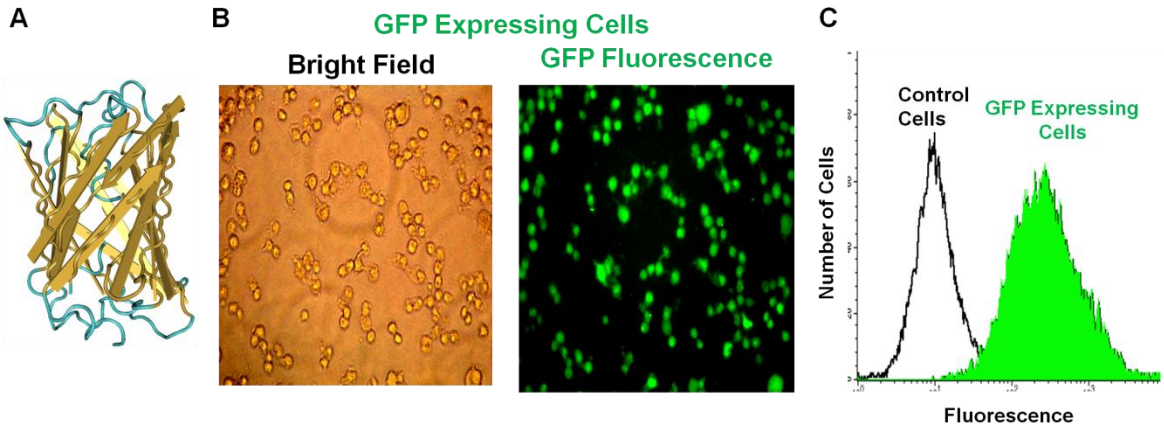

Figure 23.5: GFP represents a good candidate for reporter gene construct. (A) 3-D structure of green fluorescent protein, (B) COS-7 Cells expressing GFP and (C) Flow cytometric analysis of GFP expressing COS-7 Cells.

Lecture 24 Protein Production strategies in Expression System (Part-I)

Introduction-Heterologous expression system offers a cheap and reproducible production of the protein in large quantities for applications. Various prokaryotic and eukaryotic expression system are developed for this purpose with distinct features. E.coli as a expression system is cost effective and manipulation procedures are easy but it has limitation to express proteins with post-translation modification. Similarly, eukaryotic expression system can be able to perform post-translational modifications but it may not be cost effective. In the current lecture, we will discuss different expression system and the underlying strategies to produce foreign protein in large quantities.

Criteria to choose a expression system-Choosing a host expression system is the first step to decide the strategies to clone the gene in a suitable vector and subsequent down-stream procedures. The number of factors need to consider to choose the host expression-vector system suited for over-expression of a protein.

1. **Quantity of the desired protein-** if a protein is required in small amount, any host expression system may be suitable for the purpose but if a large quantity of the protein is required, a e.coli, yeast or baculo expression system might be more suitable than mammalian expression system.

2. **Size of the protein-** E.Coli expression system is not preferred for large size of the protein but an eukaryotic expression system is more suitable.

3. **Compatibility between source organism and expression system-** In general a close distance between source organism and expression system is preferred as it may increases the chances of getting the expression of cloned gene and presence of the protein in soluable fraction.

4. **Down-stream application-** This is the most important criteria to choose a host-vector system. If the protein production is for generating antibodies, any expression system may suit well for this purpose but if the protein is required for activity or for ELISA, then a compatible expression system is preferred.

In this series of lecture, we will discuss 4 different expression system:

1. *E.COLI* as a Expression System-

2. Yeast as a Expression System-

3. Insect Cell line as a Expression System-

4. Mammalian cells as a Expression System

***E.COLI* as a Expression System**

An Over-view of protein synthesis machinery in *E.coli*- Protein production in prokaryotic system is a multistep process and an over-view of these steps are as follows:

1. Binding of RNA polymerase to the promoter elements to start the transcription to form m-RNA.

2. As soon m-RNA is synthesized, a translation machinery start the synthesis of protein. Protein synthesis start usually at the start codon AUG and ends at the stop codon (UAA, UGA or UAG). In bacteria transcription and translation occurs simultaneously and translation start as soon as m-RNA comes out from the RNA polymerase.

The details of these steps can be followed from any molecular cell biology text book such as, B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, P. Walters, Molecular Biology of Cell, 5th Ed, Garland Publishing, 2007.

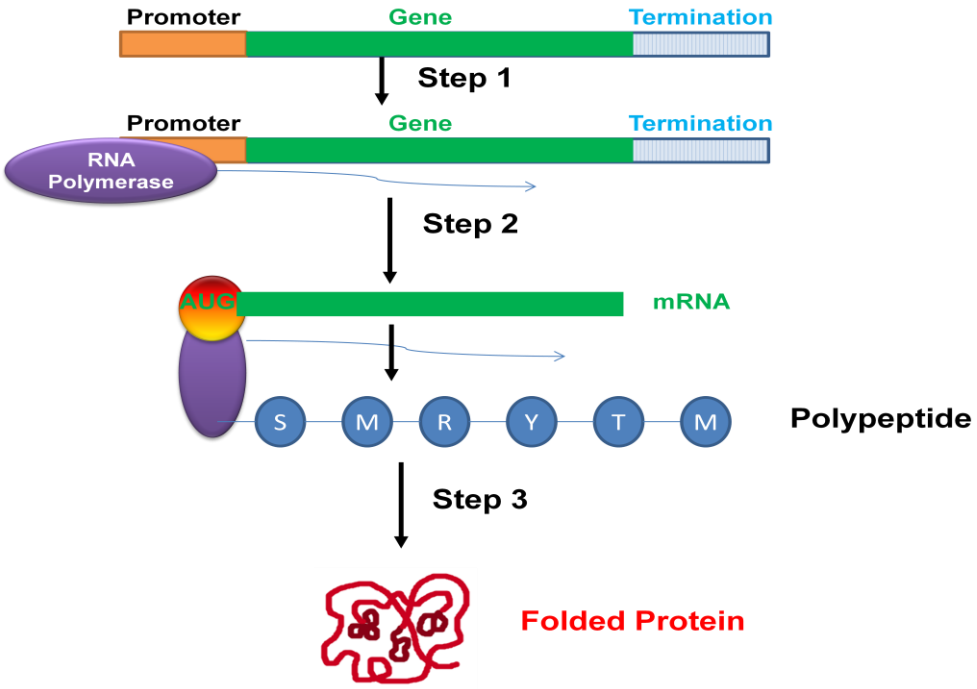

Figure 24.1: Steps of protein synthesis in e.coli expression system.

Typical Components of an *e.coli* expression vector- In our previous lecture we discussed the salient feature of cloning vector of e.coli but additional structural features are essential for an expression vector.

1. Promoter-This is upstream sequence to the gene and provides the docking site for RNA polymerase.

2. Ribosome binding site- Ribosome binding site including Shine-Dalgarno sequence is the docking site for assembly of ribosome.

3. Termination site- it terminates the synthesis of m-RNA.

4. Affinity tag- The presence of affinity tag either before or after gene sequence provides a mean to purify the protein using affinity chromatography.

Promoter regulates the production of protein- The structural elements of an E.coli promoter is given in Figure 24.2. The sequence at -10 and -35 are crucial to facilitate RNA polymerase and consequently determine the strength of the promoter. The nucleotide substitution in this region severaly affects the turn over of RNA polymerase binding events or transcription initiation events. Subsquently a number of promoters are designed for over-expression of protein in E.coli using a strong or a weak promoter to suit the over-expression strategy.

