Eukaryotic Gene Expression
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Eukaryotic Gene Expression:
Basics & Benefits
Lecture No. #32
Eukaryotic protein expression systems - III
Gene expression in mammalian cells using viral vectors.

Welcome to this lecture series on eukaryotic gene expression basics and benefits. In the last 3 or 4 lectures we have been discussing about the various eukaryotic expression systems, we began by discussing how genes can be cloned and expressed in yeast and insect cells, then we moved on to mammalian cells, and in the last class we discussed about, how you can introduce genes and express them using non-viral vectors.

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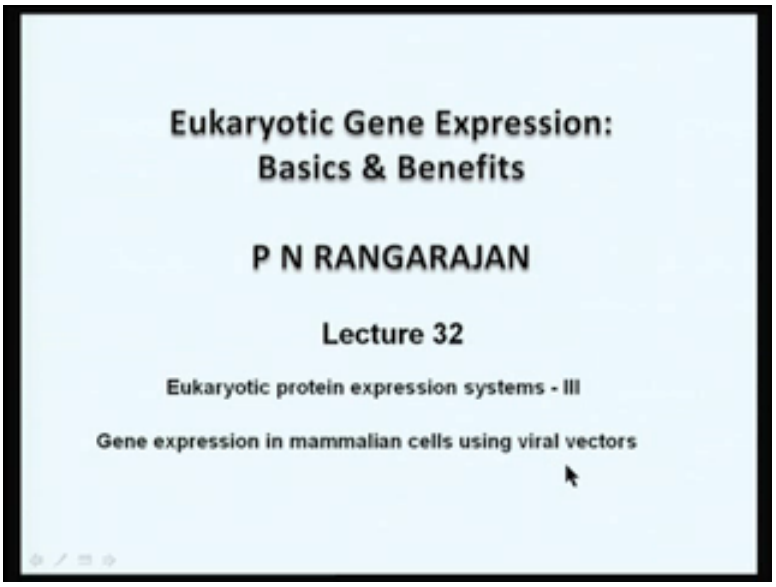

And today we are going to discuss, about expressing genes in mammalian cells using viruses as gene delivery vehicles or gene transfer vehicles or viral vectors.

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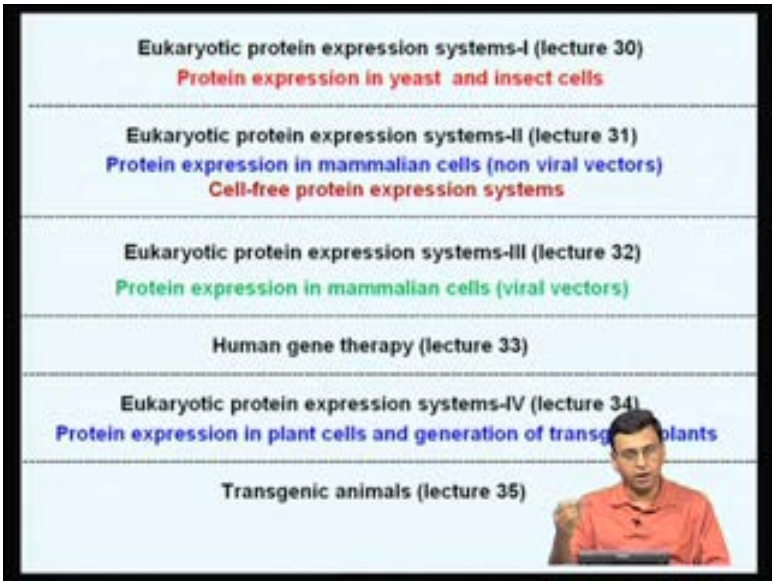

So, these are summary of what we have been discussed in the last few lectures, we discussed in lecture number thirty about protein expression in yeast and insect cells. Then we discussed about how non-viral vectors can be used for transecting genes and plasmids in to mammalian cells. Then we also briefly discussed about the various cell free protein system that are available, and how we can actually make cell free system like rabbit reticulocyte cells, weed germ extracts and more recently you can also make using cell free systems, huge amounts of proteins, even milligrams or even grams amounts of vectors proteins can be made using cell free expression systems. So, today the focus is going to be on, how we can introduce genes and express them, so that your protein of interest can be produced in mammalian cells, but our focus is going to be on how viruses can be used for introducing genes into mammalian cells and expressing them very efficiently.

This lecture is very important; because both this lecture and previous lecture actually form the basis for a very fascinating area of research, which has taken off in the late 1990's named as a human gene therapy. So, unless we understand how people are trying to introduce genes into mammalian cells using non-viral vectors and viral vectors. We will not be able to understand some of the important advances, and very exciting development that are taking place in the area of human gene therapy. So, let us spend some time to understand, how viruses are being used for delivering genes into

mammalians cells, and how you express your gene of interest so that the proteins can be made in mammalians cells.

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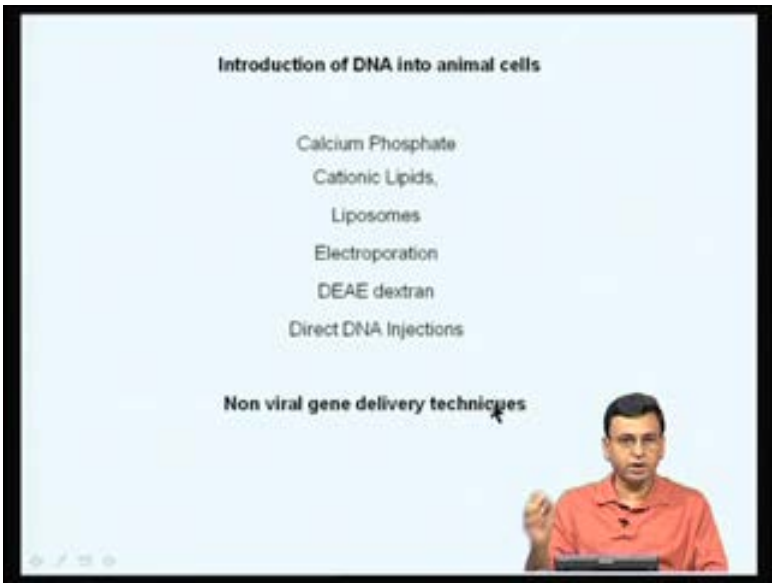

So, in the last class we basically discussed about, a number of non-viral techniques as well as non-viral vectors, basically plasmid base vectors, using either viral promoters or certain cellular promoters for expressing your gene of interest, and we discussed a variety of methods like the calcium phosphate precipitation, using cationic lipids and liposome's, electro oration there are also many other methods; like D E A E dextran directly injecting D N A, and some of these methods will discuss much later in this lecture series. Basically, what we discuss is that a number of transfection protocols have been establishes to introduce the expression plasmid of your interest into mammalians cells, and these are all termed together as non-viral gene delivery techniques.

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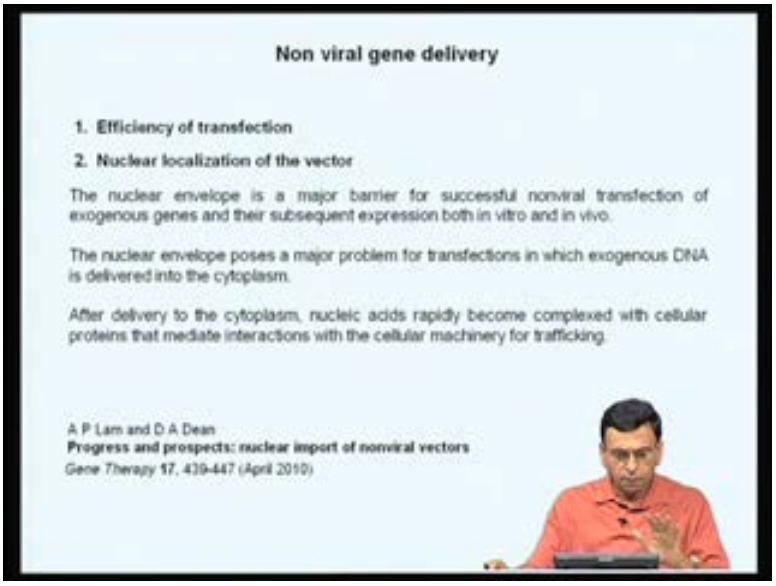

Now why do you have to now discuss about using viruses as gene delivery vehicles, what is wrong with non-viral gene delivery. The 2 major problems with non-viral gene delivery mechanism was; one is the efficiency of the transfection, when you use reagents like liposome's or even electro oration or even calcium phosphate precipitation. The percentage of cells in to which you can introduce your plasmid or expression plasmid is rather low. So, compare to viruses for gene delivery, the non-viral vectors are not as efficient as the viral vectors as for as the efficiency of the transfect ion is concerned. So, if you really want to express a huge vast majority of cells with your gene of your interest, and produce large amounts of this protein in the non-viral gene delivery techniques posses certain problems.

The other major problem with the non-viral gene delivery mechanism is that the expression plasmid that have introduced in to the cells, you are only introducing to the cytoplasm, but now you have to take them into the nucleus. So, unless you're expression vector goes inside the nucleus, your gene will not be transcribed and R N A will not be translated to your interest cytoplasm. So, there are number of the nuclear actually itself is the major barrier for successful of non-viral transfect ion of exogenous genes, as well as their subsequent expression both in vitro and in vitro, and the nuclear envelop poses a major problem for transfect ions in which exogenous gene is delivered into the cytoplasm. So, after the delivery into the cytoplasm, the expression vector or the nucleic

acid becomes complex with cellular proteins that mediate interactions with cellular machinery for traffic into the in and out of the nucleus.

But, there is a very nice review in gene therapy about the progress and prospects nuclear import of non-viral vectors, where you actually discuss number of problems centered with delivering your vector into the nucleus, but today in fact we have mechanisms or we have devices and methodologies for actually introducing your D N A or your nucleic acids right into the nucleus, these are called as nucleofection techniques. There are operators which you can buy and you can actually introduce your vector right into the nucleus using this nucleofection vectors. So, the non-viral vectors have now gain some momentum with technologies such as nucleofection techniques.

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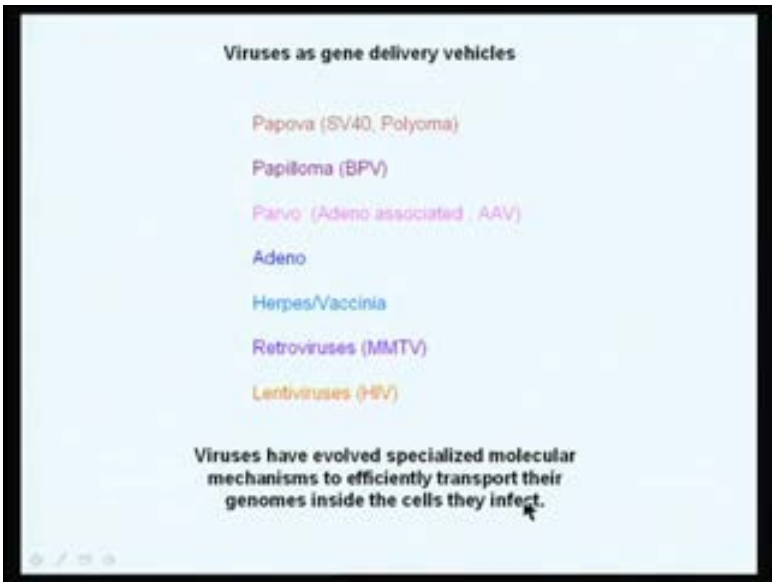

But with some of these limitations, people started to wondering, how we can introduce genes more efficiently in vast majority of cells, and one thing that came into the mind is, why we cannot use viruses as gene delivery vehicles. The reason is, a number of viruses belonging to a variety of families of viruses, variety of classes of viruses, like pap ova viruses, papilloma viruses, parvo viruses, adeno viruses, herpes viruses, retro viruses, lenti viruses they all infect humans and animals, and these viruses have been infecting us for time immemorial. And, therefore they have also devised a variety of mechanisms by which they can attach to cells, pushed there genomes, inside the viral, inside the

mammalian cells, and the viral genome can efficiently transcribe their genes, and also they can replicate, and they can come out of the cells, either lysine or without lysine.

So, since the viruses have devised natural mechanism, they can infect cells, mammalian cells, well people thought use these viruses as natural gene delivery mechanisms or gene delivery vectors. So, since viruses have a evolved specialized molecular mechanisms for efficiently transporting their genomes inside the cells. In fact people started asking, why cannot we now modify these viruses in such a way that instead of they going and infecting and replicating their genome, and transcribing their genes and producing their viral proteins, can we now introduce our gene of your interest into a virus. So, that when the virus goes and infects the mammalian cell, instead of making the viral proteins it now makes your protein of your interest. So, this concept of using viruses has gene delivery mechanisms gained a huge popularity, and in fact today some of the most efficient mammalian expression system that we have, is based on viral vectors. So, let us now try to understand, how viruses can be used for delivering your genes into mammalian cells, and how you can express efficiently once the gene is introduced into the mammalian cell.

