Organic Chemistry In Biology And Drug Development
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Lecture - 33
Molecular Biology

Welcome back. In the last session, we have revisited the processes involved in the central dogma of biology i.e. the flow of information from DNA to proteins. Mechanism of various enzyme and various process have been thoroughly discussed.

Now, we will discuss the amplification of a piece of DNA. If we have a small amount of DNA how can we get thousands and thousands of copies of this DNA. Copying of DNA in the biological system is called replication.

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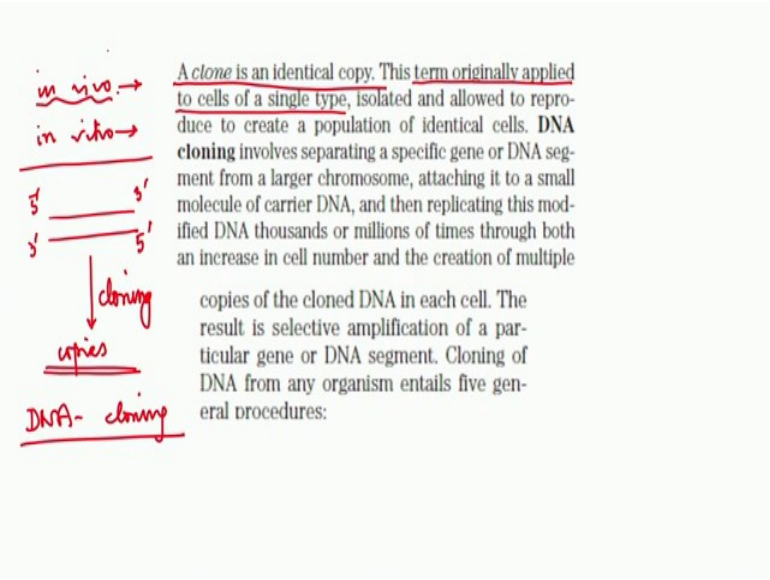

But here we are talking about something which is carried outside the living system. Here the processes are carried out in a test tube or Eppendorf utilizing the different components of the biological system that are involved in replication processes. We will show you how to do that but this is important to distinguish between the two processes. Replication is the process which takes place inside the living organism and that is is called in vivo process. The amplification carried out outside the living system is called an *in vitro* study.

If we have a copy of a double strand DNA how can we make identical copies without going into the intricate machinery of the biological system. This process is called cloning.

Cloning is basically making identical copy of anything. If you scan or Xerox a piece of paper you will get the clone of your original system. So, cloning is nothing but making or creation of identical copies.

Now, this is can be applied to a cell also. If you make copies of a particular cell it will be also cloning. But that is cloning of the cells. DNA cloning means multiplying or amplification of a piece of DNA. That is called DNA cloning. So, we are talking about DNA cloning in this case.

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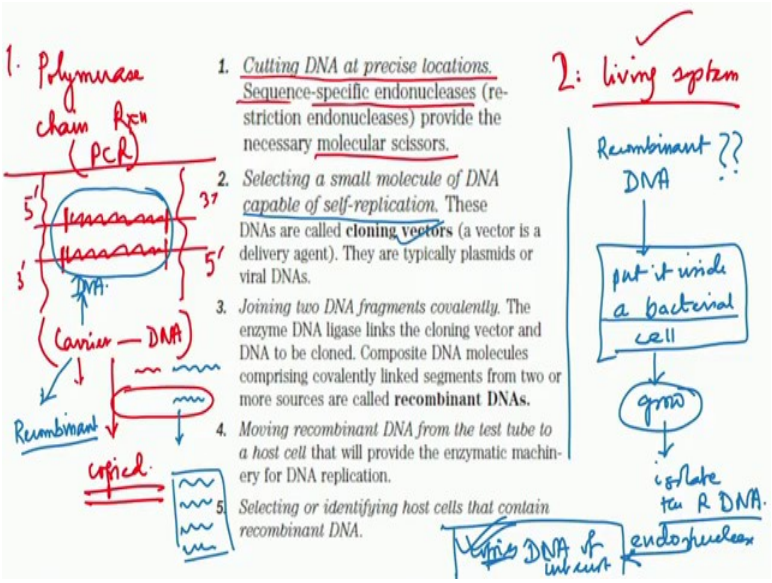

Now, there are usually two methods for multiplying a piece of DNA. One is called Polymerase Chain Reaction or PCR based method. Other method is semi in vitro process and it utilizes the cells in a living organism at some point of time. Here we will discuss the second method.

