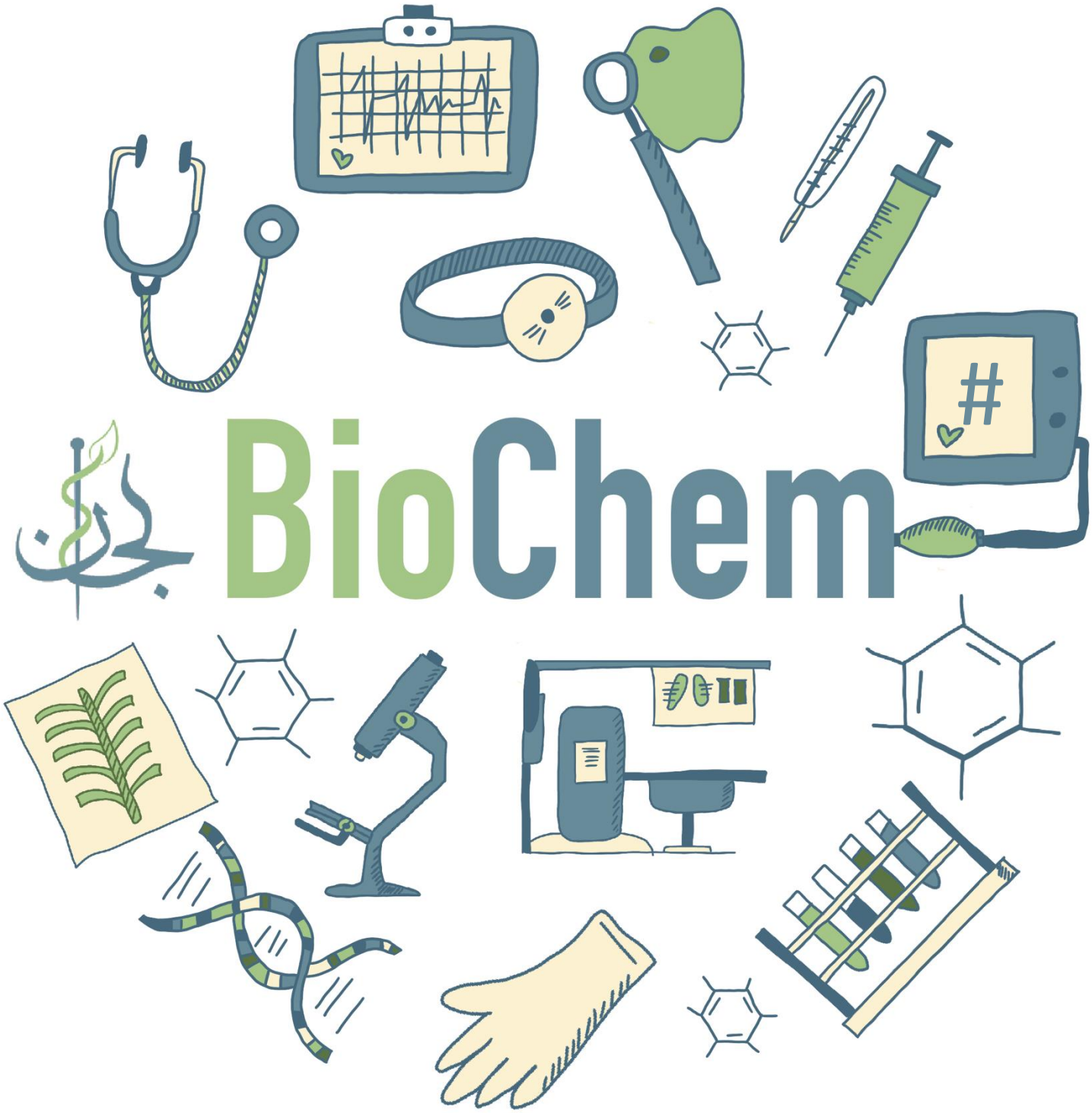
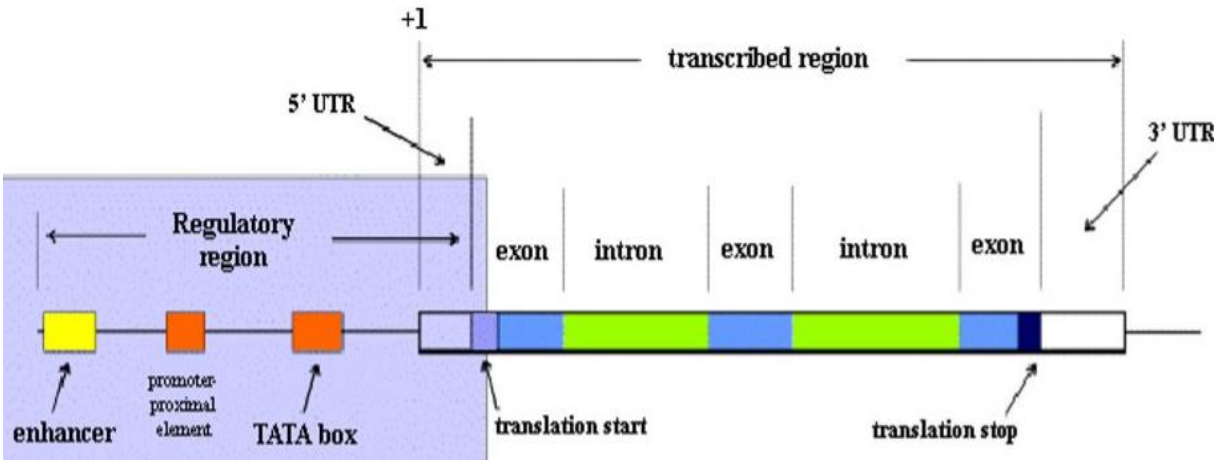


BioChem

Writer:	Salsabel aljwabreh
Science:	Naemah abuhantash
Grammar:	Naemah abuhantash
Doctor:	Mamoun ahram

In this lecture we will talk about (how we can use the recombinant DNA technology and recombinant DNA molecules to Analyse DNA regulatory sequences and to determine if two proteins interact with each other or not).