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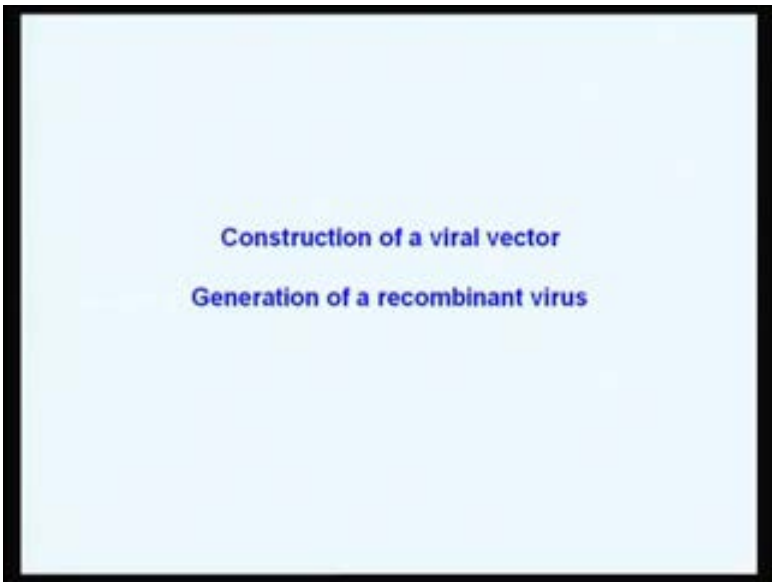

So, the focus is going to be on how you construct a viral vector, so you have a viral genome which codes for a number of viral proteins. Now, you have to take out some of the viral genes, and put your gene of your interest and this constitutes what is called as a construction of a viral vector. Now, once you have a viral vector, form this you have to

generate what is called as a recombinant virus, so that this virus can now go and efficiently infect your cell of your interest. So, there are 2 strategies 2 steps in using viruses as gene transfer vehicle or virus mediatory gene delivery into mammalians cells. The first step is construction of a viral vector where you can introduce your gene of you interest, and once you have a recombinant viral vector ready form that you have to generate a recombinant virus, how do you achieve this.

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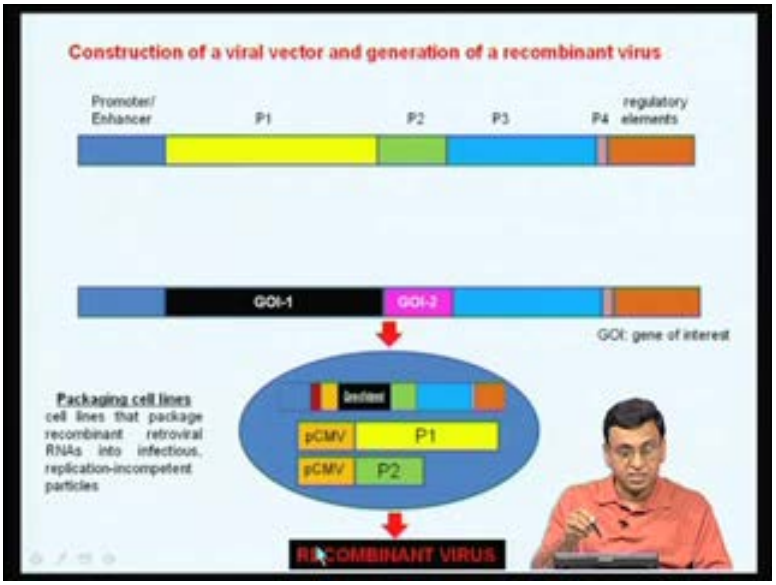

The general strategy that is employed for using viruses, has gene delivery vehicles is schematically shown here now. Let us take a normal viral genome, it can be retrovirus or an adenovirus or an adeno associated virus, this is a very general simplified scheme which is applicable to all kinds of virus mediated gene transfer mechanisms. Normally, any viral genome will contain promoters and enhancers, and through which transcription factors usually the host transcription factors go and bind to this promoters and enhancers, and they transcribe a number of viral genes which are downstream, I have designated here some of the genes has p 1 p 2 p 3 p 4 etcetera.

So, usually the viral genomes will have a regulatory element that is necessary for transcription of the viral genes, and also at the 3 premed you will also have something like polyrelation signal processing of the R N A and so on and so forth. So, the general strategy of now converting this retro, this virus genome into a viral vector is, you now delete some of the viral proteins which probably or not very essential or which are

actually called as a structural genes, which actually code for structural components of the virus, like the cap side envelop and so on and so forth, delete those genes in this case. For example I have shown that I have deleted p 1 and p 2 from this viral genome, and clone your gene of your interest in the place of a p 1 and p 2, and if you now have a backbone in which it is as a e coli origin of replication, and a selection marker such as anti-biotic marker. You can now once you legatee your gene of your interest into a plasmid, you can easily replicate this plasmid in large amounts in e coli. So, you'll now have generated a recombinant viral vector.

The first step has been achieved now, so by removing certain viral genes and introducing your gene of your interest, you have now generated a recombinant viral vector. Now, from this how do you generate a recombinant virus what you necessary do is, you now take this gene and then introduced into a cell line called as packaging cells. What are packaging cells; packaging cell lines are nothing, but cell lines that package the recombinant retro viral, can be retro viral or can be any viral R N A's or viral genomes into infectious replication in competent particles. Let us now try to understand what this all, what you normally do is, I told you in generating a recombinant viral vector, you have actually deleted certain viral genes, in this case p 1 and p 2. So, what you do is you now take any cell line, any mammalian cell line and clone this p 1 gene and p 2 gene under a mammalian expression vector, and generate a stably transected cell line, which is now constitutively expresses this viral proteins p 1 and p 2, this is called as a viral packaging cell line, that means here you have a mammalian cell line which is constitutively expressing viral proteins such as p 1 and p 2.

Now, into such cell line if you now introduce to your recombinant viral vector, which lacks the p 1 and p 2, but in their place contains you genes of your interest I have designated G O I 1 and G O I 2. You can put 1 or 2 genes depending upon your need, so once you transfect your plasmid recombinant viral vector into this packaging cell line, what happens. This recombinant viral genome will now be packaged into virus particles, infectious virus particles, because the virus protein is necessary for the viral packaging is now produced by this cell line. So, what comes out of this cell line is actually recombinant virus, but there is something very different from this recombinant virus compare to the wild type virus, when you infect this genome into cells you get a recombinant replication competent virus, because it contains all the viral proteins

required not only for infection, but also for replication of the virus. But, when you infect this, many transfect this particular viral genome, in which you have deleted certain viral genes and put your genes of your interest.

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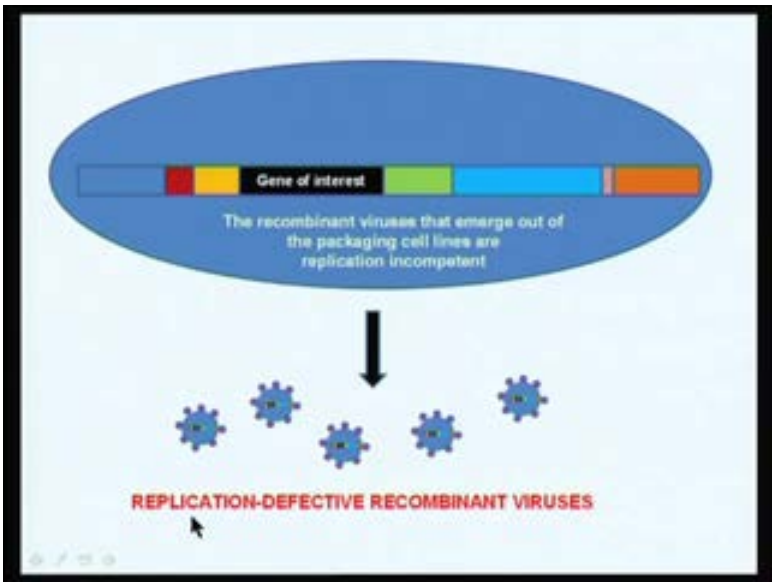

When the recombinant virus that is coming out of such cells, they are named as replication defective recombinant viruses, that is because this viruses which are emerging out of this packaging cells, they contain a recombinant genome, and this recombinant genome lack certain viral genes which are necessary for viral replication. So, the viruses that emerge out of a packaging cell line, following the transfect ion of a recombinant retro viral vector are termed has replication defective recombinant viruses. Now, what do you mean by this, when you now take these viruses infect another cell line

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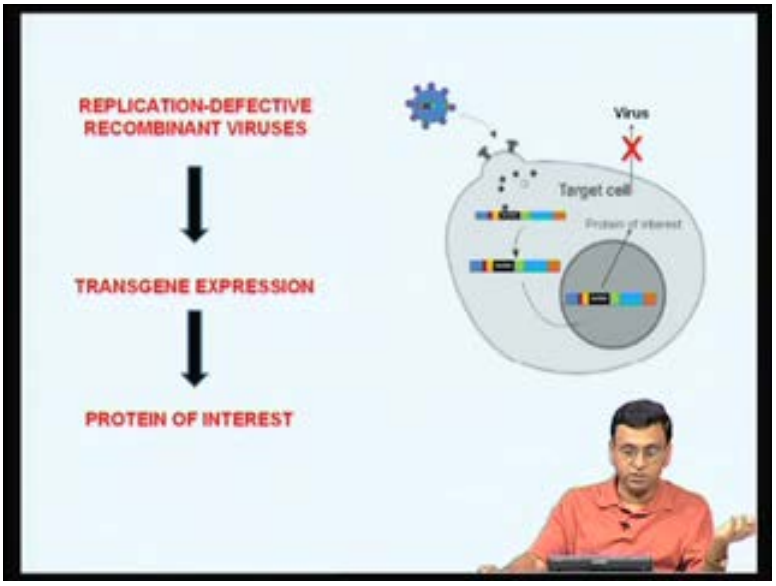

These viruses can go and infect, because these cells contain usually specific receptors through which the viral surface proteins interact, and once the virus infects the viral genome goes inside the cells, but instead of making the viral proteins, now your gene of your interest will be transcribed in the nucleus and the protein of your interest to be made. But, since no viral proteins are being made inside the cells, a virus cannot emerge out of this infected cell, therefore the virus cannot replicate and a infectious viral particle cannot came out. So, this is like abhimanyu getting into a chakrayuwa. He can go only inside, but cannot come out, so the virus can infect efficiently the mammalian cells, and push the genome inside and the gene can be transcribed efficiently. But, a virus cannot emerge out of the cell, because the viral genes necessary for successful multiplication of the virus and producing the viral progeny are missing in this replication defective viral particles.

So, by this strategy you can actually generate a huge amount of these replication-defective recombinant viruses, and if you now simply add these viruses to any cell line of your interest, the virus will infect those cells. Your protein of your interest will be made in large amounts, but no virus will come out of this cell line, so you're very safe. So, this is a general strategy by which viruses can be used as gene transfer vehicles for expressing genes of your interest in mammalian cell lines in large amounts. I hope the concept is clear now. So, basically when the recombinant replication defective recombinant viruses infect these mammalian cells, the genome is pushed inside, the

transgenic will be expressed and your protein of your interest will be made, but no virus will come out of the cell, because in this case proteins such as p 1 and p 2 are missing and without that virus cannot successfully replicate and recombinant viruses cannot come out of this cells.

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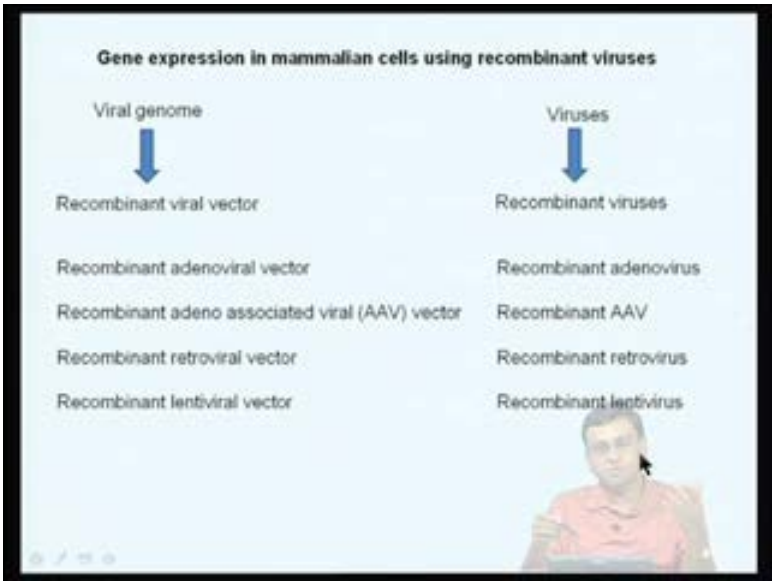

So, having understood the general concept by which one generates recombinant viruses. So, basically you first clone your gene of your interest into a retro viral vector in which certain genes are missing, and once you get a recombinant viral vector, you produce this virus in large (( )) large amounts by transforming this Plasmid in to e coli, and once you get this plasmid in large amounts, you take this plasmid transfect them into mammalian cell line, which is actually called as a packaging cell line, because this cell line is actually producing certain viral proteins that can package your recombinant viral genome into infectious viral particles. But, the difference between this infectious virus particle and the normal virus infection is that, these virus particles are called as replication defective viral particles, because they can only infect, but they cannot replicate inside the mammalian cells. So, this is the basic strategy by which one can use virus as gene delivery vehicles, and one can generate recombinant replication defective recombinant viral particles.

So, using this strategy let us now try to understand, how people have been generating a number of different viral vector recombinant viruses for transecting mammalian cells and

expressing your genes of interest in large amounts. So, there are two steps in using the recombinant viruses for expressing genes mammalian cells; one is the generation of a. Retro viral vector from a viral genome, and once you have a viral vector clone your gene of your interest, and then you can actually now convert a normal virus infectious replication competent virus into a replication defective recombinant virus. These are the two major aspects by which viruses can be used as gene delivery vehicles.

So, what I am going to do in the next few minutes is to discuss with you, how people have generated recombinant adenoviral vectors and recombinant adenoviruses, recombinant adeno associated viral vector and recombinant adeno associated virus. Similarly, recombinant retroviral vector, recombinant retrovirus recombinant lentiviral vecto,r recombinant lenti viruses; there are many other types of viruses, but since time does not permit discuss everything, we will use these four different viral systems, I think I will also briefly mention about vaccinia vectors, how vaccinia viruses can be used for generating recombinant vaccinia viruses for making your proteins in large amounts in mammalian cells. So, let us go 1 by 1 and see how this viral system have been developed.