Suppose you have this piece of DNA and I want to copy this region only. I do not require the full copy of the DNA. At first step, you have to cut the DNA at precise locations.

So, you have to cut the DNA at precise locations, but the cutting should not involve the segment that you are interested to amplify. This cutting is done by sequence-specific

endonuclease enzyme because it is done inside the DNA strand. They are called the restriction endonuclease or the restriction enzymes.

Restriction enzymes are the endonucleases. If you read the other strand from the same 5 prime to 3 prime end you will get the same or identical sequence. It has got a local C_2 symmetry. If you turn it upside down you will get the same thing.

But remember this is local. You cannot turn the whole DNA this way to get the same thing. These types of palindromic sequences are present and they are recognized by these restriction endonucleases. They are molecular scissors. After chopping this off you need a vector. It carries this DNA and its own DNA. Then ultimately that carrier plus this DNA of interest can be copied. So, this is not polymerase we are talking about in general what is done, we are talking about the second one.

In the living that means, amplification and living system we are talking about that in the beginning. So, you have the DNA piece of DNA. This is your region of interest and you cut the DNA from the two sides using restriction endonuclease or the restriction enzymes. Incorporate a carrier DNA into it. I will talk about this carrier molecule. This carrier and then the DNA becomes an integrated part of this carrier which can be copied when that is put inside a bacterial cell.

If you put this carrier molecule inside the bacterial cell you will have bacterial cell containing this carrier DNA hybrid when the bacterial cell grows. This is actually called recombinant DNA. The carrier molecule is made up of DNA and this is also DNA. So, this is called recombinant DNA.

A vector is nothing but a delivery agent. For example, in case of malaria transmission of one microorganism into the human body is occurring via the mosquito. So, the mosquito is a vector for malaria, encephalitis.

So, vector is nothing but it is a delivery agent. It takes up the thing that I need to deliver and then brings it to the bacteria. They are capable of self-replication, but not by itself. When it is put in the bacterial cell then bacterial cell grows and there will be cloning of the cells. This recombinant DNA also makes its own copy in different cells. If you want to isolate this piece of DNA, we have to chop the outside layer of the bacteria. Then take

it out recombinant r DNA and utilize the same endonuclease to cut it off from the from the carrier molecule.

This has got one advantage. You can put it in the bacterial cell, now this cell you can preserve. Whenever you need this recombinant DNA you can ferment the bacteria which will make copies and then you can isolate the DNA.

You have the bacterial strain containing this recombinant DNA. Then you can always get hold of the copies of the DNA.

And the other important factor is that if these copies of if this DNA piece is ultimately after transcription and then translation, it makes a particular protein which you are interested. Then you will although all the time you will get, you do not have to break the cells, because you are not interested in the DNA any longer.

If you are interested only of the protein that this DNA makes, then you just grow the cells and then isolate the protein from the cells. In many cases, you can lyse the cell to isolate the protein.

I will give some example here. Diabetes are two types- one is insulin dependent, another is insulin independent. So, some people who are suffering from diabetes, they have to take insulin that is the insulin dependent. Earlier insulin was obtained from the cow/the bovine. So, it was bovine insulin that diabetes patients were using.

Later it was found that it is not a very good practice to slaughter a cow. It is not human insulin. There are some differences in the bovine insulin and human insulin although there is good homology. The sequence of the amino acids in the protein is very similar.