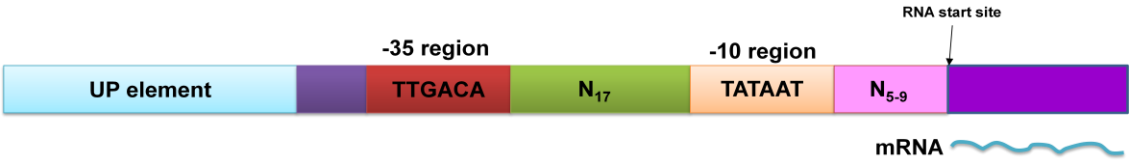

Figure 24.2: A generalized promoter structure in E.coli

IPTG inducible promoter- IPTG is an synthetic analogue of lactose and it is an inducer for lac operon. The lac promoter is very widely been used to construct different expression plasmid to express protein in E.coli. The different vector contains lac promoters or its derivatives:

1. The lac promoter- example of plasmid, pUC, pGEM etc.

2. The trp-lac (tac) promoter- it is a hybrid promoter where -10 region from lac UV5 promoter is fused with the -35 region of trp promoter. Ex. of plasmid is pKK223-3

3. The trp-lac (trc) promoter- it is similar to tac promoter except that distance separating -10 and -35 region of promoter is different from the tac prommoter. Ex. of plasmid pTrc 99A.

Bacteriophage λ P_L promoter- This promoter keeps the tight control over the protein production. It is regulated by the presence of repressor cIts857 to either repress the transcription or not. cIts857 is temperature sensitive and degraded at high temperature and consequently in a temperature depenent fasion it represses the transcription at low temp but not at high temperature. This promoter is useful in the cases where the protein is toxic in nature.

Bacteriophage T7 Promoter- Similar to Bacteriophage λ P_L promoter, T7 RNA polymerase promoter is used to design plasmid with tight control on the protein production. These vectors contains most of the structural blocks from pBR322 and MCS is in front of the T7 promoter to drive the transcription of the insert. Hence, vector contains foreign gene in front of the T7 promoter for expression. The host E.coli also need modification to suits the T7 promoter expression system. Host E.coli is either transformed with a plasmid which carries the T7 RNA polymerase gene or the T7 RNA polymerase gene is integrated into the bacterial chromosome. In few host strain T7 RNA polymerase is placed under the control of IPTG inducible lacUV5 promoter to tightly control the production of T7 RNA polymerase. A schematic is given in Figure 24.3 to explain the control mechanism in T7 promoter based expression system. After induction with IPTG, the inducer binds the lac repressor and stimulate the production of T7 RNA polymerase using E.coli RNA polymerase. The T7 RNA polymerase binds to the T7 promoter and drive the transcription of the target gene to eventually give large amount of protein.

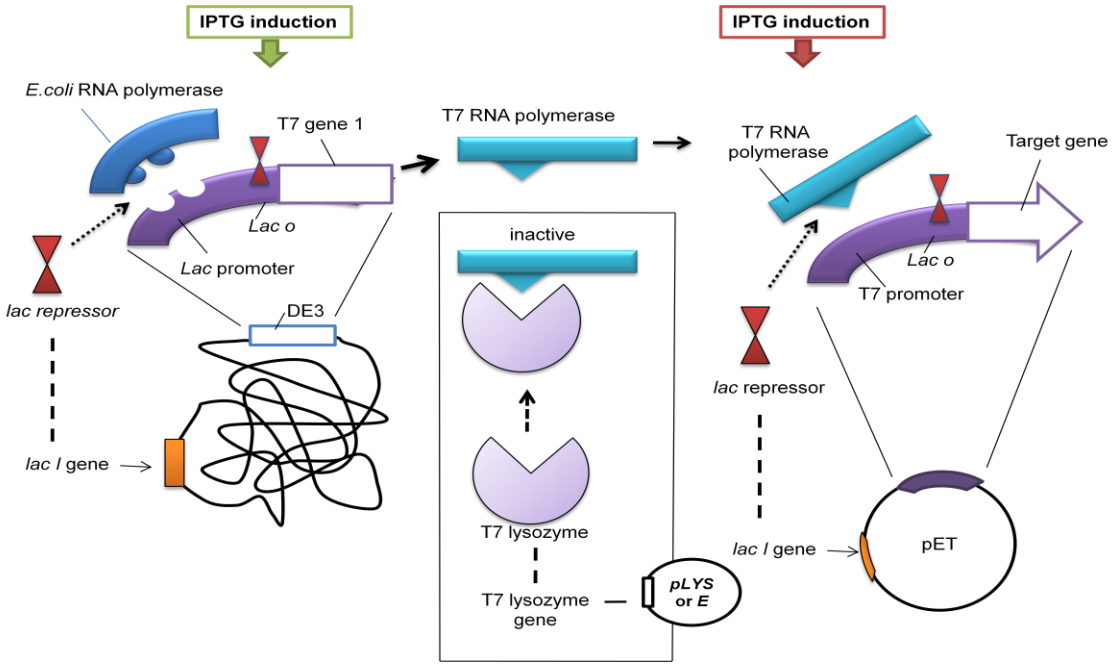

Figure 24.3: T7 Promoter mediated protein production control mechanism in expression host.

Expression of gene using E.coli expression system- The steps in an expression of a gene is outlined in Figure 24.4 and it has following steps:

1.Transformation- As discussed we can use multiple methods to transform the host with a recombinant clone containing suitable selection marker.

2. Growth of the bacteria- A single colony of the transformed colony is inoculated in the suitable media as discussed before upto the log phase (OD=0.6-0.7).

3. Induction- The bacterial culture is now induced with IPTG (0-1mM) for 3-6 hrs to produce the protein.

4. Recovery of the bacteria and analysis of protein expression- Bacteria can be recovered from the culture with a brief centrifugation at 8000-9000 RPM and analyzed on a SDS-PAGE. The details of SDS-PAGE will be discussed in a future lecture. The SDS-PAGE analysis of a particular expression study in *E.coli* is given in Figure 24.4 and it indicates a prominent expression of the target protein in the induced cells as compare to the uninduced cells.