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Let us first discuss how gene transfer and expression has been carried out using adenoviruses and adeno associated viruses, these are both are D N A viruses, so the adenovirus, adeno associated virus as well as the vaccinia virus are the example for the D

N A viruses, how D N A viruses can be used for expressing your genes of your interest. The retro viruses and lenti virus are examples for R N A viruses or retro viruses as a gene transfer vehicles.

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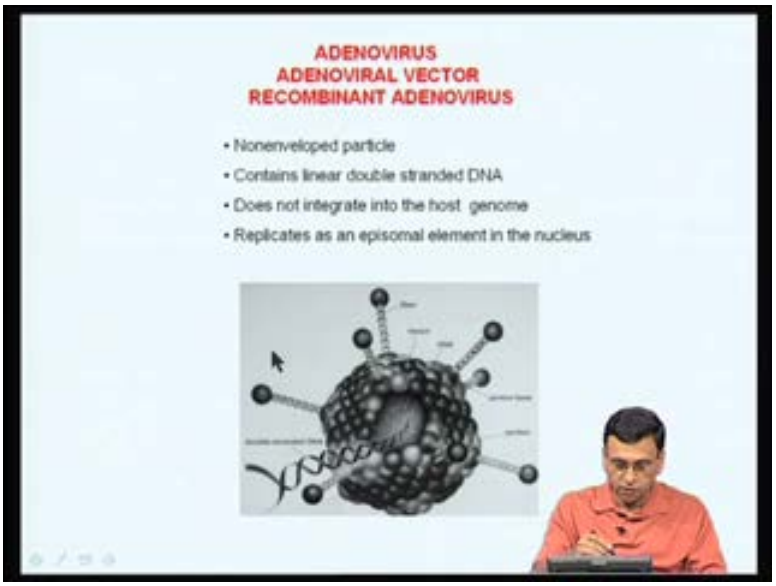

Now, what is an adeno virus, a adenovirus is a non-envelop viral particle. It contains a linear double standard D N A and once an adenovirus infects any mammalian cell it infect a wide verity of mammalian cells. The viral genome does not go and integrate the host genome, it remains has an episome or an extra chromosomal D N A, and it replicates inside the host cell nucleus has a extra chromosomal D N A. So, the viral D N A will be transcribed, viral proteins will be made, and then the viral D N A will be assembled and infectious viral particles emerge out of the cell. This is just the structure of a normal adenovirus.

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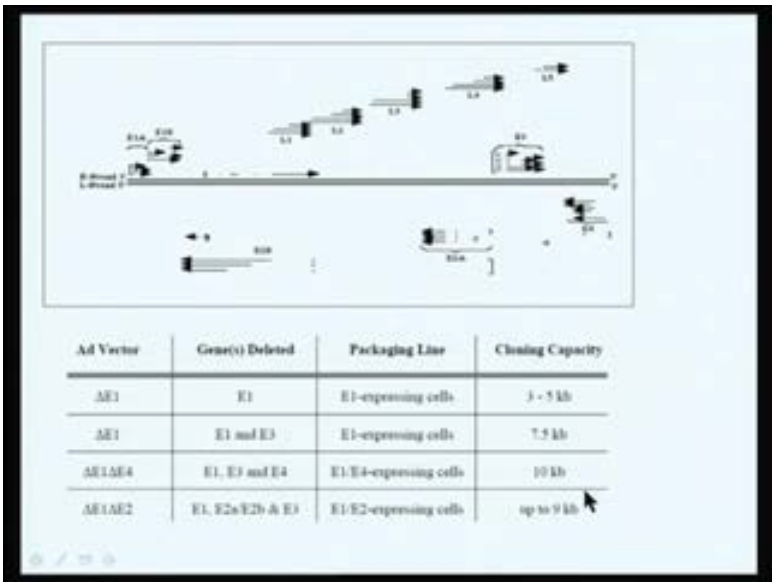

And if you look at a adenoviral genome, it contains a number of transcription units or number of genes which are transcribed usually in 3 different phases of the virus life cycle, genes which are transcribed immediately after the virus infection of a mammalian cell are known as immediate early genes, this is what genes, this is a kind of e 1 a and e m b actually are immediate early genes, they usually. These immediate early genes are nothing, but transcription factors which are required for the transcription of the next phase of the viral genes, namely the early genes. So, once the immediate early genes are transcribed (( )) genes, now go and bind to the promoter of viral promoters and transcribe the trigger the expression of the early genes, and these early genes, then following the early gene expression you get what is called as a late genes which are transcribed, and following the expression of all this immediate early genes early genes are late genes finally the virus replication as well as virus transcription is over, and then the viral D N A gets assembled into the viral proteins and a infectious viral particle emerges out.

So, the strategy for converting such an adenoviral vector adenoviral genome into an adenoviral vector is, you basically delete the immediate genes, such as the even a even b or even the e 3, and in their place put your gene of your interest. So, this is what the some of the normal very popular adenoviral vectors have been generated here. For example you have an adenoviral vector in which the e 1 gene has been deleted, so it is known as the delta e 1, and what you do if you want a recombinant adenovirus to generate from such a vector, you have to take this e 1 deleted adenoviral vector, and put them in a packaging cell line that expresses e 1 protein.

Therefore, you will get a recombinant retro virus, which is again replication defective, that is these recombinant adenoviruses can multiply only in those cell lines which can express e 1 a, in the cell mammalian cell does not express e 1 then the virus will not replicate, it can only infect and your transgene of your interest will be expressed, but the virus will not replicate. Such e 1 delete adenovirus vectors can actually carry about 3 to 5 give k b of your transgenes, if you have a gene bigger than that you cannot use the e 1 deleted adenoviral vectors. So, to accommodate larger fragments of D N A people have started deleting other genes, and started putting these genes in packaging cell lines, so that these proteins can be provided in trans. So, like that you have e 1 and e 4 deleted e 1 and e 2 deleted or e 3 deleted and so on and so forth. So, basically you delete these genes from the adenoviral genome, and clone your transgene of your interest in their place, and then transfect them into packaging cells which are now expressing those missing proteins, therefore you get a recombinant adenovirus. The more you delete from the adeno genome, larger fragments can be cloned, so up to 10 to 9 k b of your genes can be cloned into this kind of adenoviral vectors.

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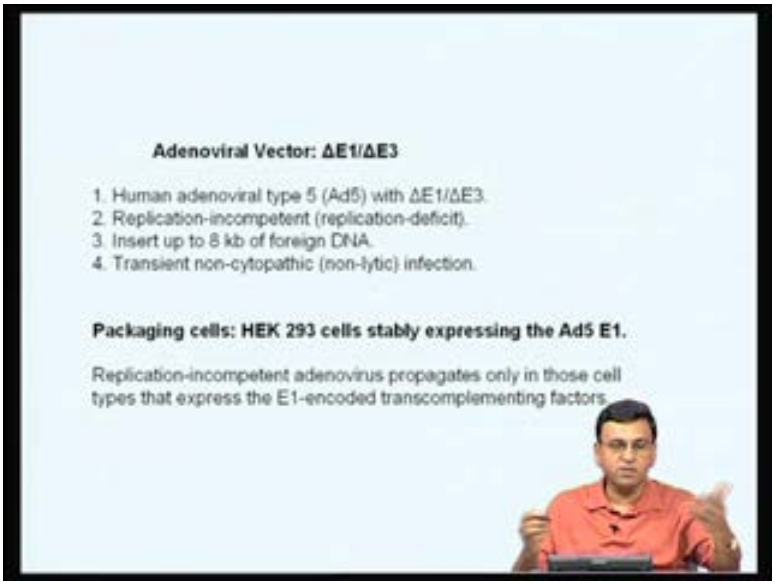

So, the strategy has been shown here, the human adenoviral type 5 is what is normally used for making these adenoviral expression systems, usually have either e 1 deleted virus or an e 3 deleted virus. When, you do this they become replication incompetent, it cannot replicate in mammalian cells which do not express e 1 or e 3 proteins up to 8 to 10 k b of foreign D N A can be inserted in to such kind of a replication defective

adenoviral vector genomes, and usually when you take such replication defective adenovirus infected cells, they do what is called as a non cytopathic or non-lyric infection, that is adenovirus do not lies the mammalians cells, but they bud out of the mammalian cells, therefore the cells still remains alive. So, the strategy usually is to generate this kind of a replication defective adenoviral vectors, and transfect them to packaging cells. One of the most popular cell lines which is used of generating a recombinant adenovirus is called as a H E K 293 cells, these H E K 293 mammalian cells actually express the e 1 protein of the adenovirus. So, when the replication defective adenoviral vector is introduced into the cell line, you get a replication defective adenovirus emerging out of these cells.

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Now, today we have a number of companies which actually sell these kinds of adenoviral expression systems, so you do not really have to construct; adeno recombinant adenoviral vectors or generate packaging cell lines. You can just go and buy these expression systems from number of commercial companies, you just take and clone your genes in to those adenoviral vectors systems, and take the packaging cell line that is provided by the company, and you can generate your own replication defective adenoviruses. For example one of the companies which is very popular which sells these kinds of a expression system is called clone tech.

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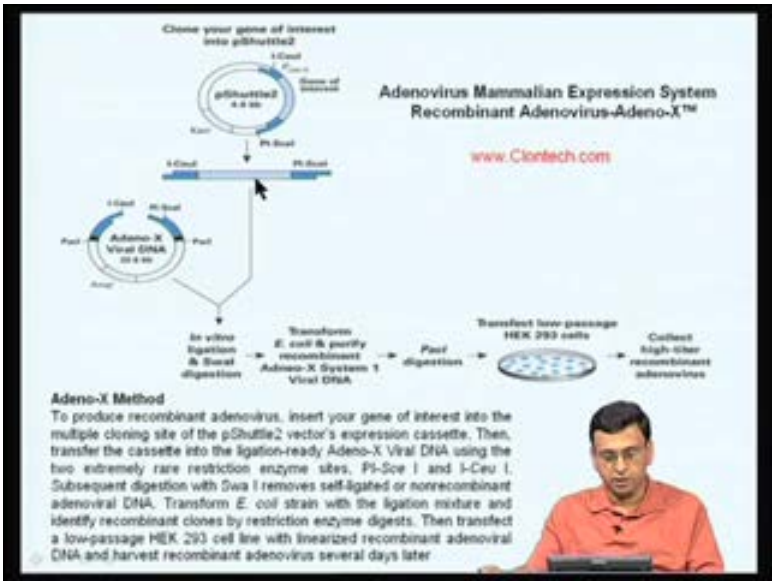

And I am just listing some of this things, for example these companies sells an adenovirus mammalian expression system known as the recombinant adenovirus adeno x. The company gives a complete a kit for generating a recombinant adenovirus, where you actually clone your gene of your interest in a shuttle vector, and make the shuttle vector in large amounts in the e coli, then liberalize this vector using these two specific enzymes. And they also supply the entire adeno viral genome, which is the called as the adeno virus gene, you can see it is about 32 point 6 k b D N A, which is also digest the same enzymes as your digested your shuttle vector, and now you ligate this and this, and you get and then transfect them into the H E K 293 cells produce e 1 a, and finally, you get replicationed effective recombinant virus which expresses your gene of your interest, kits are available all that you have do is to buy these expression systems, and clone your gene in to those vectors, generate a recombinant virus and express what are protein you're interested in.

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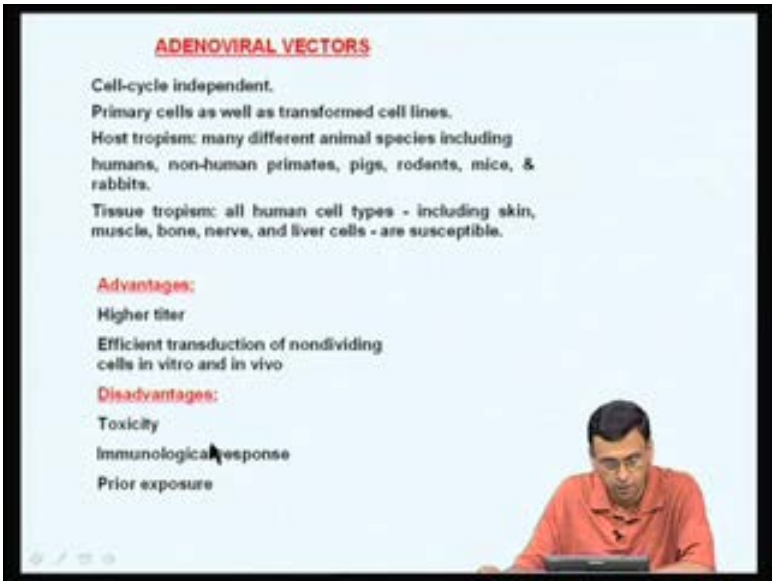

So, the adeno viral vectors have a number of advantages, they can infect in a cell cycle independent manner, they can infect primary cells as well as transferred cell lines. They have a wide broad cost tropism. They can infect many different mammalian species including humans, non-human primates, pigs, rodents, mice and rabbits, they also have a broad tissue tropism. They can infect virtually any type of human cells, such as skin, muscle, bone, nerve, liver, cells etcetera. So, once you have a recombinant viral vector, recombinant adenovirus, you can actually infect a number of mammalian species as well as a number of mammalian tissues or cells with this kind of adenoviral vectors. The advantage this is you have now very good expression systems available commercially, so you can generate a very high titer of the adenovirus. They are very efficiently transducer both dividing as well as non-dividing cells is very important, when you come to retro viruses retro viruses can only infect dividing cells, they cannot infect non-dividing cells, whereas the adenoviruses can infect both dividing as well as non-dividing cells, but there are certain disadvantages. We will discuss some of the disadvantages in the next class when we talk about human gene therapy.