Later on it was found that we can take the human insulin gene as a recombinant DNA and put it inside a bacterial cell via this cloning technology. It has got the insulin gene. So when it grows it will make the insulin. Now whatever insulin that are available in the market are all recombinant insulin. It is a perfect human variation of the insulin.

So, you see that this technique is so important. If you want a particular protein the proteins are made in the cells in very small amount. If you want large amount of the proteins try to identify what is the corresponding gene of that protein. Then take the gene

and then put it as a recombinant DNA. You attach it to the carrier DNA or the vector DNA, and then put it inside bacteria.

If you are successful in that then all the time you can get the protein. As the bacteria grows it makes the protein. That is the modern day technology for any laboratory who are doing proteomics or enzymology i.e. protein related biology.

You have a vector DNA and you attach your DNA of interest. How will you ensure that it has been attached? When you have this vector DNA some of the vector DNA will remain as without any attachment from outside.

Some of the vector DNA will be attached to this external DNA. It is very similar when I to catalytic antibodies. There was this type of problem that some immortal cells are made up of cancer cells. Which are actually hybrid of the cancer cell and the other foreign cells.

That is the spleen cells which are making the antibodies. So, they have to select which are hybridoma and which are non-hybridoma cells. Which DNAs are recombinant DNA and which DNA are only the vector DNA.

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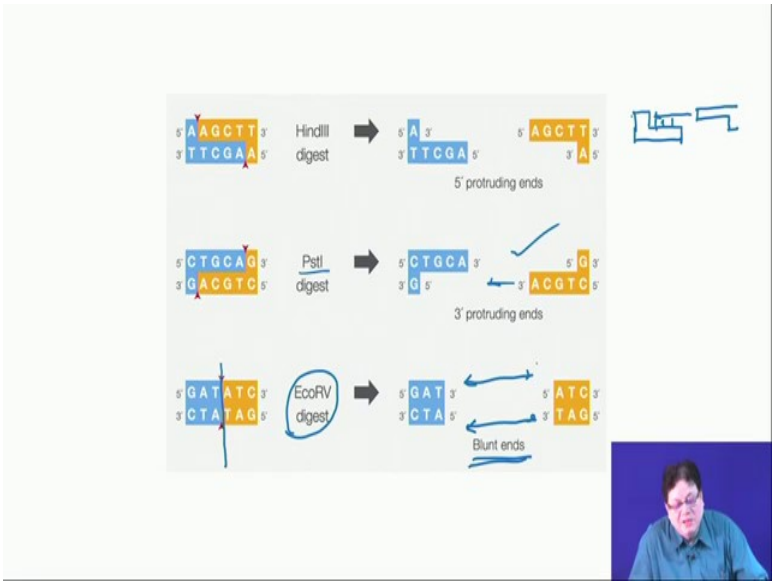

You have to identify that. It is very important and we will discuss how it is done. Before that, let us talk this restriction enzyme. Restriction enzyme recognizes palindromic sequence. This is the palindrome A A G C T T and if you write read from this side A A

G C T T same. That is a six based palindrome and the enzyme that recognizes it is called HindIII.

So, HindIII recognizes this sequence and it cuts at this point. Between the two As the phosphodiester bond is cleaved and that produces this type of fragments. This is called 5 prime protruding ends or the 5 prime overhang on this side. Overhang means where there is no complementary strand present. As there is no complementary strand present so that is called the sticky cuts.

What is Sticky cut? If you think this is a block of wood kind of thing this part goes inside because there is perfect matching here. So, it goes inside and you can again do the ligation.

So, you need a ligase to ligate these two pieces. There is a overhang which recognizes each other.

So, similarly there is this Pst1 another enzyme which recognizes C T G C A G. This is a palindrome C T G C A G and this also gives a 3 prime overhang i.e. 3 prime end is extended. There is another type of cleavage that is called blunt cut. It is done by EcoRV. EcoRV recognizes G A T A T C which is a palindromic sequence. It is a blunt cut. It cuts straight away at the same point and that gives blunt ends.

It is easy to ligate these two because they have a recognition arm. There is no recognition arm because it is a blunt cut. There are ways to join this together. That is a much more complicated once if you have blunt ends.