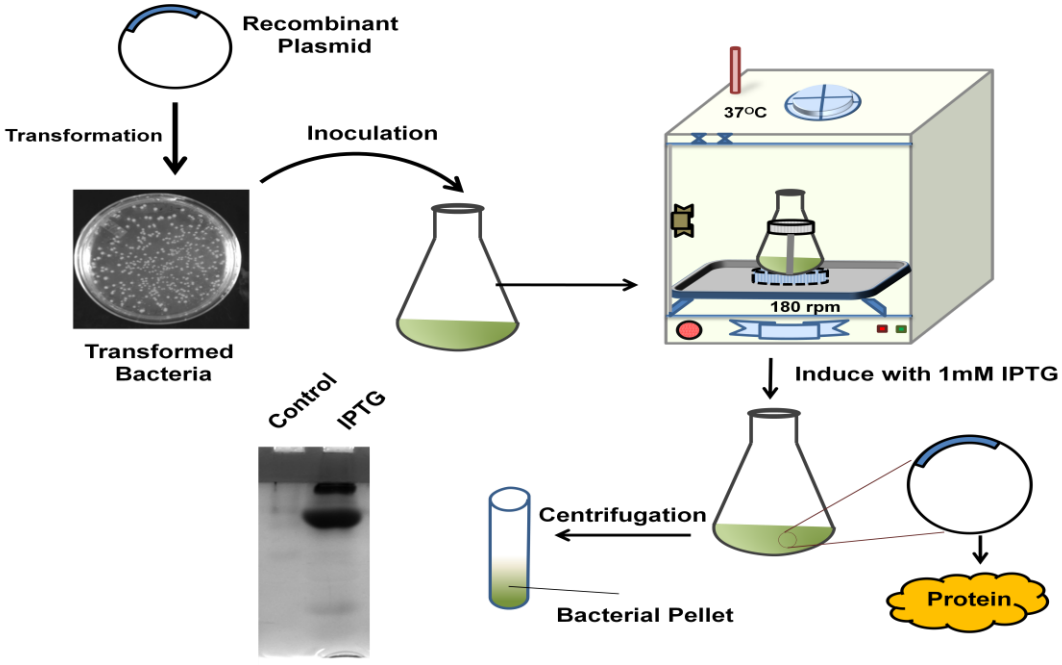

Figure 24.4: Protein Production using E.coli as expression system.

Lecture 25 Protein Production strategies in Expression System (Part-II)

Factor govering expression of foreign protein in E.coli

Translation efficiency: Translation efficiency is goverened by composition of promoter especially the sequence of '**shine dalgaro sequences**' which enables the binding of ribosome protein production machinery. In addition, the distance between shine-dalgaro sequence and the start codon is also important for efficient translation start. More-over, secondary structure of promoter elements also affect the efficiency of gene expression.

Codon Usage: Genetic codes are degenerate and there are 61 codes available for 20 amino acids (Figure 25.1). As a result, organism has a preference towards a set of genetic codes. Expressing these sequences require t-RNA to recongnize the genetic code. But if the host expression system has no t-RNA or low level of a particular t-RNA then it will either delay the synthesis or stop the synthesis at the particular amino acid. Consequently either it will produce less protein or a truncated protein.

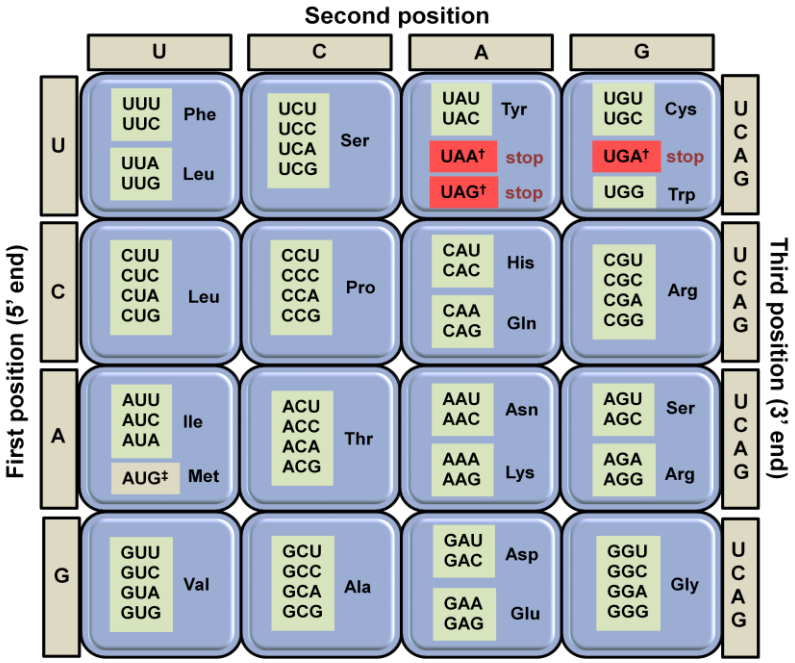

Figure 25.1: Genetic Code table

Growth Conditions: Growth media has dramatic effect on the gene expression. Either the media components provide the raw material for the synthesis of amino acid or provide amino acid for synthesis of a protein. In addition, growth media rich with carbon source may provide high cell mass and as a result it will give more protein.

Expression of Fusion proteins-The proteins in E.coli expression system can be expressed as a hybrid protein where reading frame of two gene (one for fusion tag and other is for foreign gene) are in frame. The fusion tag can be placed either at N-terminus or C-terminus. A list of commonly used fusion tag is given in Table 25.1.

The advantages of fusion proteins:

1. Easy Purification: A detail of this aspect will be discussed in the future lecture.

2. Targeting to the cellular compartment- A fusion protein can be targeted to the different cellular compartments for various reasons. Such as periplasm targeting sequence will allow the protein to accumulate into the periplasm and hence can help to easy isolation.

3. Half life of protein- In many cases a fusion tag hides the potential protease sites on the foreign protein and enhances its half-life.

4. Soluability- Keeping a tag at N-terminus direct the protein synthesis and hence help in increasing the soluability of the foreign protein.

Table 25.1: Selected List of Fusion tags and their applications.

S.No.	Fusion Tag	Vector	Features
1	β-galactosidase	pUC, pBluescript, pGEM	Blue-white screening and affinity purification
2	Maltose Binding Protein (MBP)	pMAL	Affinity purification
3	Thioredoxin (trx)	pTrx	Affinity purification
4	Poly His_6	pET series	Affinity purification
5	GST	pGEX	Affinity purification and reporter gene assay
6	Alkaline phosphatase	pTA1529	Reporter Gene assay

Removal of fusion Tag- For many biotechnology applications, a protein is expressed as fusion protein with a N-terminus or C-terminus tag, to easily purify the protein. But after purification the tag needs to be removed for down-stream applications such as vaccine or protein crystallographic studies. A list of reagent is given in Table 25.2 to cleave the fusion protein to remove the tag. In general fusion protein junction point has either the protease cutting site or the site is sensitive to the chemical treatment. Treating the fusion protein with protease or chemical agent cuts the fusion protein to release the target protein (Figure 25.2). Passing the cleavage mixture allows the binding of the tag into the affinity column whereas target protein does not bind and comes out in the flow through. Target protein free of fusion tag can be collected and used for down-stream applications.

Table 25.2: Selected List of reagents for cleavage of fusion protein.

S.No.	Reagent	Cleavage Sequence
1	Cynogen Bromide	-Met ↓
2	Hydroxylamine	-Asn-↓-Pro
3	Enterokinase	-Asp-Asp-Asp-Asp-Lys↓
4	Factor Xa	-Ile-Glu-Gly-Arg ↓
5	α-thrombin	-Leu-Val-Pro-Arg↓-Gly-Ser
6	Trypsin	-Arg ↓ or Lys ↓
7	Subtilisin	-Gly-Ala-His-Arg ↓

Yeast as a expression system-yeast is the simpliest unicellular eukaryotic cells available for protein production. It is easy to manipulate and the production cost is also very low in comparison to the other eukaryotic expression system. It offers most of the advantages available in a typical eukaryotic cells. In addition, a large amount of genetic, molecular and cell biology aspect of yeast is known and this knowledge has help us to design better protein production strategy and troubleshooting.

Host Species Used- There are two different varities of yeast strains available for protein production.