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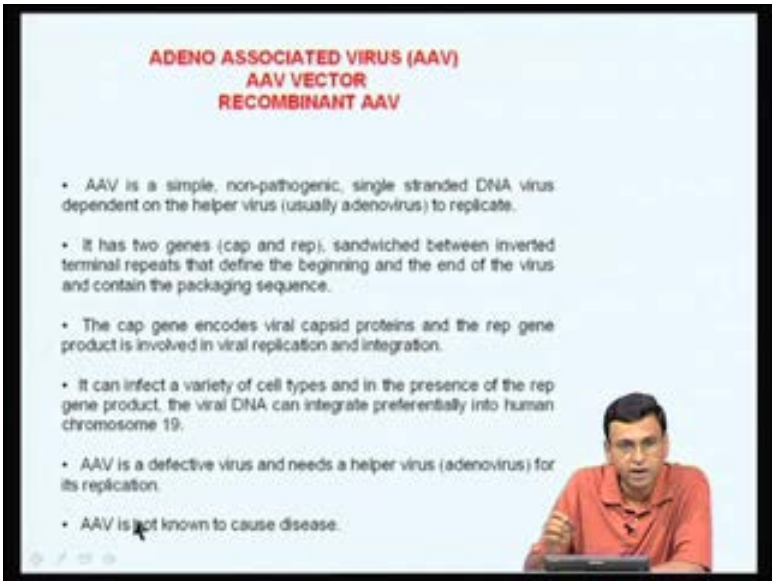

So, the major problem with the adenoviral vector system is that, it is a rather big genome almost 30 to 35 k b genome, and many adenoviral proteins still express in trace amounts when you put these adenovirus in to the mammalian cells, whereas transfecting mammalian cells is not a problem, when you take this recombinant adenovirus infect, use it for gene therapy purposes you get into lots of problems, we will discuss these problems in the next class when we discuss about gene therapy, but because of this problems associated with the adenoviral vectors, peoples went on to see, can you develop simpler expression systems, and one of the expression vector system that have developed is called as a adeno associated virus.

Now, adeno associated virus are A A V is a very simple non-pathogenic single standard D N A virus, dependent on the helper virus, usually adenovirus to replicate, since it is a very small genome, it contains a very few viral proteins, in order for adeno A A V to replicate mammalian cells. It also requires a co-infection by adenovirus, because the adenovirus actually provides some of the proteins requires for the A A V replication and transcription. So, A A V can successfully replicate and viral particles can come out of the mammalian cells only when it is co infected with adenovirus. The two major genes of the A A V genome are called as cap and rep, these are sandwiched between inverted repeats that define the beginning and end of the virus and contains a packaging sequence.

So, the viral genome contains what is called a packaging sequence, and codes for two major proteins called as cap and rep. The cap gene encodes viral capsid proteins, and the rep gene product involved in viral replication and integration of the A A V virus in to host genome. The adenovirus remains extra chromosomal remains as a episome, whereas the A A V actually goes and integrates in to the host chromosome. In fact the A A V can infect verity of cell types and in the presence of the rep gene product; the viral in D N A can integrate very preferential into human chromosome 19. This was one of the biggest advantages of using adeno associated virus as a gene delivery vehicle, because when you infect a recombinant adenovirus adeno associated virus in to mammalian cell in presence of the rep protein, A A V goes and integrate at a specific region in chromosome 19, it does not go and randomly integrate. It goes and integrates a specific region in the chromosome 19 which has very important advantages, in the next class when we discuss gene therapy. So, A A V is a defective virus and needs a helper virus for its replication, and so for A A V does not cause any human disease.

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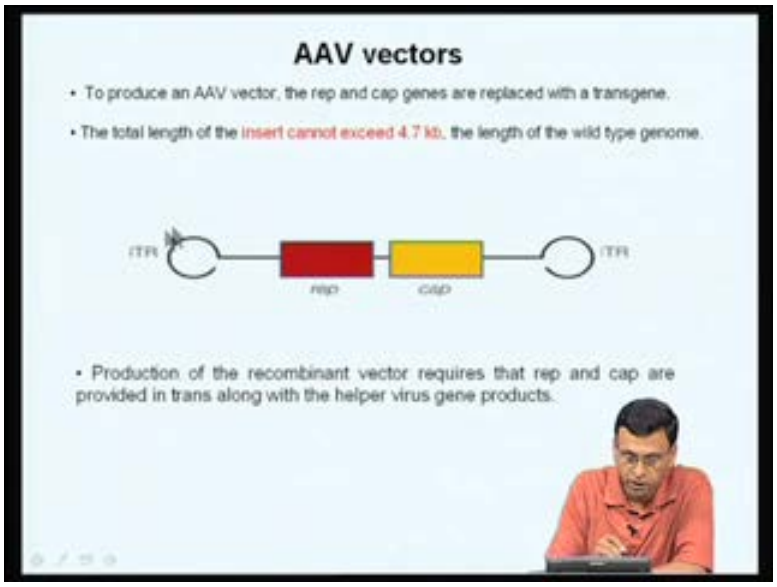

So, this is the general strategy for generating A A V vector, so to produce an A A V vector, the rep and cap genes are replaced with your transgene that is what I have shown here. You have the inverted repeats of the A A V genome, and you have the tube genes coding for rep and cap all that you have to do is, introduce your gene of your interest in the case of the rep and cap.

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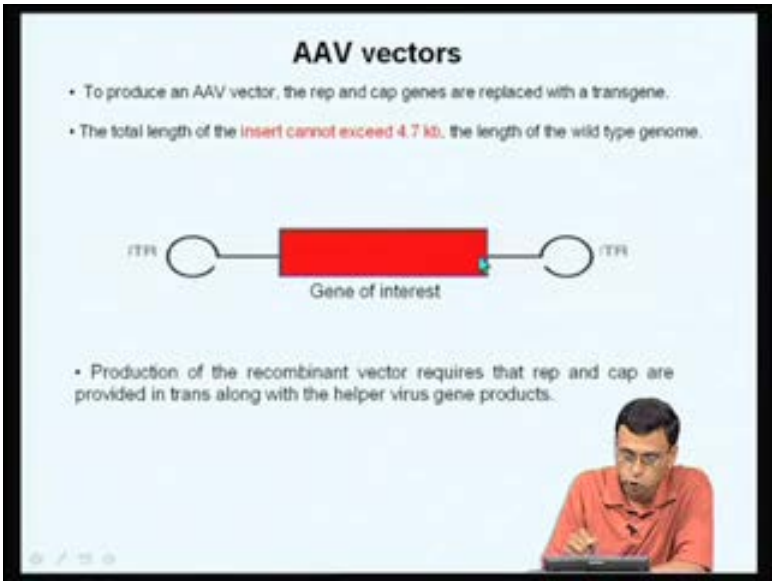

So, you have generated recombinant A A V vector, so you take this recombinant A A V vector and again put it in a packaging cell line which this now producing rep and cap, and along with the adenoviral protein you can successfully generate a recombinant adeno associated virus.

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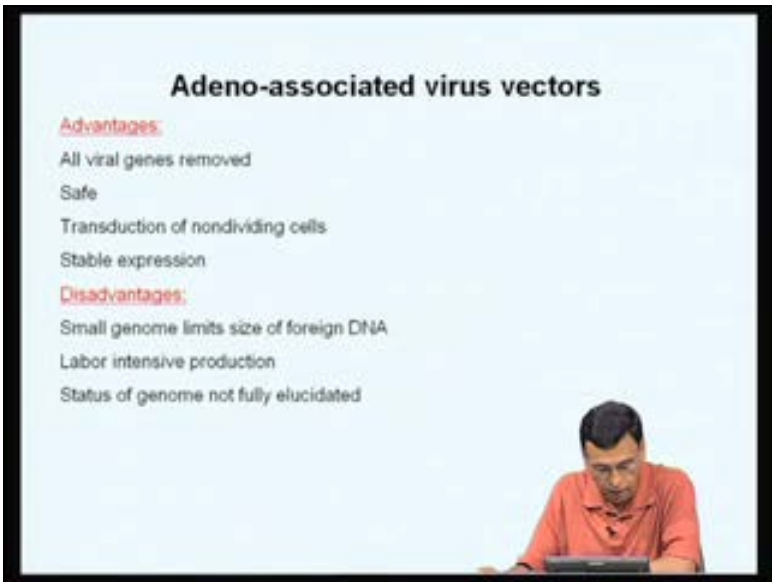

The adeno associated viral vectors have number of advantages, because it does not have any viral genes in almost all the viral genes have been removed. They are very safe, because they do not produced any disease. They can also divide just like adenovirus they

can also transfect non-dividing cells, they since the adeno associated virus goes and integrates specifically chromosome 19 in human cells, they have an advantage, because once you transfect, and cell line with adeno associated virus. The progeny will also carry a copy of the A A V gene. So, when the cells divide the daughter cells also carry adeno genome, so the daughter cells also will start expressing your gene of your interest where in the case of adino virus, since it is an extra chromosomal.

When cells divide all the cells will not inherit the recombinant adenovirus genome, so in the process you will lose (( )) only a small population will start expressing, the disadvantages of A A V is that, since the genome is very small, the amount of transgene you can put is very small, as you can say you cannot put more than 4 point 7 k b of the transgene, that is the maximum you can clone in to a A A V vector. If you have a gene of your interest bigger than 5 k b you cannot use A A V as your vector of your choice for introducing genes, it is producing A A V is more labor intensive and still the A A V mechanism of expression, life cycle of A A V is not very well understood.

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So having discussed the adenovirus and adeno associated virus, let us spend some time to see another very important D N A virus vaccinia virus, how vaccinia virus has been used for expressing genes in mammalian cells, how do you generate a recombinant vaccinia viruses expression, vector and recombinant vaccinia virus as well.

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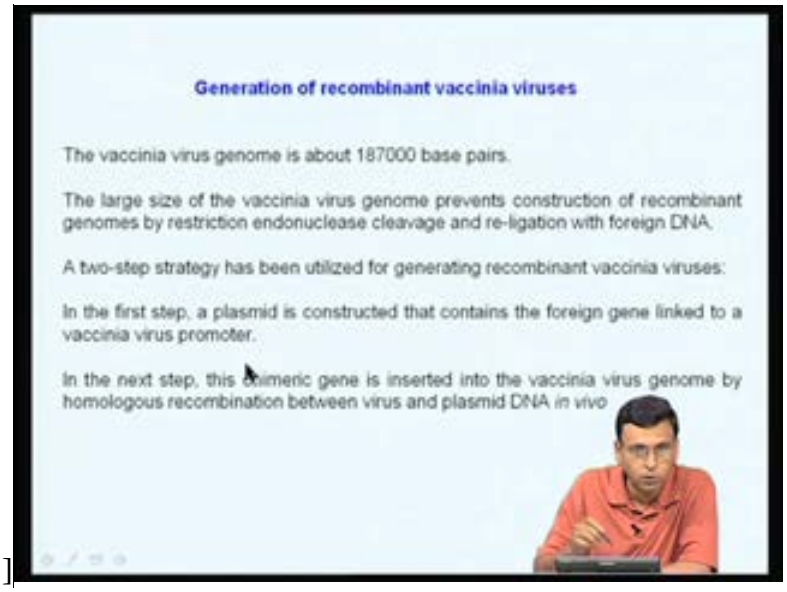

Now, what is a vaccinia virus, vaccinia virus is a huge virus, it has a genome of almost hundred and one lakh eighty seven thousand base pairs, the large size of the vaccinia virus genome prevents the construction of recombinant genomes by restriction nucleus cleavage and re ligation of the forging D N A, since the genome the retro viral genomes are very small, but the vaccinia virus genome is too huge, so you cannot take this D N A simply cuts some region of the viral genome and put your gene of your interest, you need to have a different mechanism. So, a two step strategy has been utilized for generating recombinant vaccinia viruses. The first step a plasmid is constructed that contains a foreign gene linked to a vaccinia virus promoter, and the second step this chimeric genes is inverted to vaccinia virus genome, by homologous recombination between the virus and the plasmid D N A in vivo. Let's, now try to understand a schematic representation how actually you generate a recombinant vaccinia virus.

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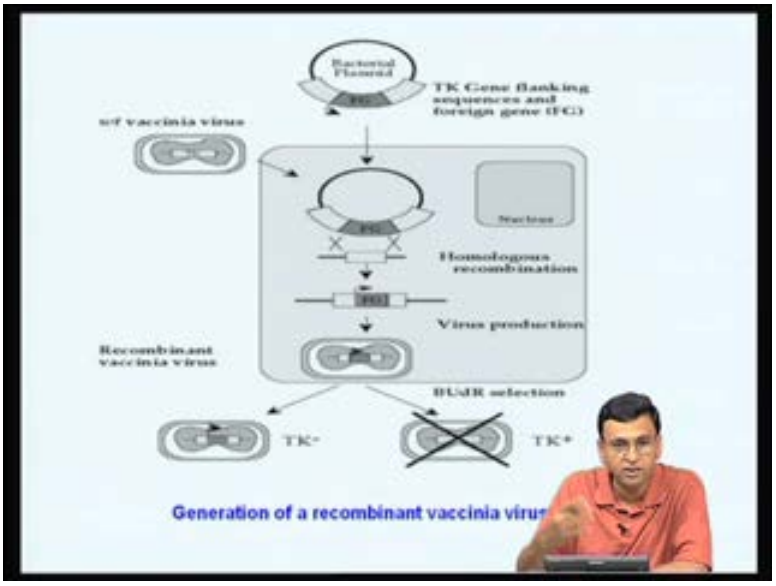

So, what you do here, first step in generating a recombinant vaccinia virus is, you clone your gene of your interest on either side of a thymidine kinase gene. This thymidine kinase gene is actually present in a vaccinia virus which is normal. So, the normal vilta vaccinia virus contains thymidine kinase gene. Now, you generate a bacterial plasmid in which this tymidine kinase is gene disrupted by your gene of your interest. Now, you take cells which are express in vactinia vaccinia viruses that is we take a mammalian cell of your interest, transfer this plasmid and also infect the wild type vaccinia virus, what happens, because of the homologous recombination between the thymidine kinase regions in the vector under thymidine kinase gene present on the chromosome of the vaccinia virus, homologous recombination takes place and you now generate a virus which now contains a thymidine kinase gene disrupted by your gene of your interest. When thymidine kinase gene is disrupted then such cells will do not express thymidine kinase gene, and therefore they are resistance to a compound called B U D R or compound called ganciclovir, they can survive, where as the wild type vaccinia viruses which are expressing thymidine kinase gene, they become sensitive to a compounds like ganciclovir or B U D R.