On the other hand people try to have sticky cuts because sticky cuts are easy to join just as the DNA ligase. They will join each other. There are many restriction enzymes that have been isolated and these restriction enzymes are present in bacteria. It is believed that the bacteria has evolved this restriction enzymes in order to protect themselves from the viral infection. These restriction enzymes cut this external DNA and chop the DNA apart. So it cannot infect the bacteria.

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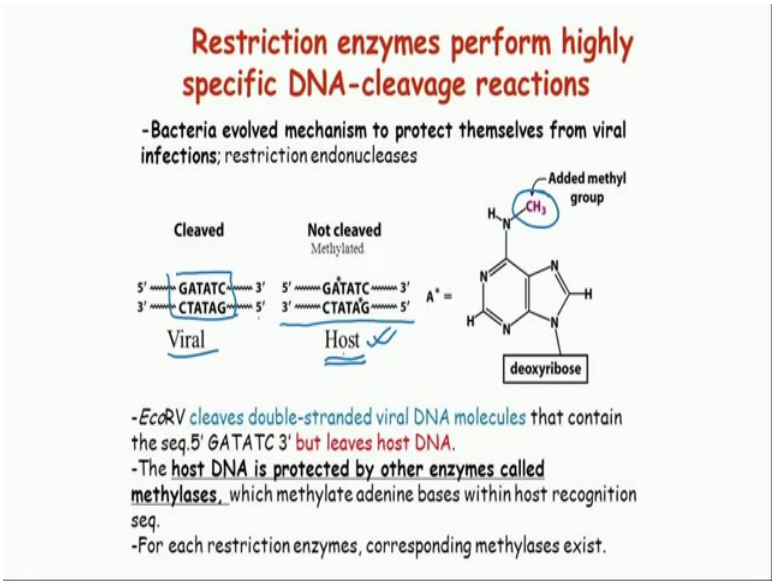

Restriction enzymes perform highly specific DNA cleavage reactions. Bacteria evolved mechanism to protect themselves from viral infections. This is the viral DNA and this is the host DNA. Now you can always ask this question that the sequence that is present in the virus has to be a palindromic sequence and that has to be recognized by a restriction enzyme. Restriction enzyme is made by the host DNA but the host DNA itself can have the same restriction site which is recognized by the same restriction enzyme.

How does it really protect itself from the self-cleavage? So, what it does one interesting point is mentioned here that the bacteria labels the A by methylation in the DNA. So, it does a methylation of the adenine and that actually indicates or gives an instruction to the endonuclease- that this is my self DNA don't cut it, cut the viral DNA which is not methylated. So that is the bacterial way of identifying self and the external DNA.

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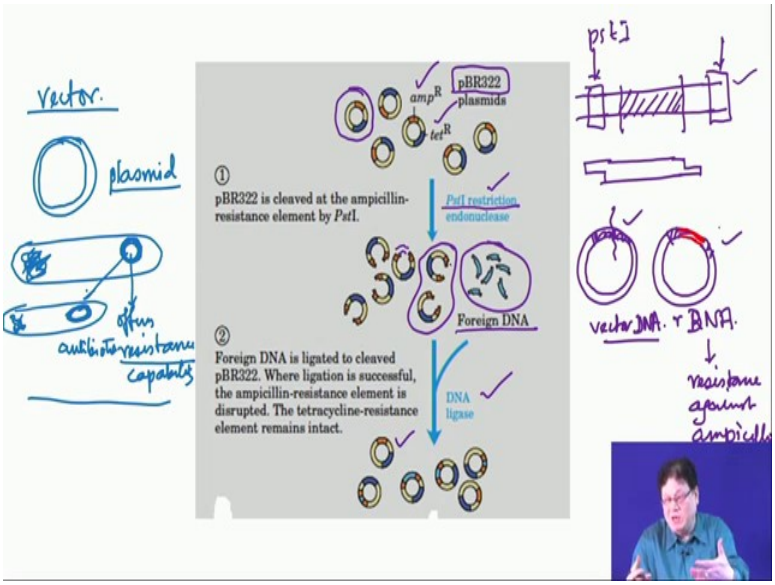

We did not talk about the vector yet. It is a piece of DNA which has which has replicability. We can replicate when it is put in a bacterial cell. Plasmids are circular DNA molecules that replicate separately from the host chromosome. Plasmid DNA is a circular DNA. It is not present in the chromosomal DNA but it is actually present within the cytosol.