Non-methylotrophic-These species don't have ability to utilize one carbon compounds such as methanol. But it can be able to utilize other carbon sources such as glucose, lactose, maltose, starch and alkane. The example of yeast in this class are *S.cerevisiae*, *K.lactis*, and *Y.lipolytica*. These yeast strains are mostly been used for fermentation to produce alchol etc. The major advantage of this class is better understanding of molecular biology, biochemistry and fermentation technology aspect of these strains. But still the technology is not eveolved to utilize this class of yeast for production of heterologous protein.

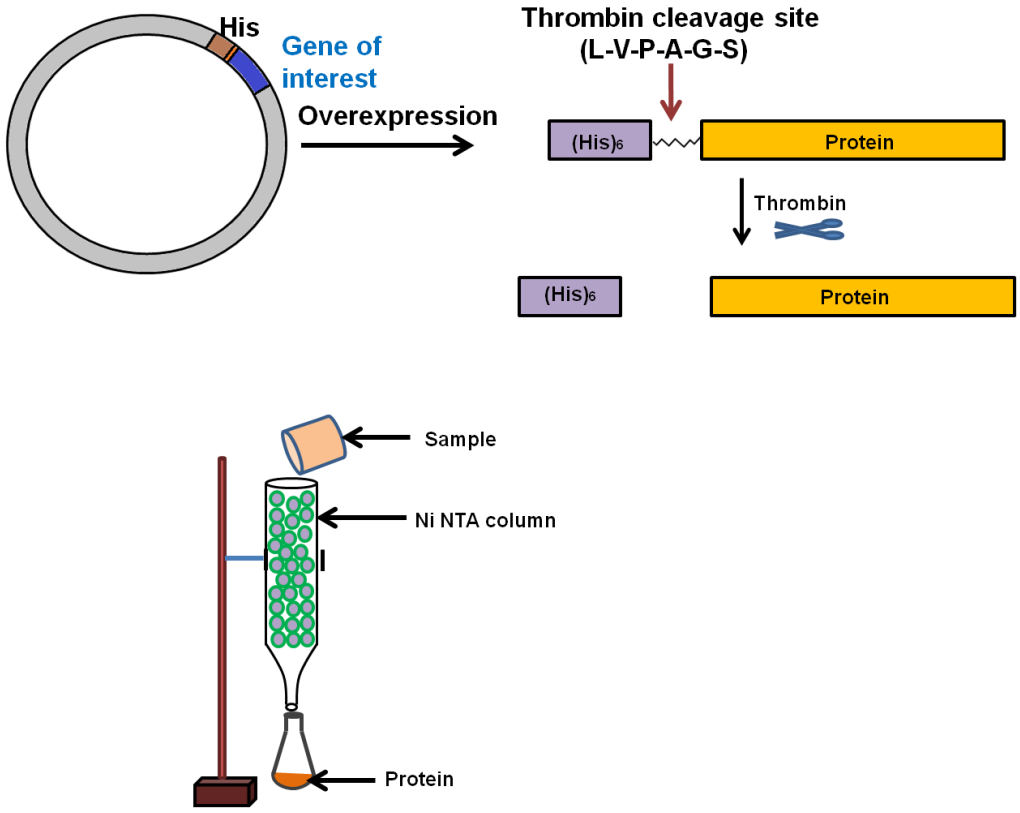

Figure 25.2: Schematic presentation of cleavage of fusion tag from target protein.

Methyltrophic yeasts- The major advantage of this class is ability to utilize one carbon compounds such as methanol as carbon and energy source. In addition, these strains have high level of methanol oxidizing enzyme and that allow them to be very strong and grow in high density. The example of yeast in this class are, *pichia pastories*, *pichia angusta*, *P.methanolica* and *C.boidini*.

Transformation- A number of transformation methods specific to yeast is discussed in the previous lecture. Lithium acetate and electroporation is the method popular method for transformation of yeast.

Vector and selection- The different yeast vector are already discussed in the previous lecture. Transformant are selected either using a auxotroph marker (such as URA3, LEU2, TRP1, HIS4) or antibiotic resistance (such as G418, hygromycin etc).

Promoters in yeast expression vector- Similar to E.Coli expression system, yeast vectors have different promoters to drive the expression of foreign protein. A list of promoter is given in Table 25.3. In general, yeast expression vector offers two types of promoters:

1. Constitutive Promoter- These promoter belongs to the house keeping gene and as a result the expression is non-inducible. The protein production starts with the growth of the yeast and as a result it is proportional to the cell mass. Example of these promoters are GAPDH, GAM1 etc.

2. Inducible Promoter- Pichia pastoris expresses two different alchol oxidases, AOX1 and AOX2 where as pichia angusta expresses methanol oxidase (MOX). The promoter of AOX1 and MOX are present on yeast vector and it has been used to drive the expression of foreign protein. The protein production is controlled by a balance of repression and induction. Presence of other carbon source such as glucose represses the transcription of AOX1 gene but in the presence of trace amount of methanol, it induces the AOX1 promoter mediated protein production.

Table 25.3: Different promoter(s) in Yeast expression system.

Strain Type	Species	Constitutive	Inducible
Non-Methyltrophic	*S. Cerevisiae*	GAPDH	UAS, ADH 1
	K.Lactis	PGK	LAC 4, ADH 4
	Y.lipolytica	TEF, RPS 7	
	S.Occidentalis	GAM1	AMY 1
	Z. rouxii	GAPDH	
Methyltrophic	*P.Pastoris*	GAP	AOX 1, FLD 1
	H. polymorpha		MOX
	P. methanolica		AUG 1

Production of protein in yeast- The protein production in yeast can be done in such as way to either express the protein in cytosol or secreted into the media supernatant.

Cytoplasmic targeted protein- The expression of protein targeted to the cytoplasm is very high but the recovery is very difficult. Yeast cell wall is very hard and high pressure homogenization is used to disrupt the cell wall. The recovery is very less and a fraction of total soluable protein comes out.

Secreted protein- Protein tagged with secretory signal such as S.cerevisiae α-mating factor signal target the protein into the secretory pathway. The signal peptide is processed in ER/Golgi vesicular transport system and appear in culture media.

It is difficult to say which pathway will be useful for over-expression of protein in yeast expression system. Irrespective of the pathway choosen the protein expression protocol in yeast expression system is given in Figure 25.3 and it has multiple steps:

(1) Transfer the transformed yeast into 5 ml medium with suitable selection marker and incubate for 2 days at 28^0C with shaking at 180rpm.

(2) Allow the culture to reach the OD_{600} to 5-7 and now resuspend the cells in a new media without carbon source.

(3) Induce the culture with a methanol of 1% (v/v) twice daily.

(4) harvest the cells and analyze the expression on SDS-PAGE.