Therefore all the vaccinia viruses emerging out of this cell line, because they produce thymidine kinase they all die, where as the recombinant viruses in which the thymidine kinase is disrupted, because they cannot produce thymidine kinase. They can efficiently grow on medium cells containing, this kind of a selection markers and therefore, you can

selectively grow only recombinant adenoviruses in which the thymidine kinase is disrupted, whereas the wild type viruses emerging out of this cells which the thymidine kinase is interactive, they all die. So, you can thus generate recombinant vaccinia viruses. One of the major advances that took place in the vaccinia expression system is generation of what are called as a non-replicating vaccinia virus.

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Specially strain, particular strain called as a modified vaccinia ankara or M V A, the problem with vaccinia is that many vaccinia virus produces number of diseases; like small pox, canary pox and so on and so forth, they are all known to produce number of diseases in humans and animals. So, what was required actually to you use this viruses safely in human cells and human systems is, to generate a virus that is defective and does not cause disease, what happen the M V A strain was actually discovered which is nothing, but it is derived for the wild type vaccinia virus strain Ankara, by over 5 hundred an seventy serial passages of the wild type virus in chicken embryo fibroblast. So, if you kept on passaging the virus in chicken embryo fibroblast over a period of time it lost at the certain region of genome, especially about thirty one kilo bases of the D N A got lost from this vaccinia virus genome.

Therefore you got a new strain of virus called as the M V A in which, because of the loss of this region of genome, it became non virulent or a virulent. So, the M V A strain lost the capacity to productively infect mammalian cells and as 6 major deletion of D N A

totaling about 31000 base pairs including 2 host range genes. So, this M V A vectors does not efficiently infect mammalian cells as a result M V A is severely host restricted, it grows well in avain cells, but not in human and most of the mammalian cells. So, this generation of this M V A vector by Burny mass lab in national (( )) states made a huge difference and a number of laboratory now started using this M V A strain of vaccinia for transfecting mammalian cells, and using this as a recombinant viruses for generating vaccinia based expression systems. Now, I will not go in to more details of vaccinia based expression systems, we have another lecture called as vaccines and recombinant vaccines and in those lecture series we will discuss, how this kind of a viral based expressions systems are being used for producing the recombinant vaccines, we will discuss vaccine in detail in that particular lecture.

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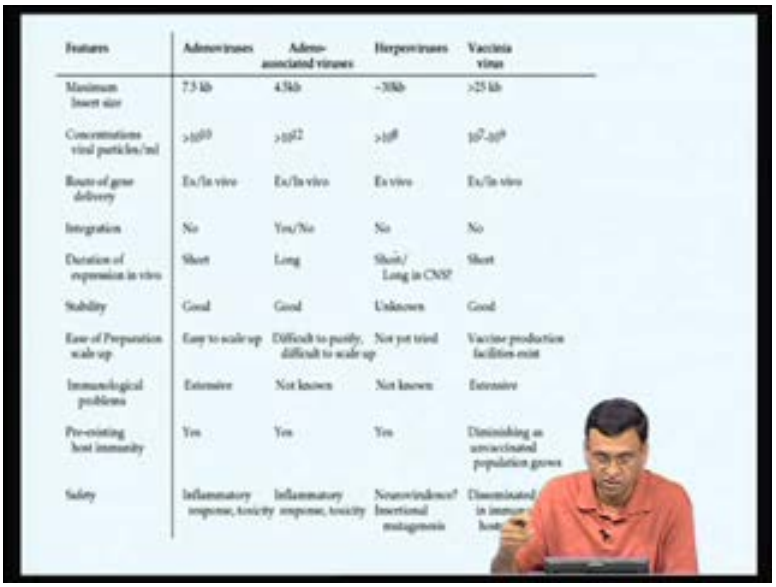

So, I just gave you 3 examples of how D N A viruses can be used for transaction of mammalian cells, and for expressing your gene of interest each one of them has its own advantage and disadvantage, this table here actually list some of the most commonly used D N A viral based expression systems, the adenovirus base adeno associate virus base, I did not discussed herpes virus based vectors, they are also being used, and also the vaccinia, and each one of them have many advantageous and many disadvantageous and one can go through this table and see what kind of a advantageous and drawbacks are there in various expression systems.

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Let us now switch gears and see, how R N A viruses can be used for as gene transfer vehicles and expressing genes of your interesting mammalian cells, we will restrict to our size to one group of R N A viruses namely the retroviruses. How retroviruses are being used as a gene transfer vehicles, and how you can clone your introduced gene retrovirus genome, generate a recombinant repair retrovirus and infect mammalian cells so that, these cells now produce your protein of your interest.

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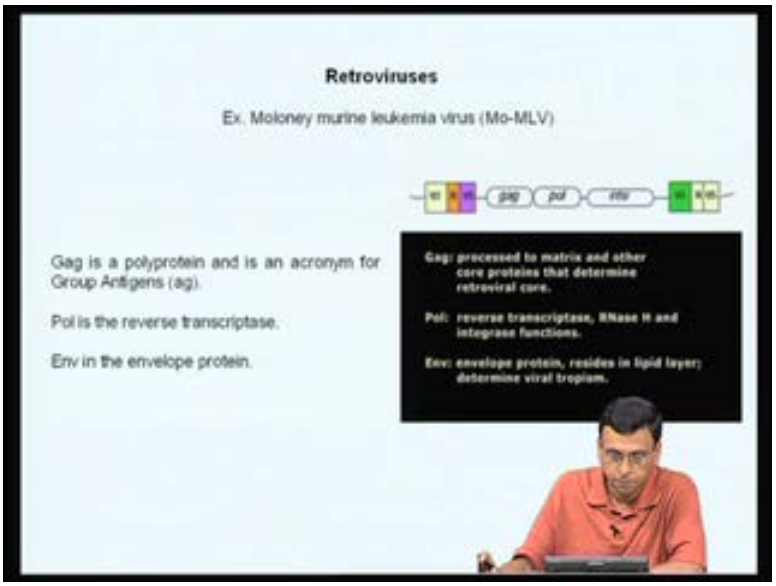

The simplest form of retrovirus is called as a Maloney marine leukemia virus, or M o M L V, this is the 1 of the most successfully used retro viruses for generating retro virus based vectors systems, if we look at the genome of this M o M L V. It contains what is called as a 5 prime l t r and a 3 prime l t r, l t r stands for long terminal repeat, the 5 prime l t r actually contains the promoters and enhancers required for the transcription of the viral genes, there are 3 viral genes called gag, pol and envelope. The 3 prime l t r, actually contains signals for poly relation for the viral R N A and so on and so forth, for the processing of the R N A, the 3 structural genes the gag is actually a poly protein which acronym for group antigens. The pol is actually reversed transcriptives and envelope is the envelope protein.

So, the gag is processed to matrix and other code proteins that determine the retro viral code. This forms the surface proteins of the retro virus, the pol actually contains reverse transcriptase R N As such as well as and integrates, because retro viruses once the infect mammalian cells. The retroviral genome goes and integrates the host chromosome, for which you require integrates function of the pol polymerase the envelope protein actually resides the lipid by layer and actually determines the viral proper some. So, depending upon what kind of envelope protein is being responses retroviruses, some of them in euranic cells some of them in only human cells and so on and so forth. So that kind of envelope protein express on the wild surface determines what kind of cells it actually infects or what was called as the determines the trapezium of the virus. Now, let us see with this kind of a simple retroviral structure, this is how the retroviral genome looks, how the retroviral when this with a retroviral genome infects what is the life cycle of a retrovirus.

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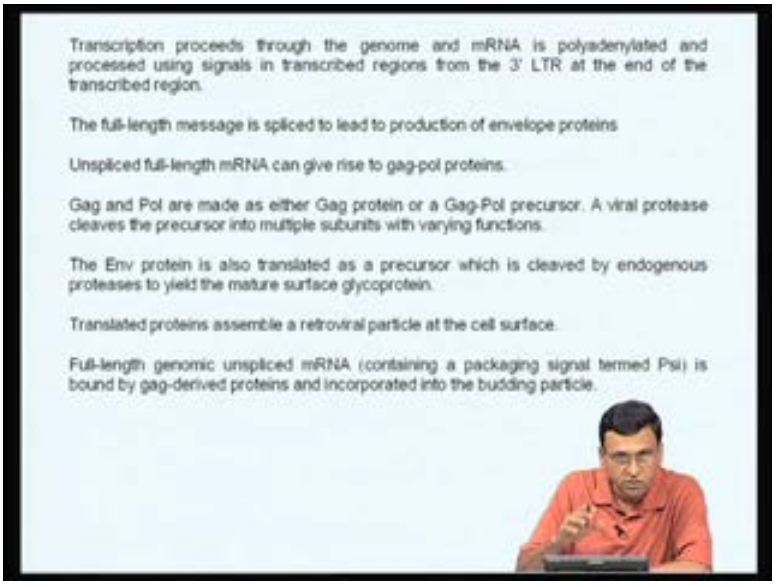

So once the retrovirus infects mammalian cell, this retroviral genome which we just now described now you just pushed inside the cell, and transcription proceed through the genome, the R N A, because as an R N A is the genetic material of this retroviruses, the full length message, the R N A is transcribed and the full length message is spliced to lead to production of the viral protein, the un spliced full length m R N A gives rise to gag and pol proteins, the gag pol proteins are either made as gag protein or the gag pol precursor, and a viral protease cleaves the precursor in to multiples of subunits with varying functions, the envelope protein is also translated as a precursor and then is cleaved by endogenous proteases to get a mature surface glycoprotein, and these translated proteins. Once all this viral proteins are made they assemble in to a retroviral particle at the cell surface of the infected cell.

The full length genomic unspliced R N A containing a packaging signal termed as psi, is bound by the gag derived proteins and incorporated into the budding viral particle, this is generally the life cycle of a normal retrovirus, so once the genome is pushed inside the cell, once the retrovirus infects the mammalian cell the genome go inside, the R N A is reverse transcribed and gets integrates the genome, and then the transcription proceeds viral protease of synthesize, and then the unspliced viral R N A is assembled along the viral protein, and the virus buds off from the surface of the infected cell.

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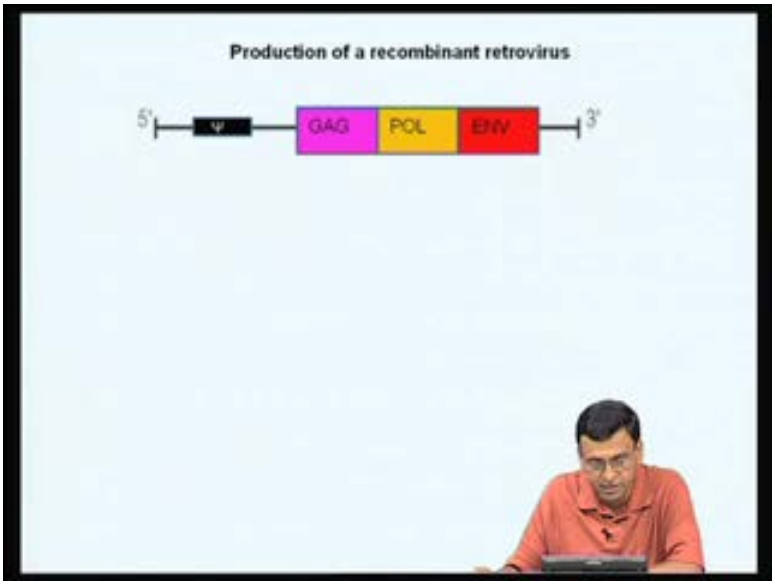

Now, let us see how this retroviral vector, as I said generation of a recombinant viruses has two steps, one is the generation of a recombinant vector, and then its generation of a recombinant virus, let see how you can generate a recombinant retrovirus, as I said the key components of a retroviral genome is what is called as a psi or the packaging signal, without this packaging signal, the genome will not be packaged in to a Varian, in to the viral particle and viral particle will not come out, so this packaging signal is called psi is very essential for the packaging of the viral genome into the virus capsid, the 3 important structural genes of a recombinant virus are gag, pol and envelope, what you do in generating a recombinant retroviral vector is, you now replace this gag, pol and envelope genes with your gene of your interest.