The usual strategy is to use a plasmid that includes a gene that the host cell requires for growth under specific conditions, such as a gene that confers resistance to an antibiotic.

Antibiotic resistance to the bacteria means the plasmids are making some enzymes which are destroying our antibiotic before showing the antibiotic action.

It is the problem if bacteria had the resistance gene in the chromosome. The time taken from chromosome to express to the protein is much more than the plasmids. The plasmids can easily replicate. They can also very quickly infect other cells which do not have these plasmids. So, there could be cross infection of this plasmid to the bacterial cell which does not have this plasmid.

How the resistance can be spread into the entire bacterial population very quickly? Because they do not have to unwind the entire chromosomal DNA in order to get the resistance gene which it is actually present in the plasmid. So, plasmids have anti-

bacterial resistance genes and also there could be many genes- one is acting against tetracycline, another is acting as penicillin. There are many classes of antibiotics.

So, there may be different antibiotic resistance genes present in the plasmid and they can cross infect. They can also be easily multiplied.

Different plasmids are made synthetically. Synthetic plasmids contain different genes expressing for different proteins.

This is called pBR322. This pBR322 plasmid has two genes- one gene confers resistance to the ampicillin, ampicillin is penicillin and there is another gene which confers resistance to tetracycline. This is my starting vector. So, this vector is pBR322. If you do not remember the name follow the principle.

The principle is that it should have the plasmid which is having two different kinds of genes expressing for two different proteins. That gives some resistance to different antibiotics. There could be plasmids where one could be antibiotic resistance gene and another could be a gene for a protein which gives color. So there could be different variations of plasmids. Let us talk about this one, this has got two types of resistance- tetracycline and ampicillin.

It has been found that this ampicillin resistance gene is inside the restriction site of restriction endonuclease enzyme Pst1. These sites where the restriction enzyme works are called restriction sites. So, this gene has a restriction site inside the ampicillin resistance gene.

Now treat this vector with Pst1 restriction enzyme. This gives a sticky cut. Pst1 gives a sticky cut, HindIII gives a sticky cut, and EcoRV gives a blunt cut. I have the foreign DNA like this and I am interested only in this part. Suppose there is a restriction site here and these are recognized by Pst.

Then you will have pieces where is the restriction site of the foreign DNA. Remember this part should not be disturbed at all. So, you are cutting little bit far on this side and little bit far on this side. Then this will have similar kind of sticky ends.

This part now can ligate with the protruding part or the over end part of this end, and the other end can ligate with the overhang part of the vector DNA which is already treated with the same restriction enzyme.

You should use the same restriction enzyme to cut your external DNA and also to cut the plasmid. The plasmid is cut in such a way that your ampicillin or any of the antibiotic resistance marker genes is cut inside because these two are not joined. This is a disjointed gene. So, that will not express the enzyme that destroys the penicillin i.e. ampicillin. So, that is the reason why you got inside the ampicillin resistance gene.

Now you add your foreign DNA with the same sticky ends and this is your vector DNA with the same sticky ends. Now, when you add DNA ligase some of the vector will just again bind to each other because they can recognize each other. So, this is the recombinant. Now, some of the vectors are not taking any external DNA but this external DNA can recognize that part.

Now, some of the vector will remain as earlier. So, they just join with each other again but some of the vector will take this external piece of DNA. Suppose, this is the external piece of DNA, and then you complete the other part. This is your vector DNA. So, you have now two types of DNA- one is the normal and the other one is the recombinant DNA.