We will start with **Analysis of transcriptional regulatory sequences and we will talk about the role of enzymes specifically an enzyme that is called (luciferase).**



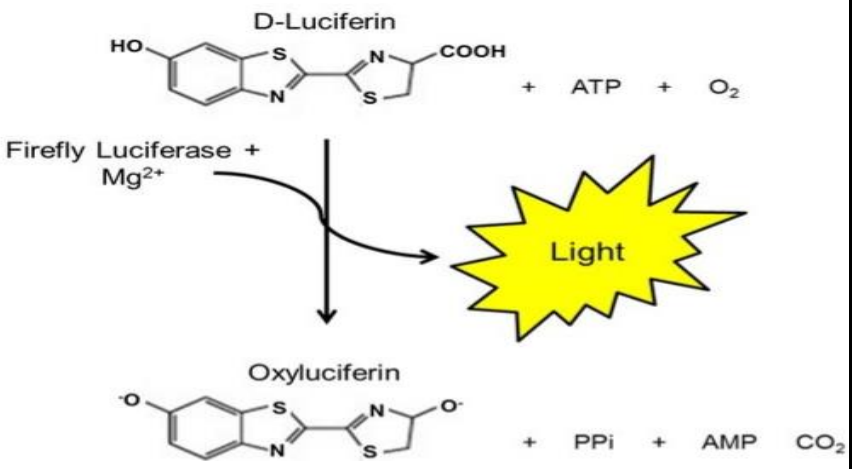
Basically, here we have eukaryotic gene composed of exons and introns, notice the transcriptional start side in red (it is different from the translation start side which is marked in the diagram by an arrow), and this gene is under the control of number of regulatory sequences, so it has the core promoter which is composed of TATA box (the -35 region) in green, and it has the promoter proximal element in blue, it might also have an enhancer (the yellow box), it might also have a silencer or a suppressor, so we can have activating regulatory sequences or inhibitory regulatory sequences.

We want to study the regulatory region (in light blue) rather than study the gene itself, we will concern about what regulatory sequences does a specific gene have.

We take advantage of an enzyme that is called the **luciferase** which was first isolated from the firefly .

There is a light coming out from the end of the firefly body (it is fluorescence) and the scientist were able to determine how firefly fluoresces) the story is:

There is a molecule that is called **luciferin** and this molecule will be converted into **oxyluciferin** by an enzyme called **luciferase** and this enzyme is monooxygenase so it is an oxidoreductase (it puts one atom of oxygen in a molecule to produce the oxyluciferin and another oxygen atom in another molecule to produce the carbon dioxide) the idea is when the luciferase catalyses the reaction the light will be released, light will come out and the firefly will fluoresce.

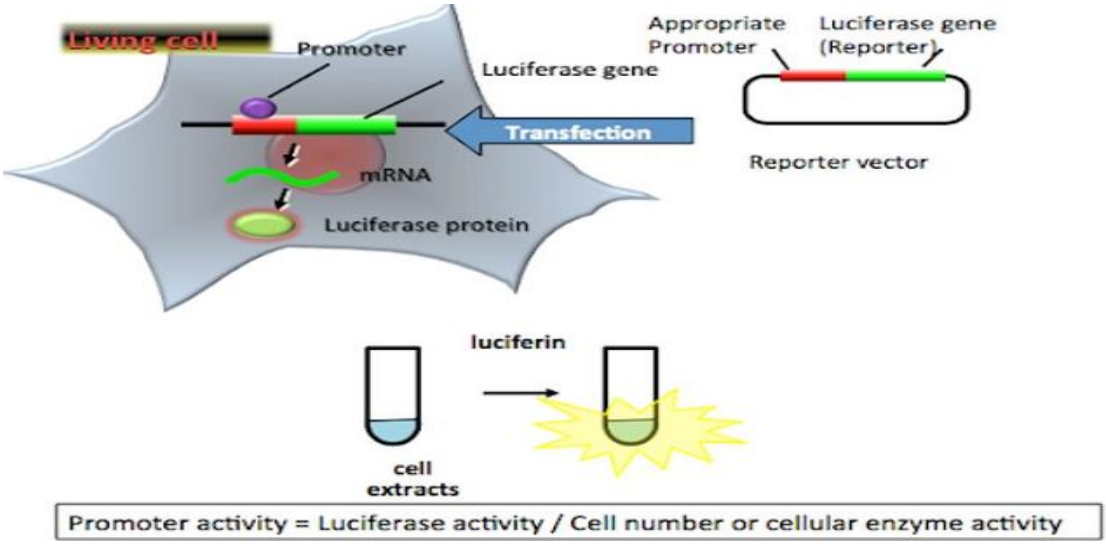


The scientists were able to take advantage of this enzyme: the idea here is The Luciferase Assay (assay means something like experiment or steps that we use to experiment something or to measure something) so the idea here that we want to **analyse the activity of a specific gene**, we want to study the regulatory sequences we don't care about the gene itself rather we care about the promoter region or the regulatory sequences, so what we do is making an expression vector (we engineer an expression vector) and we insert the luciferase gene inside it, except that this gene will be under the control of a promoter region of another gene that we want to analyse

***maybe you are confused about this piece of information so the doctor said this following sentence*:**

"really this gene (he meant the gene that owns the promoter region) and the transcriptional factors that bind to the promoter region don't care what the gene (he meant the luciferase gene) they are transcribing, all they care about that they bind to a promoter and start transcription" **