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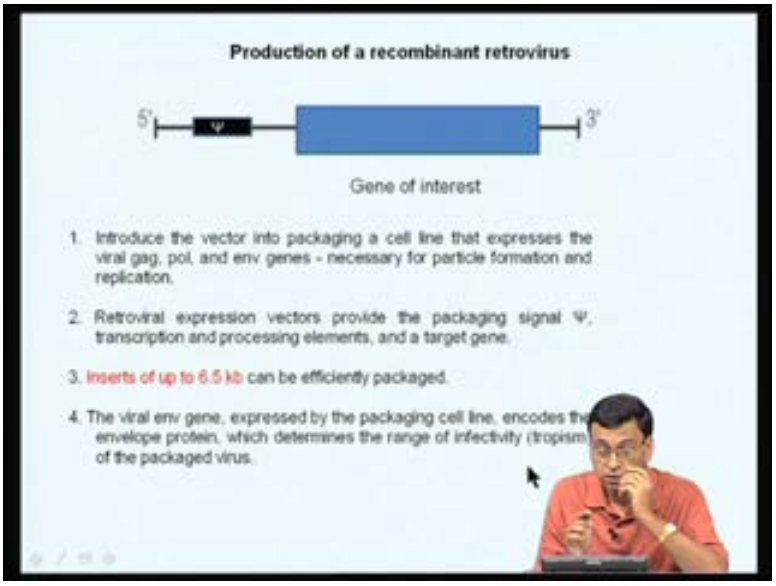

So, you now have a recombinant retroviral vector again I by putting a antibiotic marker gene and equally origin of replication you can actually clone this recombinant D N A into equalized cells, and make large amounts of this recombinant retroviral vector, so once you've made a large amounts of recombinant retroviral vector, you then introduce this vector into a packaging cell line that expresses the virus gag, pol and envelope genes. So, the retroviral vectors provide the packaging signal psi, transcription processing elements and a target gene, that is gene of your interest, and since the packaging cell line is not producing all the structural proteins, your viral recombinant genome will now be assembled, and from this cell line infectious retroviral particles actually can be produced.

So, using this kind of a retroviral expression system, up to 6 point 5 k b genes can be clone into this retroviral vectors, and the viral envelope gene express with a packaging cell line, codes for the envelope protein which determines the range of the infectivity or the tropism, this I have already mentioned, so this is how you generally generate a recombinant retrovirus, you first clone your gene of interest recombinant retroviral vector in which the gag, pol and envelope proteins are not made, and once you made sufficient amount of recombinant retroviral vector in electrode coli system, you now transfect a packaging cell line, which is actually producing this retroviral proteins namely the gag, pol and envelope, and now your recombinant retroviral genome will now be packaged, R N A will be packaged inside this viral particles, and you can

generate a replication defective retrovirus coming out of this packaging cells. Now, what are the major concerns when they developed this recombinant retrovirus is, the chance of a wild type virus emerging out of your packaging cell line, let see what is this problem.

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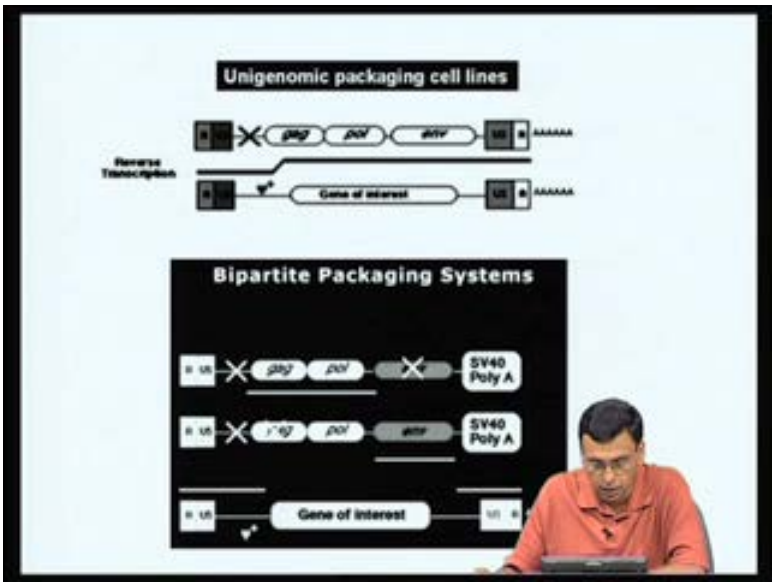

As I told, you have a packaging cell line in which the 3 structural proteins of the recombinant retrovirus is being expressed from a promoter in a packaging cell line. Now, into such a cell line you have now introduced the same l t r's, but instead of gag, pol envelope you have your gene of interest, which contains a psi signals. Now, by a single recombination since both this genomes are in the same cell, and there is a homology between the l t r's, by single recombinant event you can actually generate a wild type virus, although recombination is a rare event among let us say million viral particle that is coming out of this packaging cells, 1 or 2 may be a wild type virus.

So, there is a chance that by a single recombination event, you can actually generate a wild type retrovirus instead of a recombinant replication defective retrovirus, this is very dangerous, especially if you want to use this kind of a recombinant retrovirus for human gene therapy applications, which will discussed in next class. So, to prevent this kind of a problem, this kinds of a cell lines in which all the viral proteins are being expressed from a single construct are known as the unigenomic packaging cell lines, from this people went on to developing what are called as the biparted packaging systems, where in you produce all the viral proteins not on one single vector, but at two different vectors.

For example in this case the gag and pol, envelope is expressed from one particular construct and envelope is being produced from another construct. So, you have a packaging cell line in which gag and pol is produced by one viral l t r, envelope is produced by another l t r, and if you now introduce your construct recombinant retroviral into such a packaging cell line at this two recombinant events are required for generation of a wild type virus, which is much more rare then the single recombination events, so to develop much more safer vectors, people actually started replacing this viral l t r expressing this with mammalian promoters such as C M V And so on and so forth, so that there is no homology between the promoter regions and recombination will not be possible.

So, a number of safe guards have been developed in generating this kind of a recombinant replication defective retroviruses, and using this kind of safe features first generation, this unigenomic packaging cell lines are called as the first generation retro viral vectors, the bi parted is called as the second generation, and the third generation much more modifications were made into the packaging cell lines, like I said you replace a viral l t r with that of a mammalian promoter so on and so forth. So, a number of safety features have been incorporated, so that the chances of a wild type retrovirus emerging out of this packaging cell lines is very minimum, so they become very safe and such viruses can now be used not only for expressing genes and mammalian cells and culture, but also can be used successfully for human gene therapy.

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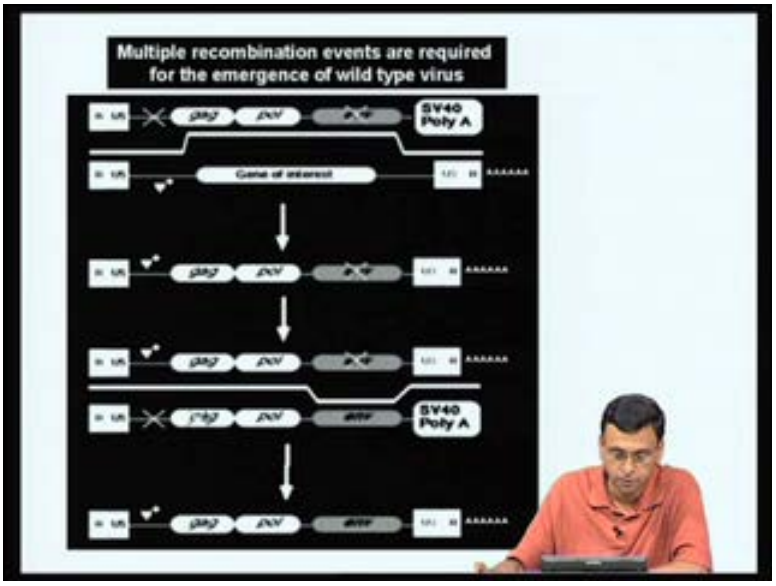

So, as I said multiple recombinant events are required for emergency the of wild type virus used this bicarbonate biparted vectors systems, the first case your gene the vector containing gene of interest as to now recombine with this particular construct, so that you will now have a viral genome expressing will be gag and pol, now that allow is not enough for emergence of virus, then again this as now recombined with a construct containing the envelope protein then only you will get a all the 3 viral proteins in a single viral genome, and then only the chance of a wild type virus emerging out is possible, so by using this kinds of a modified packaging cell lines, the chances of a wild type retrovirus emerging out cell lines was thoroughly minimized.

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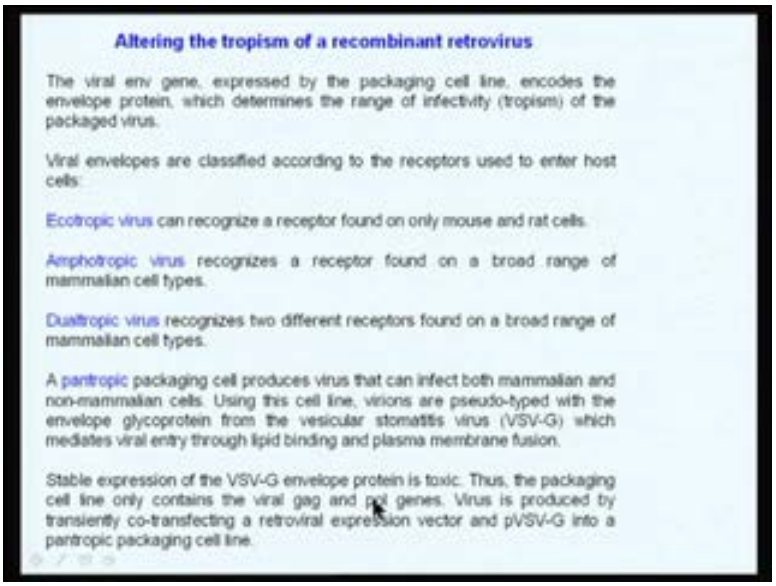

Another important advantage that took place is in the area of developing recombinant retroviral expression systems is, altering the tropism of the virus, I told you the envelope gene of the retrovirus determines what kind of a cell lines it goes and infects, so if you can alter suppose have a packaging cell line which is actually producing one kind of an envelope protein, now instead of producing envelope protein, If you now produce a different envelope protein and virus that is emerging out of that particular packaging cell line will have a different tropism. So, by changing the envelope protein which is being expressed in a packaging cell line, you can actually produce viruses which have different tropisms, so based on their tropisms viral envelopes are classified according to the receptor use to enter the host cells, ecotropic viruses can recognized a receptor found only on mouse and rat cells, that means these ecotropic retroviruses can only recognize

murine cells either mouse or rat, they cannot recognize human cells, so they will not infect human cells.

Whereas, amphotropic viruses, they recognize a receptors found on both mammalian cells that is both murine as well as human cells, so they can infect both human as well as murine cells, dual tropic viruses recognizes two different receptors found on a broad range of mammalian cell types, a pan tropic packaging cell line this is very important, this pan tropic pan tropic packaging cell line actually produce a virus that can infect both mammalian and non-mammalian cells, and using such cell lines virions are pseudo type with envelope glycoprotein from vesicular stomatitis virus or v s v glycoprotein, which mediates viral entry through lipid binding and plasma membrane fusion. So, you have now a packaging cell line which is now producing a envelope protein of different virus, namely the vesicular stomatitis virus. So, when you now introduce your recombinant retro viral vector into such packaging cells, the recombinant virus that is coming out will contain the v s v glycoprotein, and this v s v glycoprotein now go and infect a broad range of host cells, so actually alter the tropism of a retrovirus, this are actually called as pseudo typed vectors, so stay I think I will not go in to the details, a number of modifications were made sure to develop packaging cell lines expressing the v s v glycoprotein gene.

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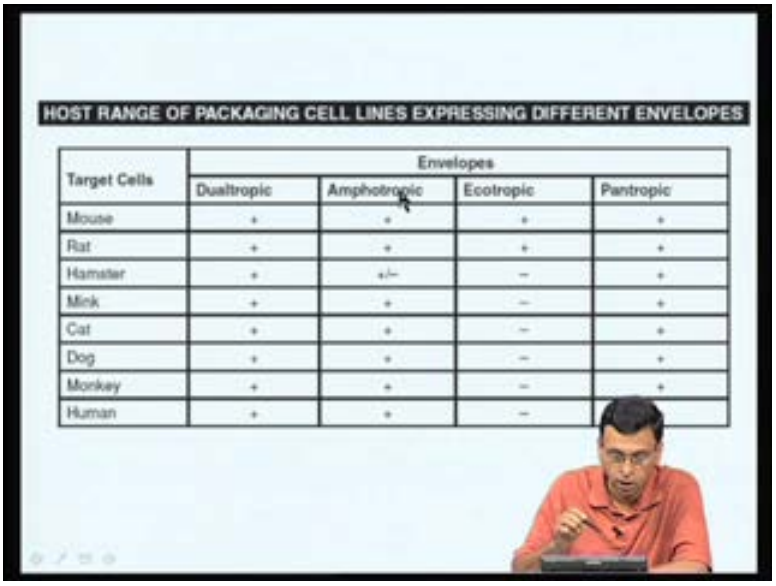

So, a number of packaging cell lines have been expressed, have been developed, each packaging cell line expressing different kinds of envelops, so depending upon what kind of envelope is being expressed by that particular cell line, you can either the generate a dual tropic virus amphotropic virus, ecotropic virus, pantropic virus, the differences between these tropisms are actually listed here, like for example ecotropic viruses can only infect the murine cells, they cannot infect human cells, whereas the amphotropic virus can infect both murine as well as human cells and so on and so forth.