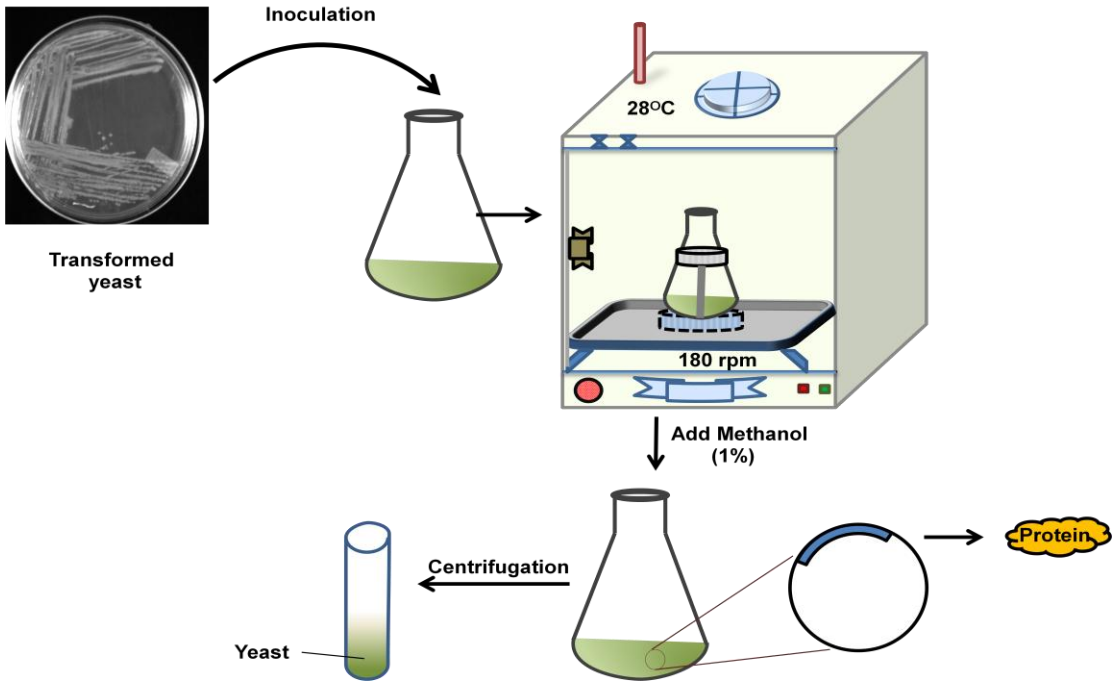

Figure 25.3: Schematic presentation of different steps in protein production in Yeast.

Lecture 26 Protein Production strategies in expression system (Part-III)

Insect cell lines as expression system: In the previous lecture we discussed the prokaryotic and unicellular eukaryotic expression system. As a eukaryote baculovirus expression system offers protein modification, processing and transport system. Compare to yeast where the down-stream processing and recovering of cytosolic protein is much easier in baculovirus system. In addition, molecular biology manipulation, transfection and the down-stream processing. The different steps needed to produce protein are as follows:

1. Cloning of foreign gene in transfer vector.
2. Generation of recombinant baculovirus vector
3. Screening of recombinant baculovirus
4. Culturing of recombinant insect cell lines
5. Protein production

1. Cloning of foreign DNA into the transfer vector-The structural components of transfer vector (Figure 26.1) is discussed in a previous lecture. It has 3 distinct structural units, (1) A polyhedrin promoter and a unstream sequence from the the virus genome. (2) A cloning site for foreign DNA, (3) polyhedrin termination site and down-stream region of virus genome. The up-stream and down-stream sequences from virus genome helps in homologous recombination. The foreign DNA is cloned in the cloning site and the recombinant transfer-vector can be propogated in E.coli.

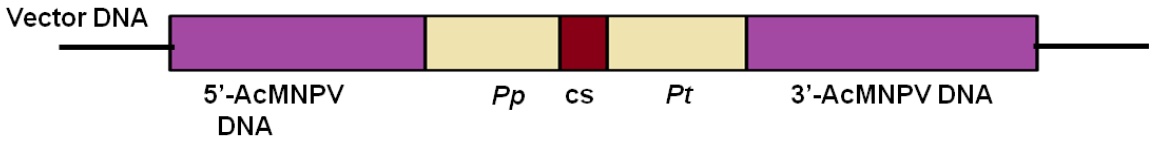

Figure 26.1: Organization of structural elements of transfer-vector AcMNPV.

2. Generation of Recombinant baculovirus: There are two approaches to construct the recombinant baculovirus:

Approach 1: In approach1, insect cell lines are first transfected with baculovirus to produce transformed insect cells (Figure 26.2). It is subsequently transfected with transfer vector containing foreign DNA and allowed to grow. In one or two divisons, a double cross over event occurs between viral genome and the transfer-vector with the help of flanking virus genome sequences. As a result, the viral genome losses the polyhedrin gene and receives the DNA stretch from transfer vector containing polyhedrin promoter, foreign DNA and termination signal.

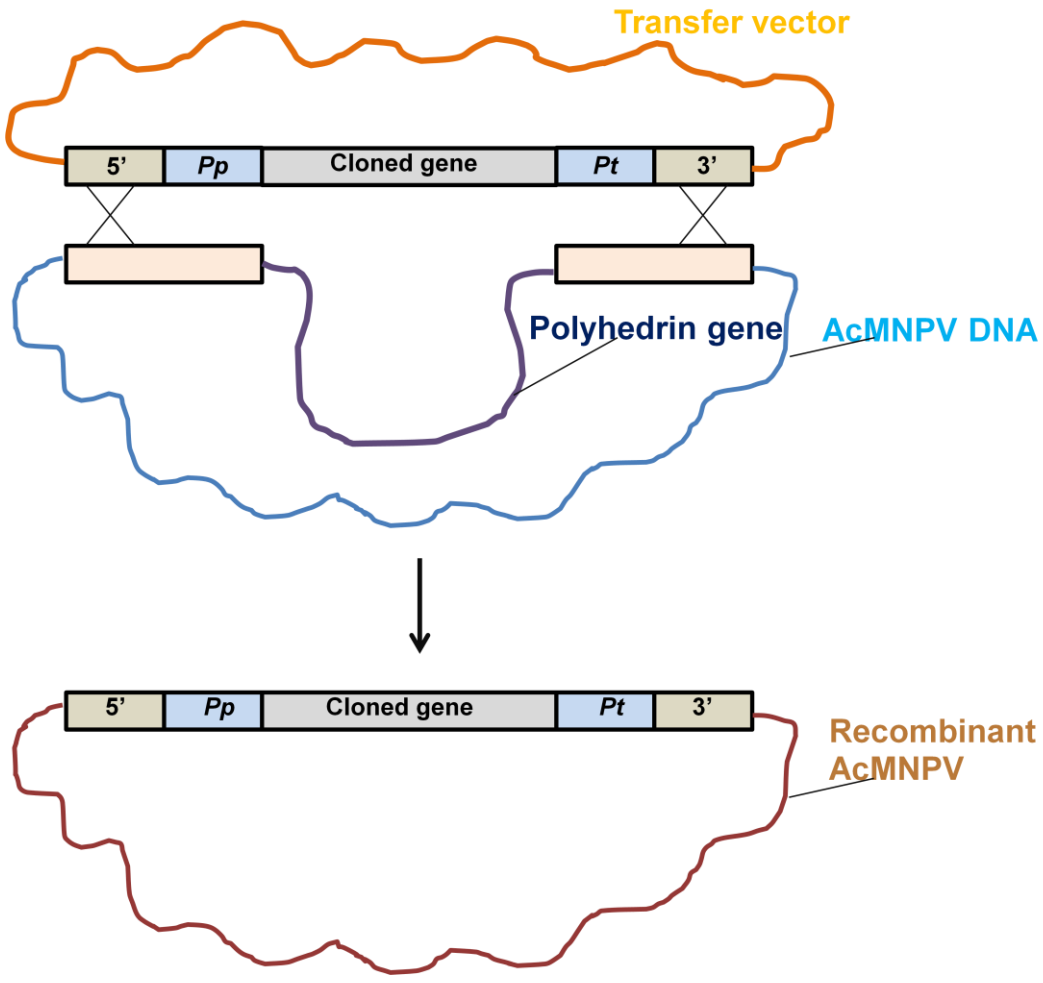

Figure 26.2: Generation of recombinant baculovirus with the double cross over event (Approach-I)

Approach 2: In approach2, baculovirus genome is engineered to introduce two unique restriction site Bsu36I introduced into the polyhedrin gene within viral genome (Figure 26.3). When modified viral genome is treated with restriction enzyme and transfected into the insect cell, no viral particle found as it is missing the function of a crucial gene ORF1629 which is required for viral replication. The linearized truncated viral genome (with missing polyhedrin gene, ORF1629 and gene 603) is transfected into the insect cells, followed by transfection of transfer vector containing foreign DNA along with the gene 603 and essential gene. In one or two divisons, a double cross over event occurs between truncated viral genome and the transfer vector with the help of flanking gene 603 and essential gene sequences. As a result, the viral genome recives the lost portion of gene 603 and ORF1629 from transfer vector and foreign DNA is incorporated into the viral genome.