Reread this important sentence to understand the whole idea **

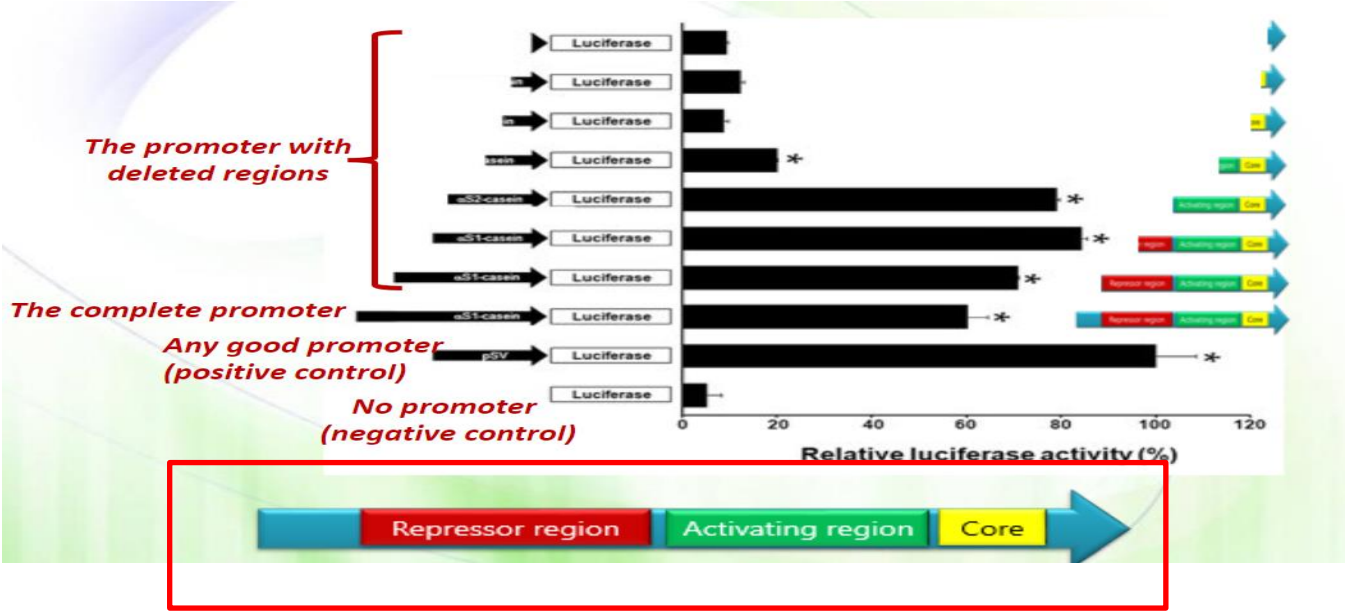


The expression vector (in the red), we inserted it into a cell, so this cell would have a plasmid inside it (this can be a human cell, it isn't necessary to be a bacterial cell…. cells don't care, all they care about that there is something that looks like the DNA -plasmid- that regulatory protein can bind to it -to the elements-).

So the plasmid is inside the cell and we want to determine if a specific gene is active or not **(againnnnn!!!! we don't care about the gene, we care about the regulatory sequences)** so we look here if the **regulatory sequences** under positive regulation that they have transcriptional domain on (we mean turned on) , or under negative regulation, so after inserting the plasmid, if you have positive regulation you should have the production of luciferase protein or enzyme and when we add luciferin to our sample, if we have a lot of luciferase that meant that there will be a signal, and that tells me that the promoter region is under the positive regulation.

If the promoter region is under the negative regulation, there will be no luciferase and so there won't be any light , and now we can tell if the promoter gene is on or off as well, we can tell if the promoter region is highly active or slightly active by the amount of light that is produced by our sample.

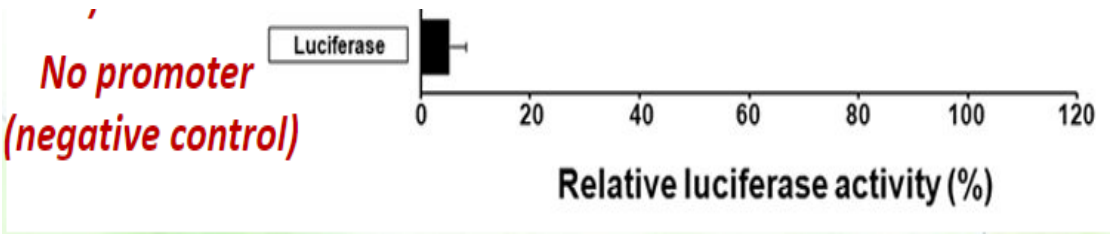
In the diagram bellow is an example of a typical experiment that is usually done in a laboratory, so we have a promoter region (in red) of any particular gene of interest, if you analyse it you will notice that there is a core promoter region (where DNA polymerase binds), we have an activating region (let us say that we have promoter proximal elements or we have positive regulatory sequence), and we have a silencer (repressor), we don't know much about this promoter region, we want to know what different parts of promoter region do exactly.



So what we do is:

constructing a plasmid (an expression vector) containing the luciferase gene that is under the regulation of this promoter in the photo, what we do then that we also produce a promoter region that contains different deletions and it will tell us what would happen if we delete a specific region in terms of the activity of luciferase and that will tell us how this region is important in terms of negative or positive regulation of this expression.

يعني راح نصنع مناطق تحكم فيها مواضع حذف مختلفة وكل موضع بحذف منطقة معينة راح نجرب كل مرة نحذف منطقة مختلفة ونشوف الأثر اللي بعملو الحذف عشان نقدر نحدد وظيفة المنطقة وراح نستخدم انزيم اللوسيفريز عشان نحدد الأثر سواء كانت منطقة النحكم خاضعة لتنظيم إيجابي أو تنظيم سلبي.