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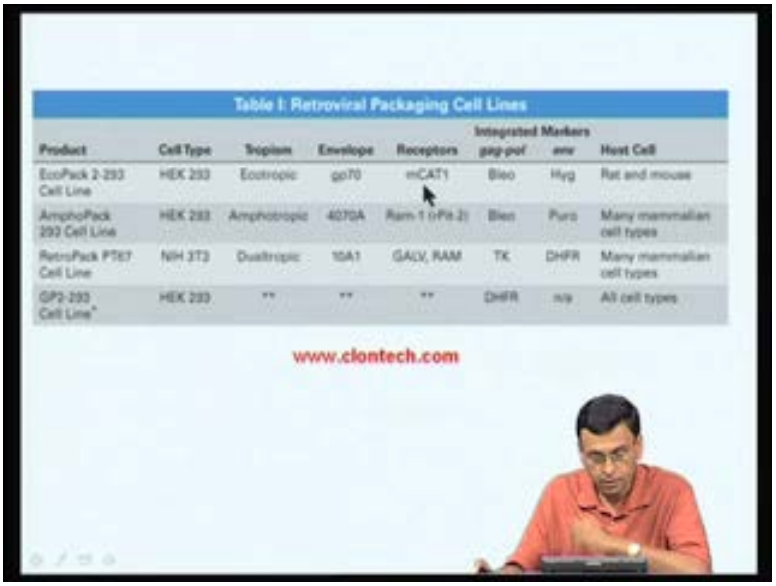

Again I just like I told you for the adenoviral systems or adenovirus expression systems, a number of companies also now selling these retroviral packaging cell lines as well as retro viral vectors, so if you want to now express your gene of interest using a recombinant retrovirus, all that you have to do you use to go to companies such as Clonetech, buy appropriate retroviral vector, and buy appropriate packaging cell line, clone your gene in to the retroviral vector, and generate a recombinant retrovirus of your choice, you can either generate by if you use an ecopack 2 to 9 3 cell line, you can generate a ecotropic retrovirus, if you use an amphopack cell line, you can use an amphotropic retrovirus cell line, and if you use this particular cell line you can use a dual tropic and so on and so forth, so you do not have to develop this cell lines these are all commercially available, all that you have to do is buy the right kind of systems that u want and generate a recombinant virus your choice.

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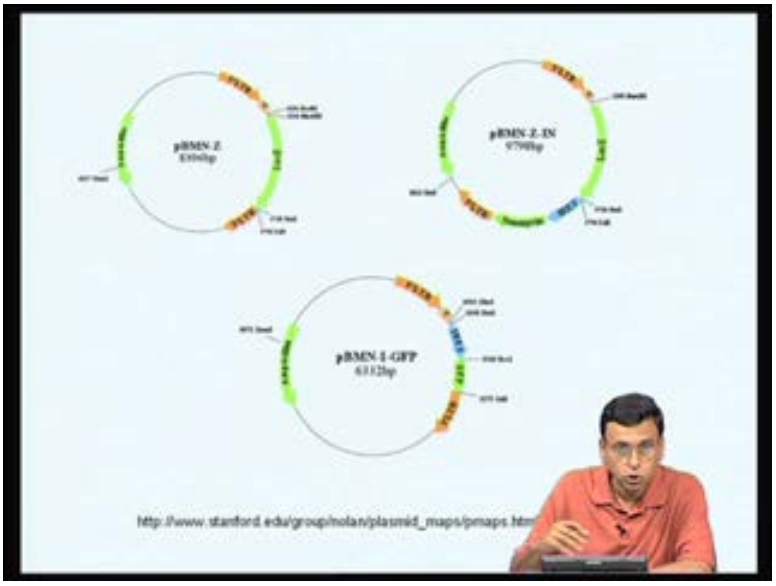

There are also number of laboratories across globe, who actually developed a number of this retro viral vector systems, for example Nolan slab in Stamford university have actually developed a number of retro viral vectors, for example here we have a retro viral vector containing the 5 primelty l t r per prime l t r with a tree primelty packaging signal expressed in the lacz, beta galactose gene, and here it not only express the lacto beta galactose gene, it also the internal ribosome intrasite containing a neomycin marker, so you can actually select cells expression the laczi gene by neomycin resistance, and you also have vectors, for example where you have a green florescent protein expressing from the internal ribosome intrasite, and so you can actually this retro viral transfer cells actually can floras, because they expressing (( )) say a number of such retroviral vectors systems either or commercial available from companies or you can actually buy from some of the investigators who actually construct this kind of a retroviral expression vectors.

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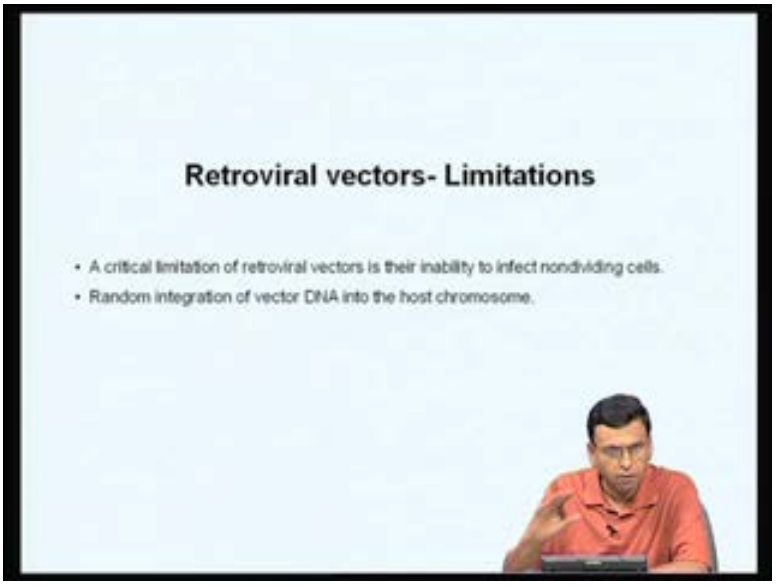

Some of the major limitations of retroviral expression system are, their inability in infect non dividing cells, retro viruses cannot infect non dividing cells, and another biggest problem is they randomly integrate to the host chromosome, they can go integrate anywhere in the host chromosome and when that happen strength, when it goes infects integrates in the oncogene or a tumor suppresser gene, it can lead to cancer so there are problems of retro viruses using especially in the case of human gene therapy.

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So to overcome some of the problems associated the retroviral vector systems, the lenti virus expression actually developed, one of the major advantage of the lenti viruses is that they can there are also retroviruses, but they can also infect non dividing cells, that is 1 of the major advantages of a lenti virus expression system, so lenti viruses are much more safer than the retro viruses.

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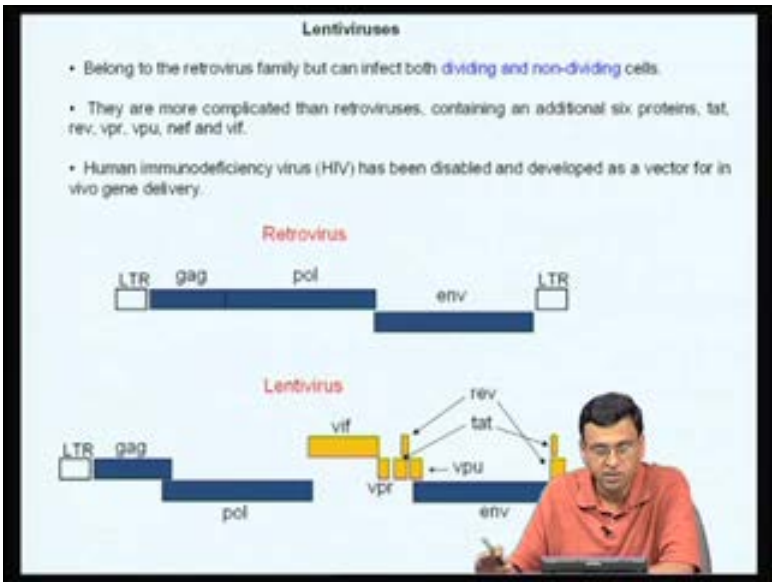

Now let us try to understand how lenti viruses can be used as gene transfer vehicles. The lenti viruses belong to the retro virus family, but they can infect both dividing and non-dividing cells, they are much more complicated in their organization, because they contain 6 additional proteins namely, r a v, v p r, v p u, n e f and v e f, I have shown here difference between a retro virus, as I said retro virus contains only 3 genes gag, pol and envelope, but lenti viruses in addition to gag, pol and envelope contains a number of other accessory genes, the best studied lenti virus is the H I V or the human immune deficiency virus. So, again using the same strategy, how can we generate antiviral recombinant replication defect antivirus, again you generate a lenti viral vector where you delete certain viral genes.

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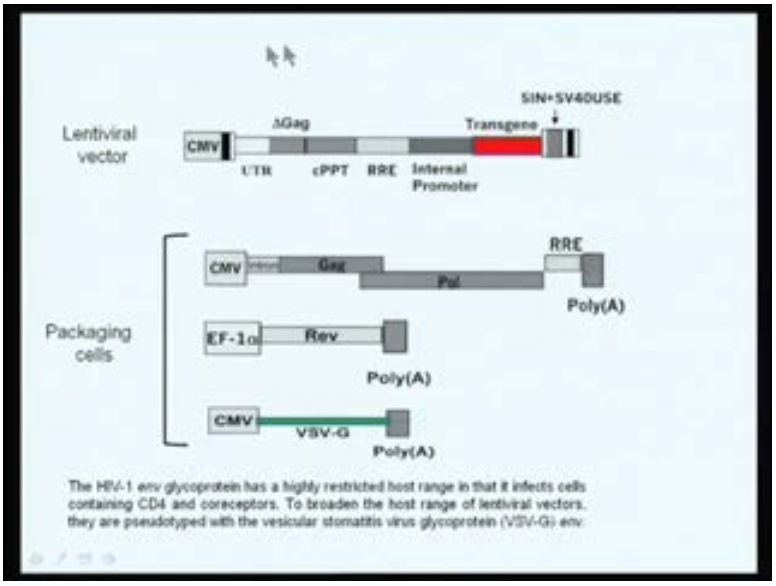

And introduce your transgene, and then you generate a packaging cells, now what is the difference between lenti viral packaging cell line and a retro viral packaging cell line. In a retro viral packaging cell line you have to express only three proteins gaga, pol and envelope, whereas in case of a lenti viral packaging cell line, you also have to express these additional proteins which are essential for the successful outcome of a lenti virus.

So, all the other lenti viral proteins are expressed by this packaging cell lines, usually all of them are not expressed in the same construct again to avoid the generation of wild type virus coming out, so they are expressed in two or three different constructs, so that chances of wild type virus emerging out is minimized.

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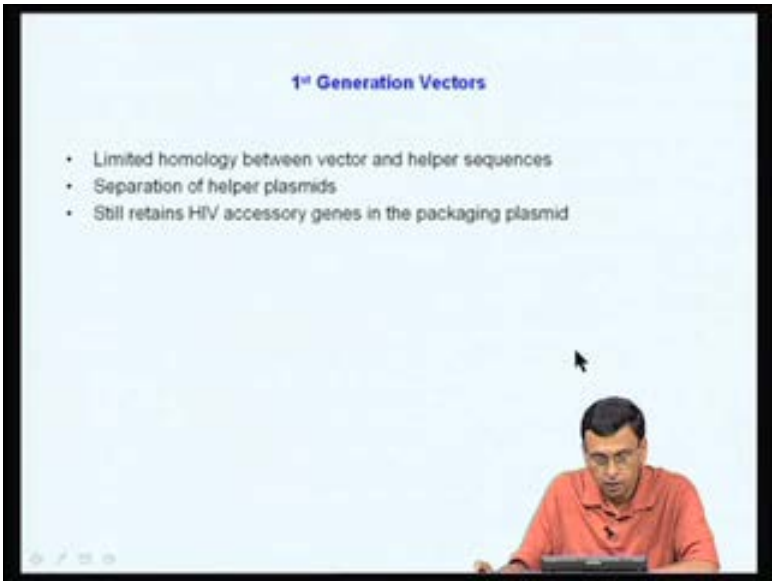

Again, just like the retro viral vector systems, a number of efforts have gone develop a very safe lenti viral expression systems, here what is called the first generation vectors where there is a limited homology between vector and helper sequences, separation of the helper plasmids, still accessory H I V accessory regions were still retained in a one single packaging plasmid, so the chance of a wild type H I v emerging was very high in the first generation vectors, in the second generation vectors, elimination of accessory genes from packaging plasmid, there is no effect on the viral vector titer, they retain the property transduction of many dividing and non-dividing cells, they are much more safer compared to the first generation vectors.

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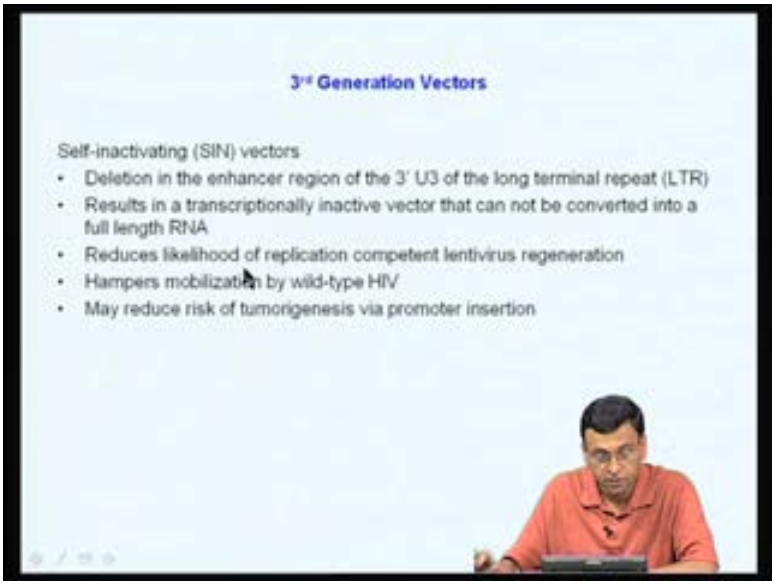

The third generation vectors are actually called as the self-inactivating vector or sin vectors, they had a deletion in the enhancer region of the 3 prime u t r, they results in a transcriptionally inactive vector that cannot be converted into full length R N A, so the chance of wild type virus emerging out of such packaging cells is completely minimized, so replication competent lenti viruses cannot come of out of such packaging cell lines, they hampers the mobilization by wild type H I V may reduce the risk of tumerogensis via promoter insertion. So, just like the retro viral cell lines, the number of developments took place to develop safe lenti viral packaging cell lines, so that safe lenti viruses can be produced from these packaging cells.