Remember this was your ampicillin resistance gene and you have cut inside. Then you put a foreign DNA in the recombinant DNA. So, this recombinant DNA lacks the resistance against ampicillin, ampicillin because your gene is disjointed and there is something foreign DNA.

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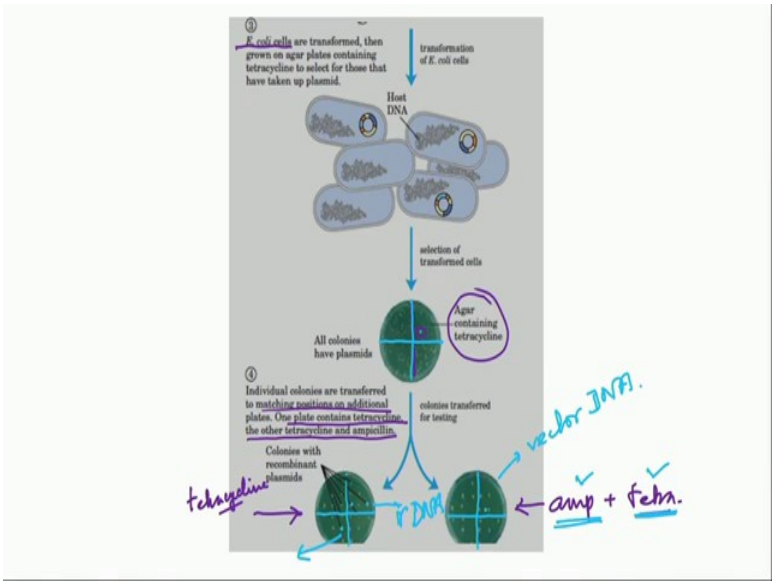

So, you take all these recombinant DNA plus the original vector DNA, and put it inside. You can transform E. coli cells that are a particular type of gram negative bacteria and you put this DNA. Some cells will have the vector DNA and some cells will have the recombinant DNA.

Now, you grow this in a agar plate where the bacteria can be cultured in a petri dish and this is tetracycline. This population is transferred here and they are allowed to grow. You have already added tetracycline in the medium. Remember tetracycline gene was not touched at all. They are in the vector DNA and it was there in the recombinant DNA. There will be bacterial colonies everywhere. All the two types of bacterial cells i.e. one containing the vector DNA and the other containing the recombinant DNA, both will grow here.

Now you take another two agar plates- one plate containing tetracycline, and the other plate containing ampicillin and tetracycline. These are both are antibiotics.

You are taking a bacterial colony from here i.e. you make a grid kind of thing and then according to the XY coordinate you place bacterial colony. You make the grid and then matching positions. The colonies are transferred from here to the same xy coordinate (the matching positions). Here you have only tetracycline.

So tetracycline resistance means everything will grow because both the vector DNA and the recombinant DNA have the tetracycline gene. But when you develop it into ampicillin plus tetracycline, then the bacterial cells will get only the vector DNA because it is the vector DNA which confers resistance to both ampicillin and tetracycline.

So, the colonies that are growing here is containing only vector DNA. You can realize that this colony is not present here. This is a recombinant colony must be having a r DNA. You take the colony and then grow in another suitable medium and then you get the copies of the recombinant DNA when the bacteria grow.

If you are interested only in the DNA isolate the DNA. If you are interested in the protein made by rDNA isolate the protein. In both the cases you have to lyse the bacterial cells and then separate the DNA nucleic acids. Then isolate the protein of interest. I told you this is kind of a semi in vitro because initially the reactions with an restriction enzymes are done in vitro. Then that is transformed into the bacterial machinery. After that it is in vivo technique.

So, this is a beautiful way. Now, many of the problems have been solved that recombinant DNA technology can generate or give copies of DNA as well as the proteins. That are the most interesting aspect that carries out all the desired reactions. This recombinant DNA technology was developed in the the early 1980s and now it has become a routine technique in any molecular biology lab.

In the next session, we will discuss the entirely in vitro method and that is called the polymerase chain reaction.