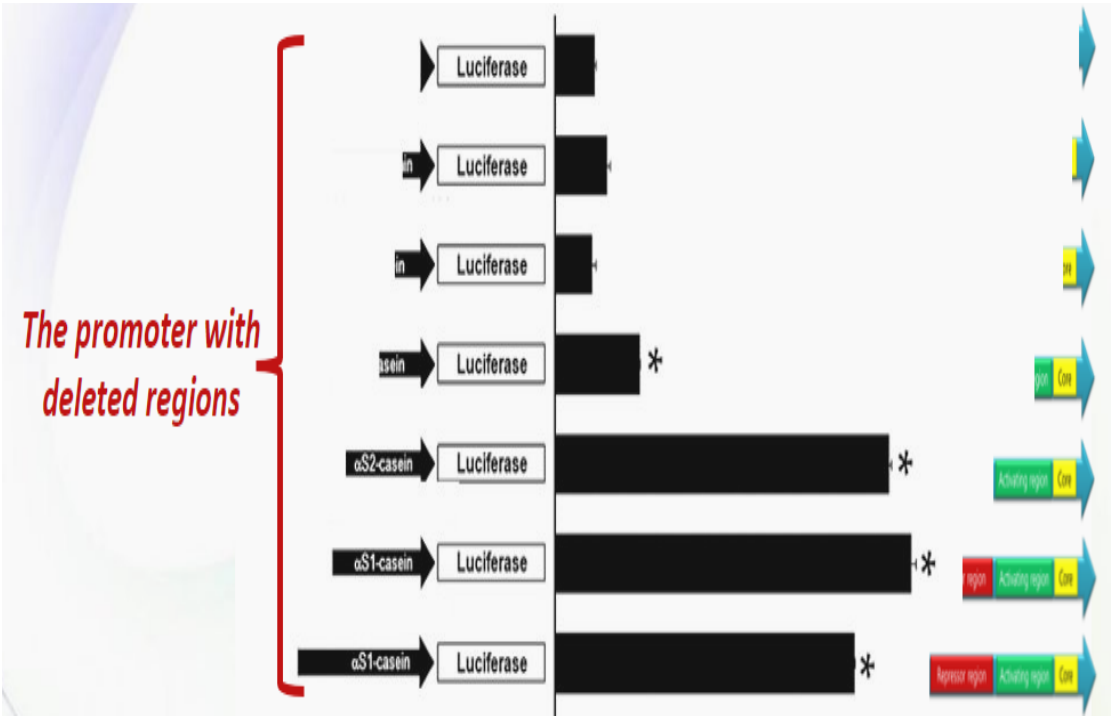


What we did in the previous photo that we transfect (insert) a plasmid that contains no promoter region (the promoter region is under the negative regulation in fact … promoter is turned off, and we are all know that the promoter is essential for the transcription), stop for a minute and remember what we said in the molecular biology course in the previous semester ((we said that even if we blocked the promoter region the transcription would happen in a slight amount due to the temporary turning on of the promoter)) *, so here we have a slight production of luciferase due to the leakage (the promoter is not tightly closed) (notice the very short black bar the percentage is almost 0%).

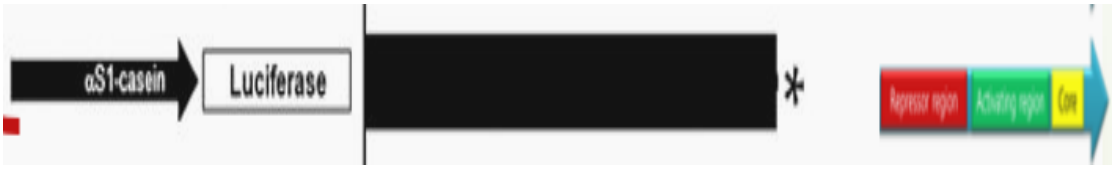
Be careful about this red piece of information*

In this photo we transfect a cell with a plasmid that contains the luciferase gene with a good promoter that is under positive control (regardless about what this promoter is, but "good" tell us that the promoter is something that make the experiment working fine), here the luciferase activity is maximal it is about 100%.

In this photo we insert a plasmid with the luciferase gene which is under the control of the complete promoter of interest (shown in the diagram bellow) without any changing or deletion, the enzyme activity is about 60%.



What we do in the following steps is to start to delete specific regions by using different deletion regions from the whole promoter and insert the luciferase gene within a plasmid in the cell, and we will observe what will happen for the percentage of the luciferase activity what will tell us about the activity of the promoter itself.



Here we use a deletion region in the promoter the is responsible of the deletion of the light blue region (marked with red circle), we observed a slight increasing in the activity comparing with the whole promoter .

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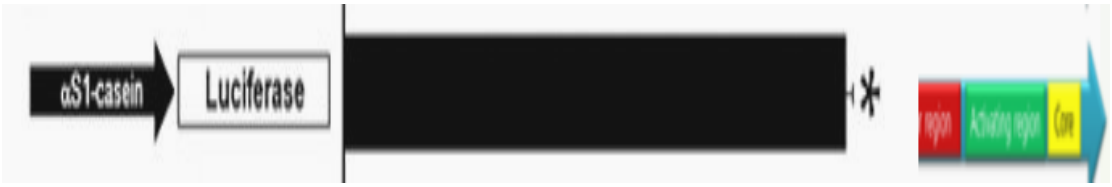
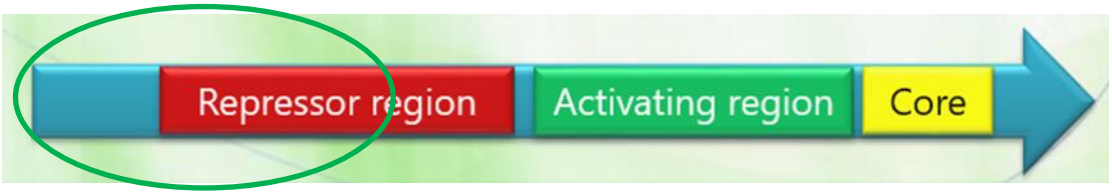
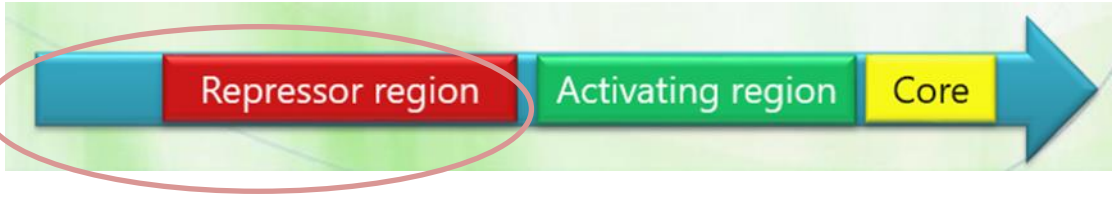
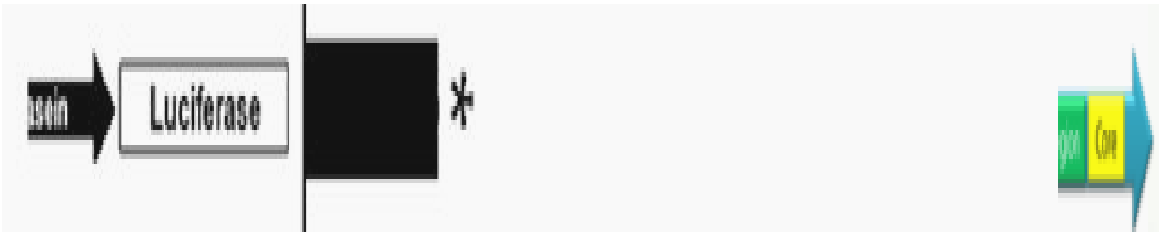


And here we cut the light blue region with a part of the repressor region * that inhibits the expression of one or more genes* colored with red in the photo (the whole deleted region colored with green), we notice that the percentage of the activity of luciferase or the activity of the promoter *or the region that it was deleted* slightly increased compared with the previous experiment and the whole promoter due to the deletion in the inhibitor repressor region.