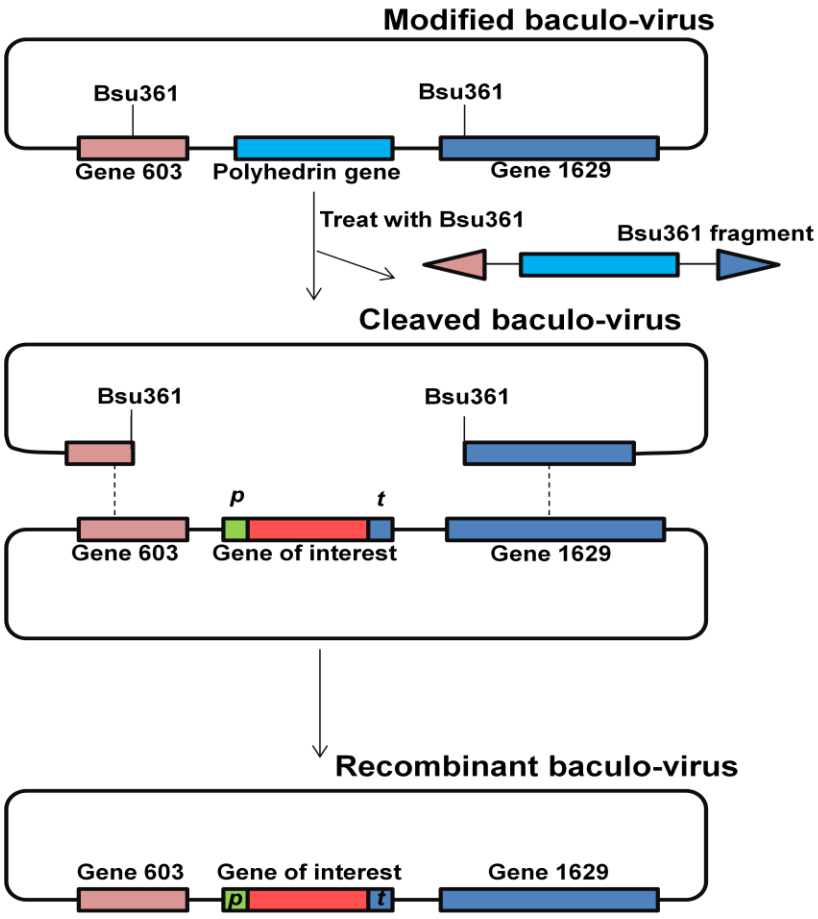

Figure 26.3: Generation of recombinant baculovirus with the modified baculo-virus (Approach-II)

Screening of recombinant baculovirus- The recombinant baculovirus can be screened by a plaque assay. It involves multiple steps as depicted in the Figure 26.4. The major steps are as follows:

1. Dilute a culture of insect cell line S*f*9 to a density of 10^5/ml.

2. Make serial dilution of baculovirus stock in serum containing media.

3. Add 1ml of viral sample to each well and incubate for 1h at 27^0C.

4. Remove the viral diluted suspension and then detect the presence of plaque. There are three popular method to detect the plaque:

(A) Over-lay a agarose and allowed it to harden. Incubate this plate for 6-8 days at 27^0C to plaque form and these can be visualized.

(B) If the recombinant virus contains a lacZ gene, then plaque can be identified by adding b-galactosidase substrate, x-gal, plaque containing cells will appear blue.

(C) In third method, cells can be stain with trypan blue, plaque containing cells will takeup the dye and appear blue where as other cells will remain colorless.

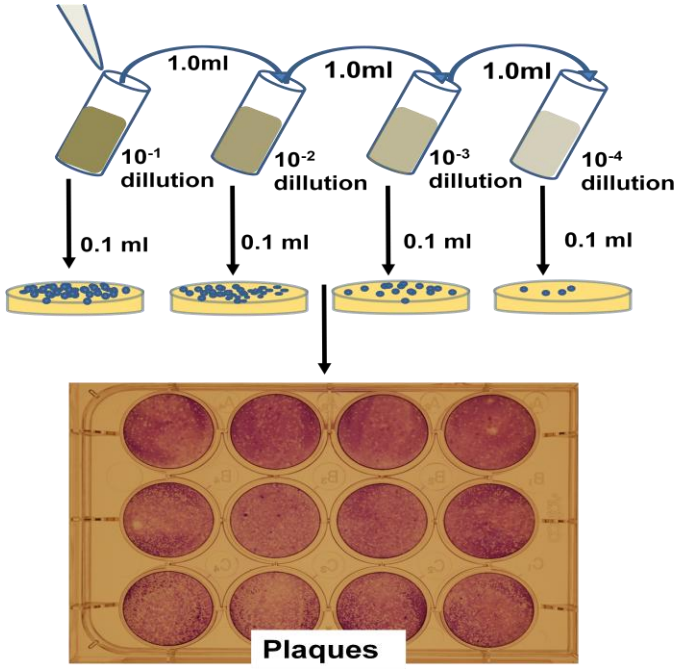

Figure 26.4: Screening of recombinant baculovirus in a plaque assay.

Culture media for growth: The culture media for growing insect cell lines is discussed in a previous lecture.

Maintenance and culture of insect cell line: Sf9 cell lines is derived from the ovaries of the armyworm (Spodotera frugiperda). It is maintained in TNH-FH insect media containing 10% foetal bovine serum (FBS) and gentamycin.

Culture media for protein production: BaculoGold or other serum free, low protein media is suitable for the secreted proteins as it facilitates easy purification.

Protein Production in Baculovirus expression system: The major steps to produce the protein are summarized in Figure 26.5. The whole process is as follows-

1. Seed 10^6 Sf9 cells in a 60mm cell culture dish and allow the cells to adhere to the dish.

2. Add 0.1 ml high titer baculovirus stock at an MOI of 1:10. Incubate the cell for 3 days at 27^0C.

3. Collect the cells and media. Centrifuge at 1000xg for 10min at 4^0C.

If the protein is secreted:

4a. Transfer the culture supernanat to new tube and determine the protein concentration with Bradford reagent.

If the protein is cytosolic:

4b. discard the supernatant and wash the cell pellet with PBS.

4c. Lysed the cells and analyzed the protein on SDS-PAGE.