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Again just like adenoviral expression systems and retro viral expression systems, number of commercial lenti viral expression systems are available I have now given here, the again clone tech actually cells, many of these lenti viral expression systems like the you can actually generate a recombinant anti-virus, which can express, which can effect either mammalian either moraine cells or human cells, because they contain the v s v glycol protein as the envelope protein or you can also generate recombinant retro viruses which are ecotropic, that means they can effect that means they can infect only mouse or rat cells, so you can decide if you want to express your protein of your interest in a mouse cells line you generate a ecotropic cell line, if you want to express your protein both mouse as well as human you generate a amphotropic cell line using the appropriate packaging cells.

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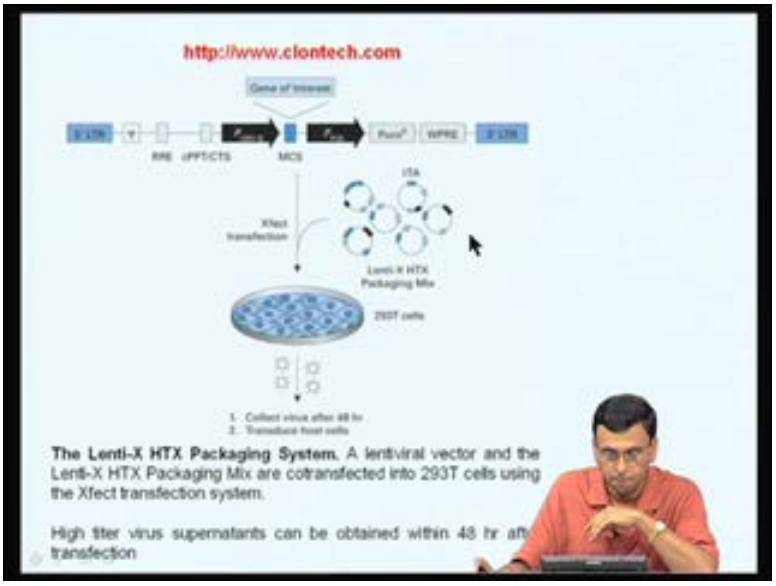

Complete details are available if you go to the company website, it actually tells you how to clone your gene of your interest, and how to transfect this packaging cells, so that you can generate the a recombinant lenti virus, all details manuals are available all that you have to do is clone your gene into vector system, and follow the protocol and you can generate a recombinant lenti virus of your choice.

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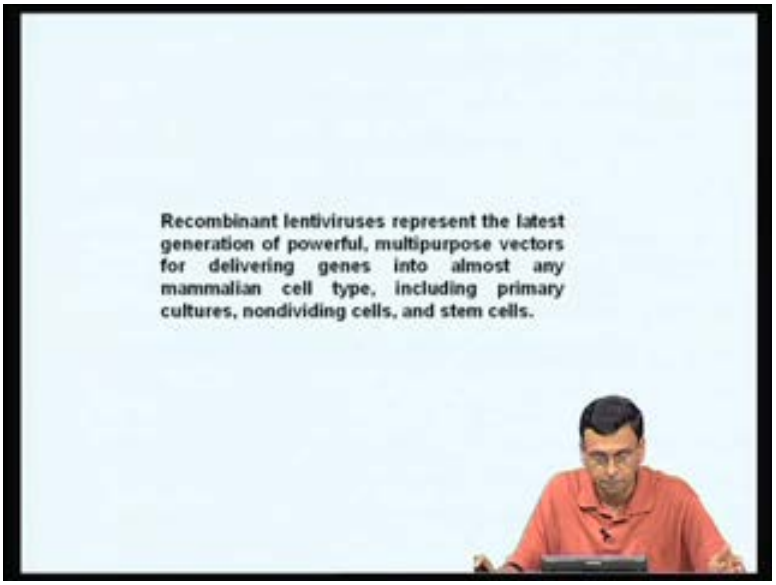

The recombinant lenti virus represents the latest generation of powerful multipurpose vectors for delivering genes into almost any mammalian cell types including primary

cultures, non-dividing cells and stem cells lenti, among all the expression systems we have studied so far the lenti virus have become the most popular, and most widely used mammalian expression systems as I speak today, and they are the most efficient of all the types that we have studied so far.

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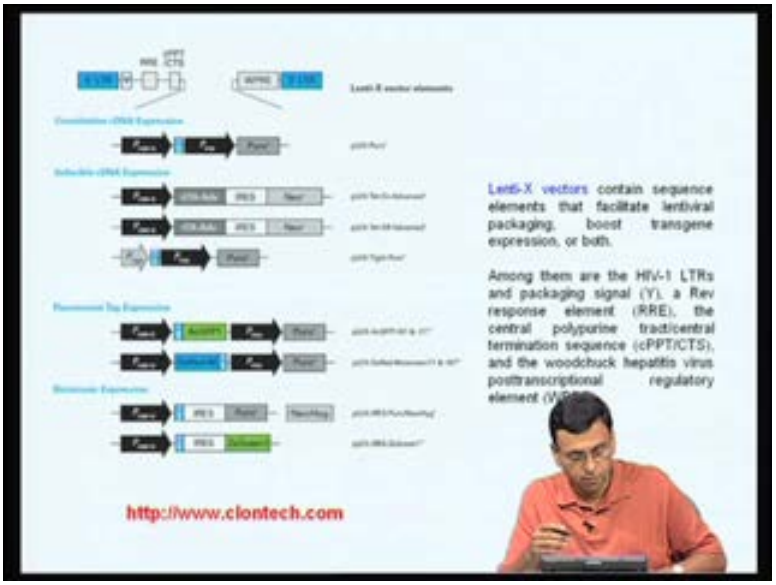

Again if you go to the company website a number of modified version of this lenti virus expression systems are available in many of this companies, you can either express your gene constitutively, you can either express your genes in inducible manner, using a appropriate induces tetracycline and so and so forth, you can have florescent tax attached to a transgenic, so that the cells which are expressing your genes will floras, because you're putting g f p, and you can also select for puromycin and so on and so forth, by systronic expression. A wide variety of lenti viral expression vectors are available from companies such as Clone tech, you can just buy and then do whatever you want.

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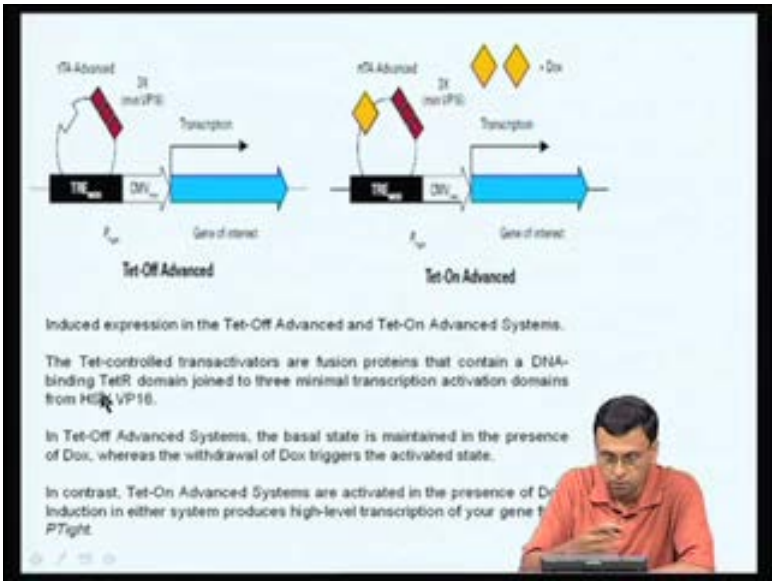

This is just another system that is sold by clone tech actually shows, how you can regulate your expression, once you generate this kind of a lenti viral vectors you actually regulate the expression of your transgene, by using molecules such as tetracycline. I will not go into the details of the system, one can just read and go through or go to the company website and look at it, these are all called as inducible lenti viral systems where the transgene expression can be induced by molecules such as tetracycline, because your gene is cloned under the tetracycline regulatable promoter.

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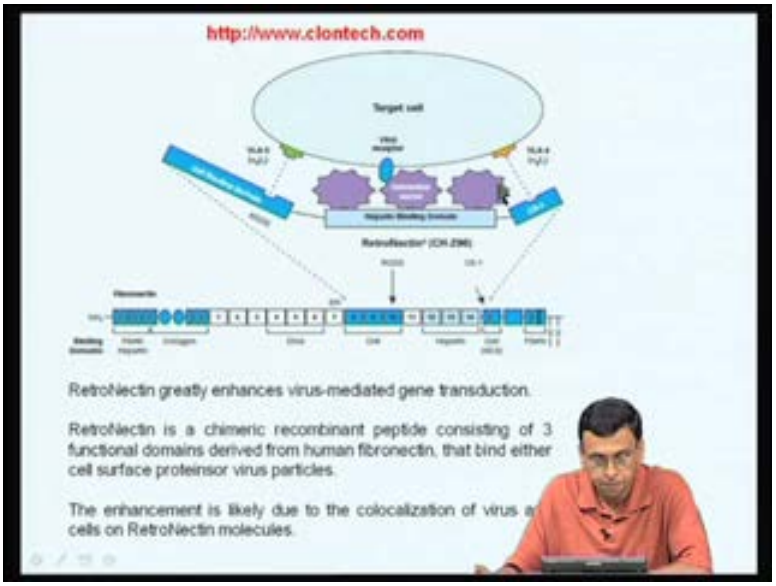

there are also molecules compounds retronectin which enhances the efficiency of transfection, so the retroviral vector, the retro virus vector can efficiently transfect the infect the cells, so that you get very high efficiency of transfection of a mammalian cells, so the retrovirus binds to a viral receptor enters, but if you have compounds like retronectin, the usually many of this mammalian cells also have a receptors for certain regions of this retronectin as well, so not only the virus will enter not only through the viral receptor, but since the virus this retrovirus viruses bind to this retronectin by multi by interacting multiple receptors efficiency of virus transfection is enhanced. So, large percentage (( )) with retroviruses if you include compounds such as retronectin, so the efficiency of gene transfection is thoroughly increased by using compounds like retronectin, again available from companies like Clone tech.

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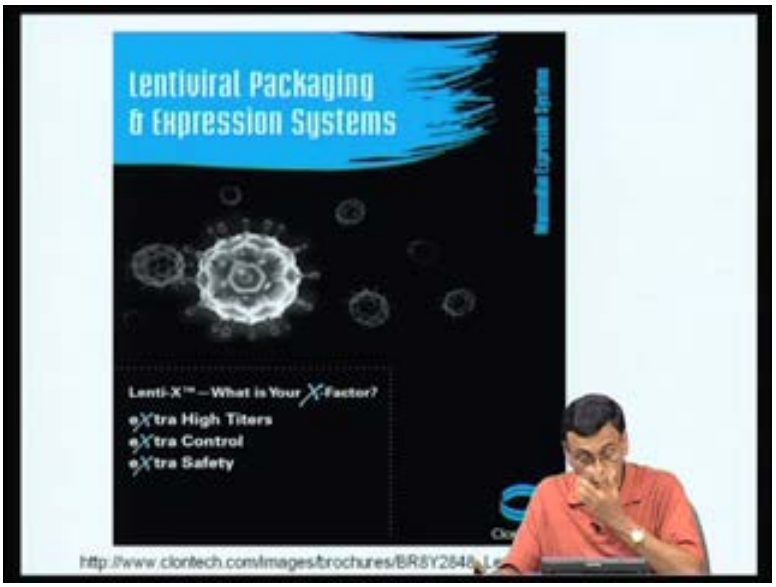

So I just given some of the very informative brochures and article that are available for you to study further, Clone tech again gives a very detailed manual on how you can use various lenti viral expressions available with them, how one goes about constructing this enti viral vectors and so on.

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Similarly, they also sell very interesting and very informative manual on retroviral gene transfer and expression one can read up little bit more, and try to understand how various retroviral vectors are being generated.

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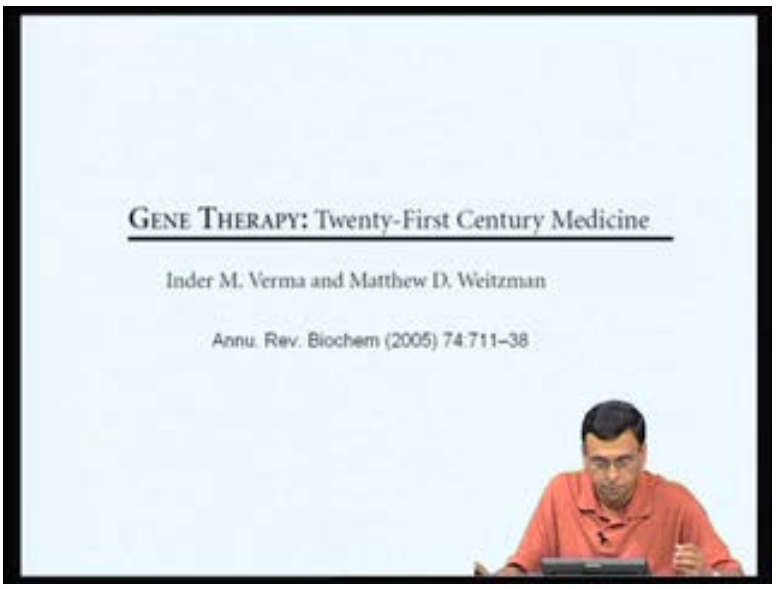

This is also very informative article in annual review of bio chemistry gene therapy twenty first century medicine, where it discuss the various viral vectors that are being generated, and how this viral vectors can actually be used for gene therapy, which is what we are going to discuss in the next class, I think I will stop here thank you.