Here we cut the promoter and delete the whole repressor region and the light blue in the left side and in between the repressor and activator region, we notice that the result would be a slight decreasing in the activity (the deletion is marked with pink).

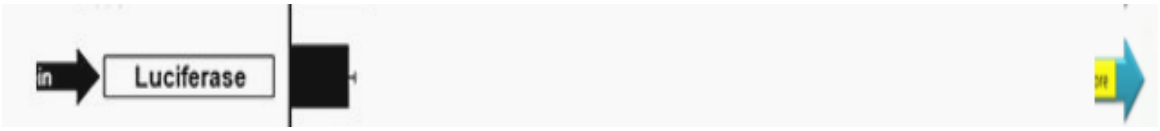




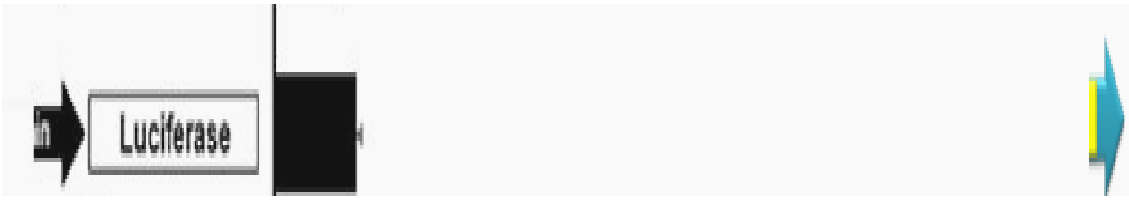
Here we cut the light blue regions and whole repressor region and a part from the activating region, we notice that the activity will decrease due compared with the previous one due to the deletion in the activating region (the deleted region marked with blue).

BE ATTENTION ABOUT THIS PIECE OF INFORMATION :

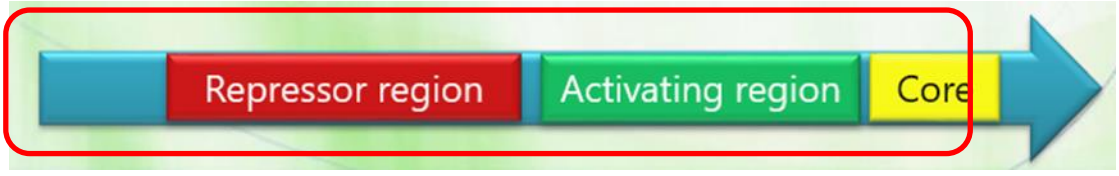
The main purpose of the repressor region is to inhibit the transcription and so any deletion in this region will cause increasing in the transcription, as well, the activating region has an essential role in activating the transcription so any deletion in this region will cause decreasing in the transcription.

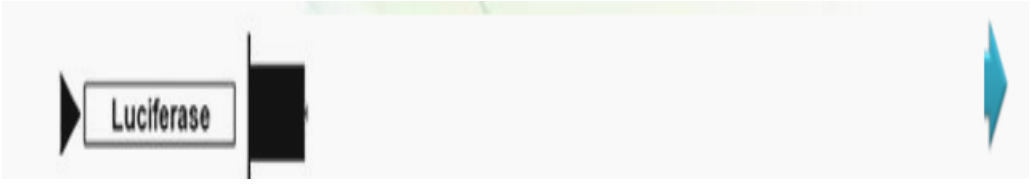


Here we cut the whole activating region with a part from the core region, we notice a decreasing in the activity of luciferase and the promoter itself (deleted area marked with black).

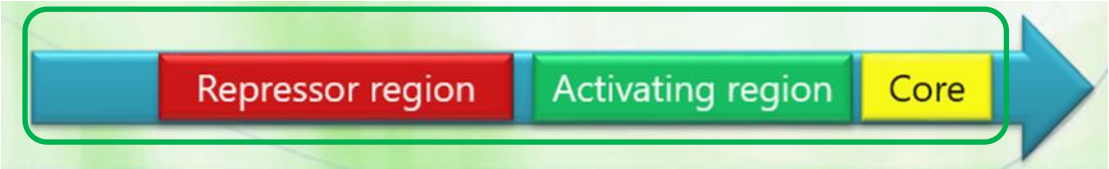


Here we cut much more part of the core region and the overall result is increasing in the activity comparing with the previous one (the deleted part in red)





Here we cut the whole promoter except a little piece, and the overall result is decreasing in the activity (deleted part in green).



Remember: such experiments are done in the laboratory

Notice:

- if we cut the core region there is almost 0% of the activity.

- The activity of luciferase will tell us about the cutting part if it was an activator or repressor.

That was what we call (luciferase assay)

We can also use recombinant DNA technology to determine if two proteins interact with each other.

In terms of determining protein-protein interaction there are two things that we can do (actually there are more) but we will concern with two:

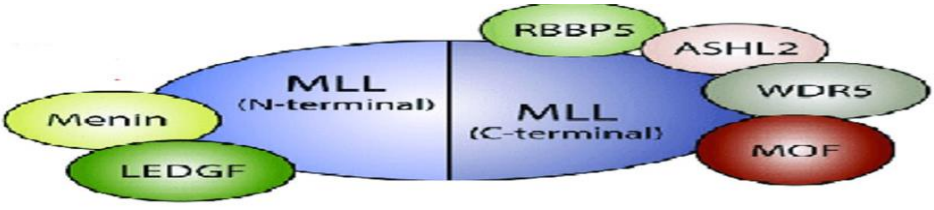
The first one is:

Immunoprecipitation

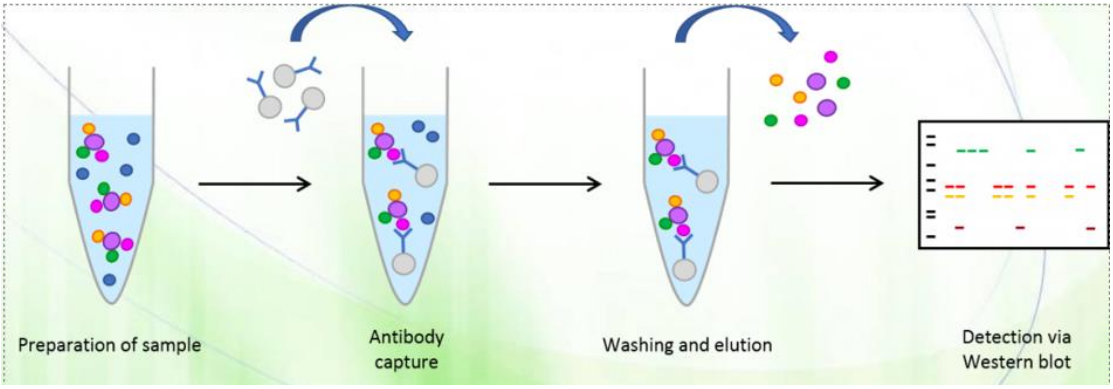
The second one is:

Yeast two-hybrid system starting from a DNA library

The idea is that you can have a protein that interacts with many other proteins, and you can have large proteins that have two domains and for example the domain in the left can interact with different proteins from the domain in the right .



And even the attached proteins can interact with each other, so our goal is to determine if the proteins can interact with each other.



In immunoprecipitation what we have that we remove all proteins from cells so we have a bunch of cells and we open these cells, now these cells have proteins interacting with each other and we preserve the protein-protein interaction, we take all proteins and we use antibodies specific to a certain protein (which is the pinky one in the figure above) so we can pull down this protein and all of the other proteins that are interacting with it, and all of the other proteins (that are not attached to the pulled protein) can be removed, so all non-bound proteins (non-interacting proteins) would be removed and we keep our protein of interest as well as all of the other interacting proteins, and then we can analyse our sample (all of the proteins that attached to the pinky one) by western blot or SDS page, for example.

This was what we call:

(co)-immunoprecipitation (precipitation: because we precipitate *pull down* proteins / immune: using the antibodies / co: because we also precipitate all other interacting proteins)

Time now is 17:22

Now we will talk about the second technique

Yeast two-hybrid system starting from a DNA library

First we have to create a **DNA library** (a collection of DNA fragments that are cloned inside a plasmid for example) (You can have clones of bacteria each containing a specific piece of DNA), so we have recombinant DNA molecules, it is almost like a book library, so you are interested with a certain book, you go for the library and for the second floor for example and go to the fifth shelf maybe and finally you would get your book) similarly, with a DNA library, if we interested with a DNA fragment and this DNA fragment is inside a plasmid and this plasmid is in a tube or a vail and this tube is in the freezer in the second shelf of the freezer and it is actually in box no.11, so what we have to do really is: going to that freezer second shelf, box 11, we take that box and get our tube from a certain location between many tubes and in a specific position we would find the plasmid that has our DNA fragment of interest.

http://www.sumanasinc.com/webcontent/animations/content/dnalibrary.html

watching this video would be useful*

there are two types of DNA library:

1. **Genomic DNA library**: we have the whole genome of a cell like the human genome, bacterial genome, etc.

We get the DNA (genome) and cut it using the restriction endonucleases for smaller pieces and we place each of DNA fragments inside plasmids (we will have recombinant DNA) and we transfect (insert) one vector inside bacterial cells and each one of them will grow carrying the plasmid inside it and then we freeze the bacterial cells, so each vail will contain a clone of bacterial cells each one of them will contain the exact same plasmid, so again when I am interested with a certain fragment I go to the freezer and pull out the bacteria that contain the plasmid of interest (in this case we have everything in the genome.. introns & exons,…).

2. **cDNA library**: it is more selective, what we do first is taking mRNA (the whole all mRNA molecules in a cell then we convert them into cDNA (complementary DNA) by reverse transcriptase (remember that mRNA is modified so we don't have introns, as a result we don't have introns in cDNA … they are all spliced out) cDNA is mature (we've already treated this issue in the previous lecture).

Then we place each cDNA in a plasmid (we clone them in plasmids) then we transfect these plasmids in bacterial cells (each cell contains a plasmid which contains a specific gene).

The difference between two types of libraries is that **the genomic includes everything inside the genome so we have exons, introns, promoter regions, enhancers, etc.**

while the cDNA contains a specific fragment of the genome (active genes or exons without introns) because we used the mRNA which is mature.

I can get the same exact identical genomic DNA library if I get the genome from, for example, stomach cell, epithelial cell, etc.

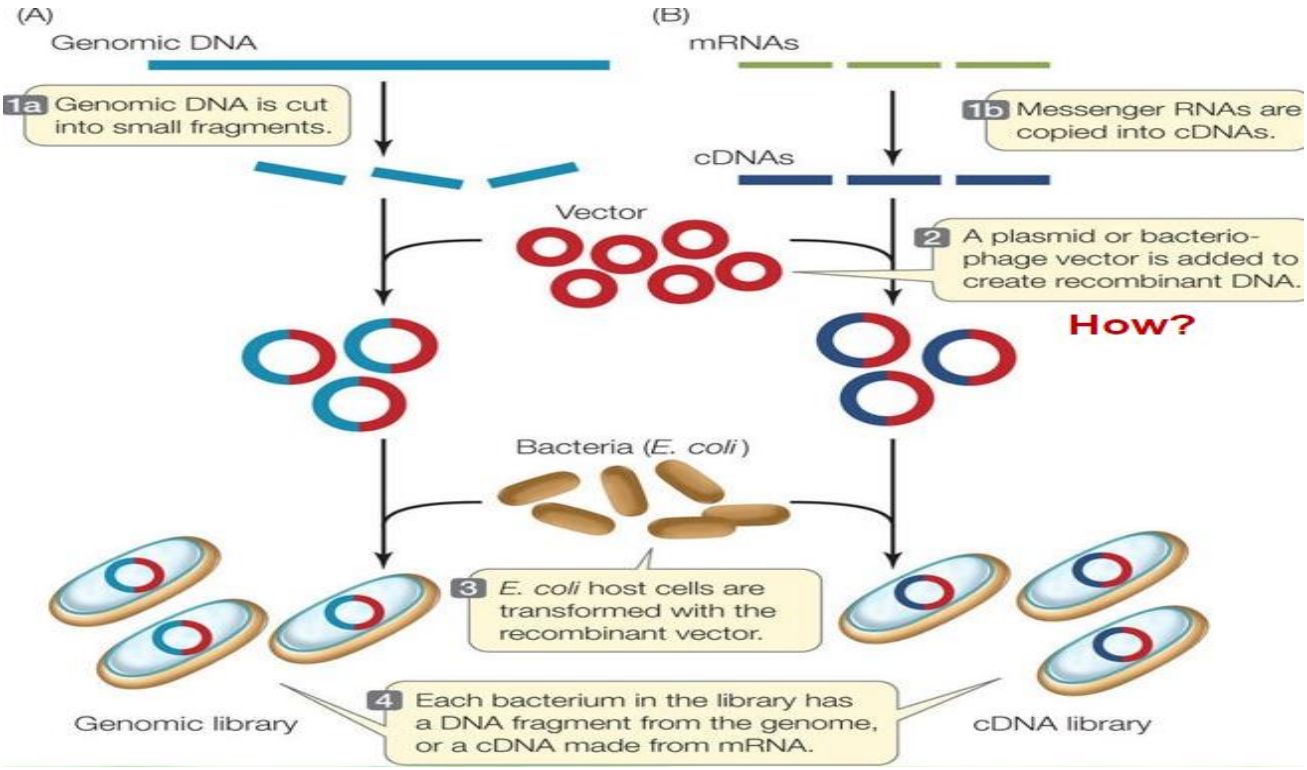
But if I create a cDNA library the source makes a difference because here we talk about an active gene (exons) so if I create a cDNA library from a stomach cell it would be different from that in the skin cells because the express different genes.