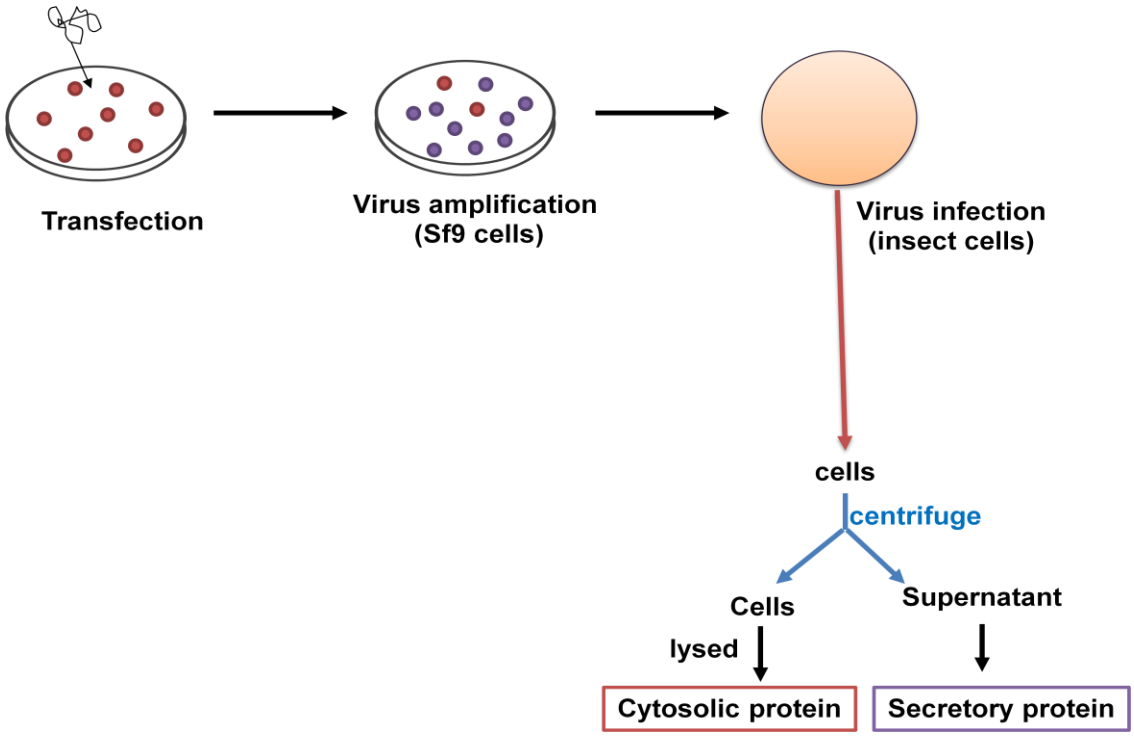

Figure 26.5: Schematic Presentation of different steps in protein production in insect cells.

Lecture 27 Protein Production strategies in Expression System (Part-IV)

Mammalian expression system: Similar to all other expression system, protein production in mammalian cell system can be achieved with either from a vector present as extrachromosomal DNA (transient) or the sequence is integrated into the genome through homologous recombination to estabilish the permamnent cell line. The expression from transient or permanent cell lines can be from a constituted or inducible promoter. Irrespective of the expression mode in mammalian system, the different basic steps needed to produce protein are as follows:

1. Cloning of foreign gene in mammalian expression vector.

2. Transfection of a cell line with recombinant construct.

3. Screening and selection of transfected cells.

4. Culturing of transfected cells.

5. Protein production.

Different Cell line for protein production: The most popular cell lines used for mammalian expression system are given in Table 27.1. The cell lines are derived from the different origin (organ of the body/cell type/animal) and consequently they are preferred to produce foreign protein with similar origin.

Table 27.1: Popular Cell lines for protein production

S. No.	Cell lines	Origin
1	CV1	Kidney
2	COS-7	Fibroblast
3	3T3	Fibroblast
4	CHO	Ovary
5	J774A.1	Macrophage
6	HeLa	Cervical
7	BHK 21	Kidney
8	HEK 293	Kidney

Growth media for mammalian cell line: The growth media for different mammalian cells is discussed earlier in a previous lecture.

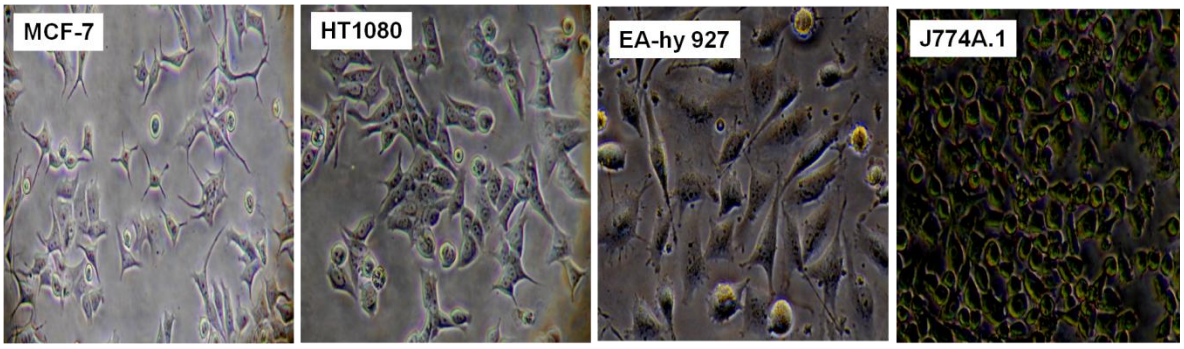

Figure 27.1: Different mammalian cell lines used for protein production.

Protein Production in mammalian expression system:

Transient expression: The expression is high but for short time period. The cells transfected with DNA express proein until 72h post-transfection. Transient expression system is used to screen cDNA library, isolation of a particular cDNA clone expressing surface antigen and to test the applicability of the recombinant construct going to use for permanent expression. There are multiple steps required to transiently express a protein in COS-7 cell line. Although we are giving the details for COS-7 but these procedures can be applied to other cell lines with slight modifications (Figure 27.2). The steps are as follows:

1. Clone the foreign DNA into the appropriate mammalian expression vector to obtain recombinant DNA. Transfection efficiency is maximum for a supercoiled circular DNA. Purify the recombinant DNA by a miniprep kit to prepare high quality supercoiled DNA.

2. Seed COS-7 cells in DMEM media supplemeted with 10% foetal bovine serum at 20% confluence in 100mm dish.

3. Transfect the cells with recombinant DNA using a transfection reagent. **[Several transfection reagent and methods are discussed in a previous lecture]**.

If expressing secreted proteins-wash the transfected cells with PBS and add serum free media. It allows to secrete the protein for next 72hr. Harvest the medium, remove dead cells and debris by centrifugation and filter the media from 0.45µm syringe filter before storage. Detect the presence of protein in media either by activity assay or by western blotting.

If expressing cell surface or intracellular protein-Transfected cells are allowed to express the protein for another 72 hrs. Remove media, wash the cells with PBS and detect the presence of protein on cell surface or in cell lysate by activity assay or by western blotting.