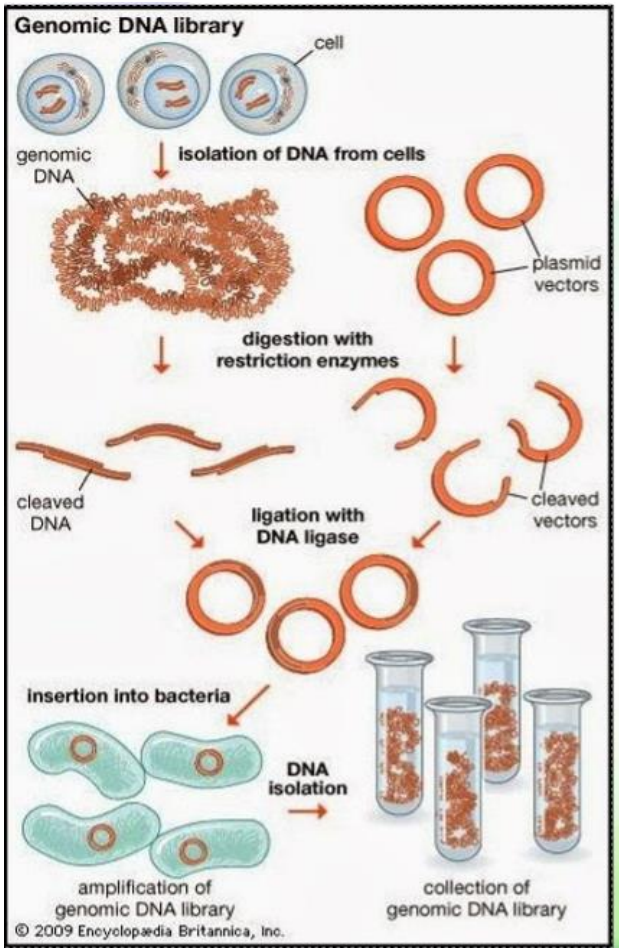
القصة بإختصار إنه الجينوم هو نفسه بكل الخلايا بكافة أنواعها لأنه نفس الكروموسومات ونفس تسلسل النيوكليوتيدات ونفس الجينات فلما استخدم الجينوميك دي أن ايه لايبراري راح أحصل على نفس النتيجة بالزبط سواء استخدمت خلية جلد أو خلية عصبية أو خلية معدة إلخ

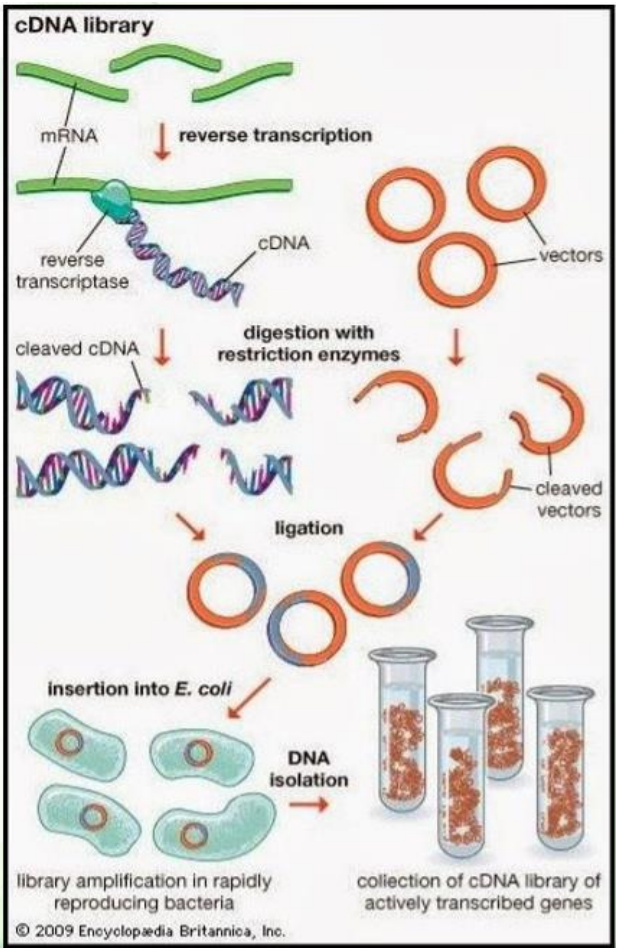
لكن في حالة سي دي إن ايه أنا راح أستخدم ماسنجر أر إن إيه اللي بكون ناضج يعني خالي من الانترونز بس بدنا ننتبه عنقطة مهمة :

لما نحكي الجينوم بكل الخلايا متماثل يعني الجينات كلها متماثلة بكل الخلايا هاد صحيح مليون بالمية لكن الجينات اللي موجودة بهاد الجينوم مش كلها بتترجم بالخلية .. مثلا بالجينوم اللي استخلصته من خلية المعدة عندي جين تصنيع حمض الهيروكلوريك اللي هو ضروري بالهضم هاد الجين راح يكون نشط يعني من ضمن الإكزونز بخلية المعدة وراح يصيرله ترجمة لأحماض امينية وبروتينات ... نفس الجين موجود بالجينوم اللي بخلية الجلد لكن ما بنحتاج حمض هيدروكلوريك عالجلد فهاد الجين راح يكون انترونز و راح ينشال لما خلية الجلد تبدأ عملية معالجة الماسنجر ار ان ايه عشان يصير ناضج و نستخدمه بالسي ار ان ايه لايبراري و عشان هيك بتختلف نتيجة السي ار اني ايه لايبراري من خلية لاخرى.