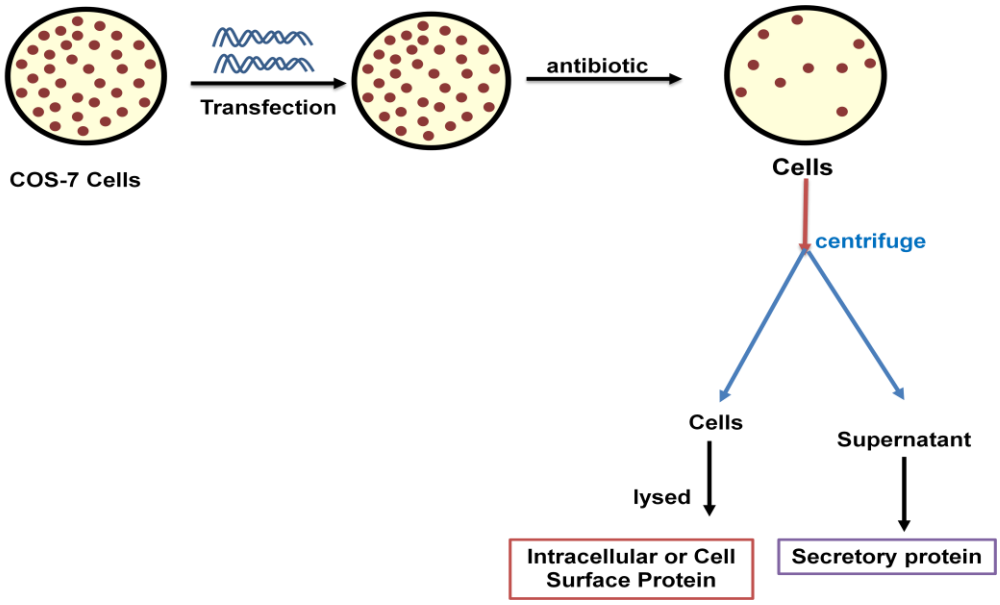

Figure 27.2: Different Steps in transient expression system.

Permanent expression: The permanent expression of a gene is possible, if it will be integrated into the chromosomal DNA. The most crucial step to estabilish a permanent expression system for a gene is the frequency of integration events rather than number of DNA uptake. In simpler word, permanent transfection depends on recombination frequency instead of transfection efficiency. Stable transformant are selected by a selection marker (such as antibiotics or auxotrophic factor or negative selection with an inhibitor) for a prolong period to ensure the integration of recombinant DNA into the genome. The steps follow to generate a stable cell line expressing foreign protein is given in Figure 27.3. The different steps are as follows-

Step 1-3 are indentical as discussed for the transient expression of a foreign gene.

4. Selection of trasnfected cells

4.1. Fourty eight hrs after transfection, split the transfected cells in a selection media containing antibiotic and allowed to grow for another 4 days.

4.2. Gently wash the cells with PBS and observe the descrete colonies.

4.3. Delineate the boundry of each colony with a marker from the back side of the plate.

4.4. Remove the media and put cloning ring to each colony. Wash the colony with PBS and add 100µl trypsin-EDTA to remove the colony.

4.5. wash the colony with PBS and transfer into one well of 24 well dish. Allow it to grow and become 80% confluent.

4.6. Transfer these cells to the 6 well dish in the presence of selection media and allow it to reach 80% confluency.

5. Take a small aliquot of the cell and test the expression of foreign protein with western blotting. In addition, integration of gene into the genome can be checked by performing a southern blotting with radioactive probe derived from the gene of interest.

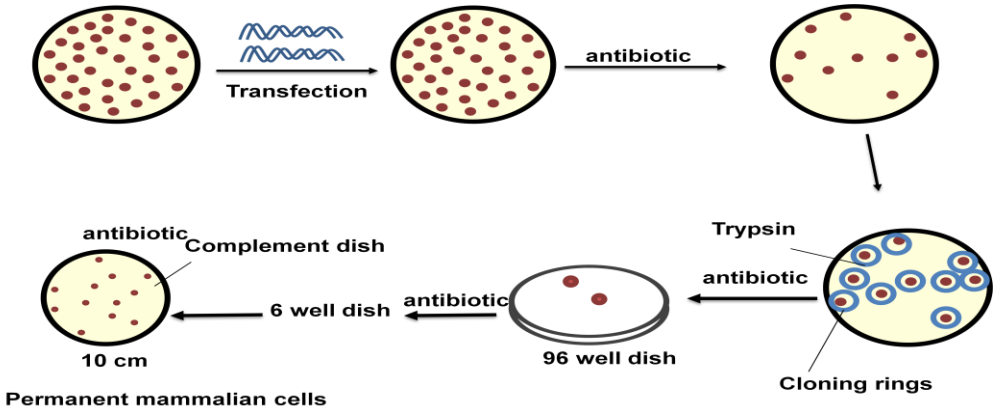

Figure 27.3: Different Steps in permanent expression system.

Inducible expression system. Inducible expression system is useful for the expression of a toxic protein or proteins with pleiotropic or non-specific effects. The tetracycline-controlled inducible system is given in Figure 27.4. In this system, seven tandenly arranged tet operator [$(Tet\text{-}op)_7$] are placed upstream to the minimum CMV promoter and transcriptional activator (tTA) gene. In another set, target gene is replaced with tTA gene. In the presence of tetracycline, the binding of tTA is blocked to the tetracycline operator. Consequently, it causes low level of expression of tTA and target gene. In the absence of tetracycline, low level of tTA binds to the operator and drive the enhanced expression of tTA which in-turn stimulates further amplification of initial signal. Transcription activator (tTA) produced in the absence of tetracycline eventually stimulates the expression of target gene.

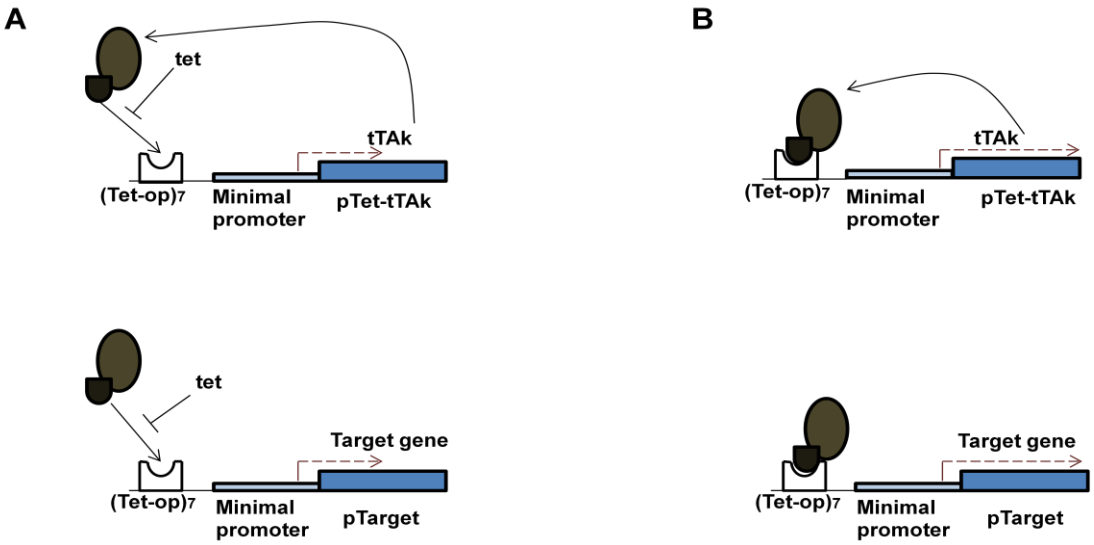

Figure 27.4: Tet inducible system of mammalian expression system. Molecular events in the presence (A) or absence (B) of tetracycline.