These photos bellow describe both libraries:


(A)
Genomic DNA
1a Genomic DNA is cut into small fragments.
(B)
mRNAs
1b Messenger RNAs are copied into cDNAs.
cDNAs
Vector
2 A plasmid or bacterio-phage vector is added to create recombinant DNA.
How?
Bacteria (E. coli)
3 E. coli host cells are transformed with the recombinant vector.
4 Each bacterium in the library has a DNA fragment from the genome, or a cDNA made from mRNA.
Genomic library
cDNA library


Genomic DNA library
cell
genomic DNA
isolation of DNA from cells
plasmid vectors
digestion with restriction enzymes
cleaved DNA
cleaved vectors
ligation with DNA ligase
insertion into bacteria
DNA isolation
amplification of genomic DNA library
collection of genomic DNA library
© 2009 Encyclopædia Britannica, Inc.


cDNA library
mRNA
reverse transcription
reverse transcriptase
cDNA
vectors
cleaved cDNA
digestion with restriction enzymes
cleaved vectors
ligation
insertion into E. coli
DNA isolation
library amplification in rapidly reproducing bacteria
collection of cDNA library of actively transcribed genes
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Now, what is **the yeast two-hybrid system?!**

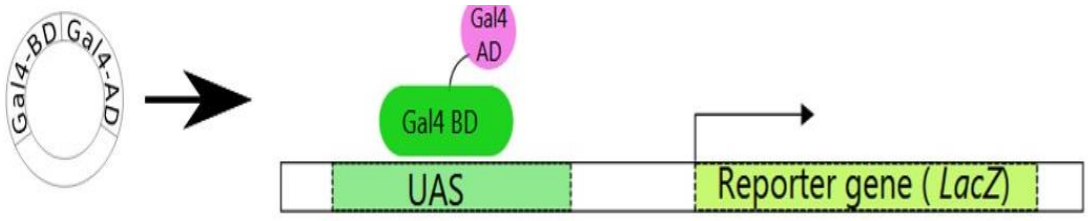
It is a technique where we take advantage of yeast in order to determine if two proteins are interacting with each other or not, so how do we do that?!

In the yeast there in a genetic system (UAS system) upstream activating sequence and this region lies upstream the reporter gene (LacZ) like galactosidase.

(by the way LUCIFERASE considered also reporter gene >>> ~~*it tells us about something*~~).

So here in the yeast we have galactosidase gene under the control of UAS system (galactosidase is the enzyme that cleaves the lactose *sugar*) … we mentioned it in the molecular biology course. As well, the **UAS** is under the control of a transcriptional factor which is composed of a DNA binding domain (**GAL4 BD**) and activation domain which activates the transcription (**GAL4 AD**).

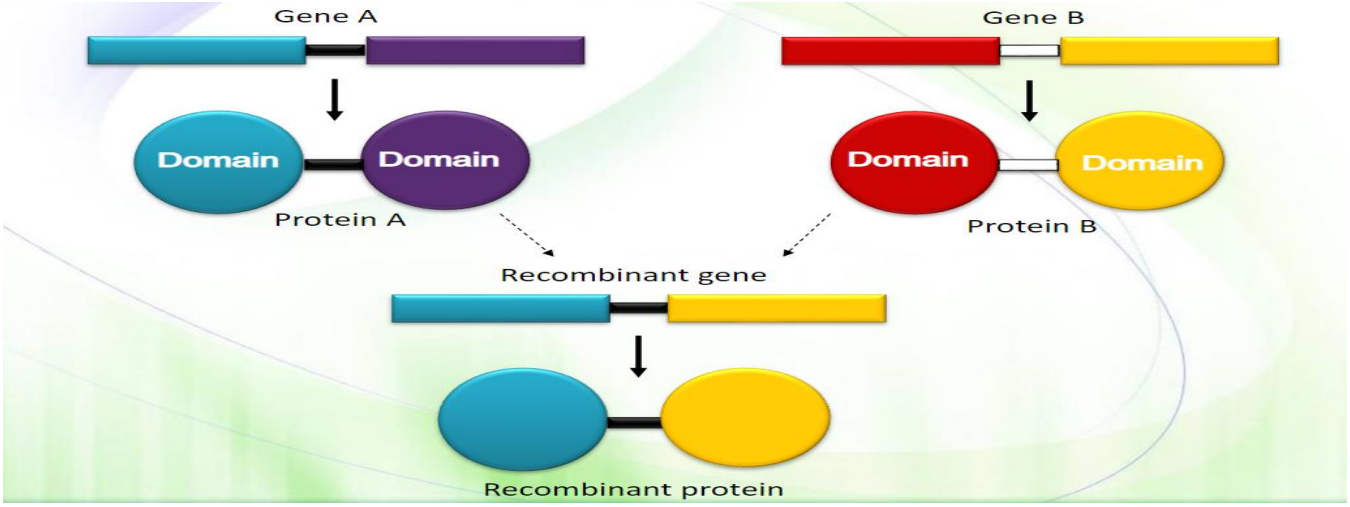
- In yeast, an upstream activating sequence (UAS) exists.
- UAS is controlled by a transcription factor that is made of two domains
  - A DNA-binding domain (BD)
  - An activating domain (AD) that is responsible for the activation of transcription.
  - Both must be close to each other in order to transcribe a reporter gene such the LacZ gene.



A. Regular transcription of the reporter gene

In order to activate the transcription of LacZ we should have the two domains (DNA binding domain & activation domain) close to each other, and we can introduce the gene of this protein which contains two domains inside plasmid (labeled with a red star in the previous photo) and then we transfect the plasmid inside yeast cell.

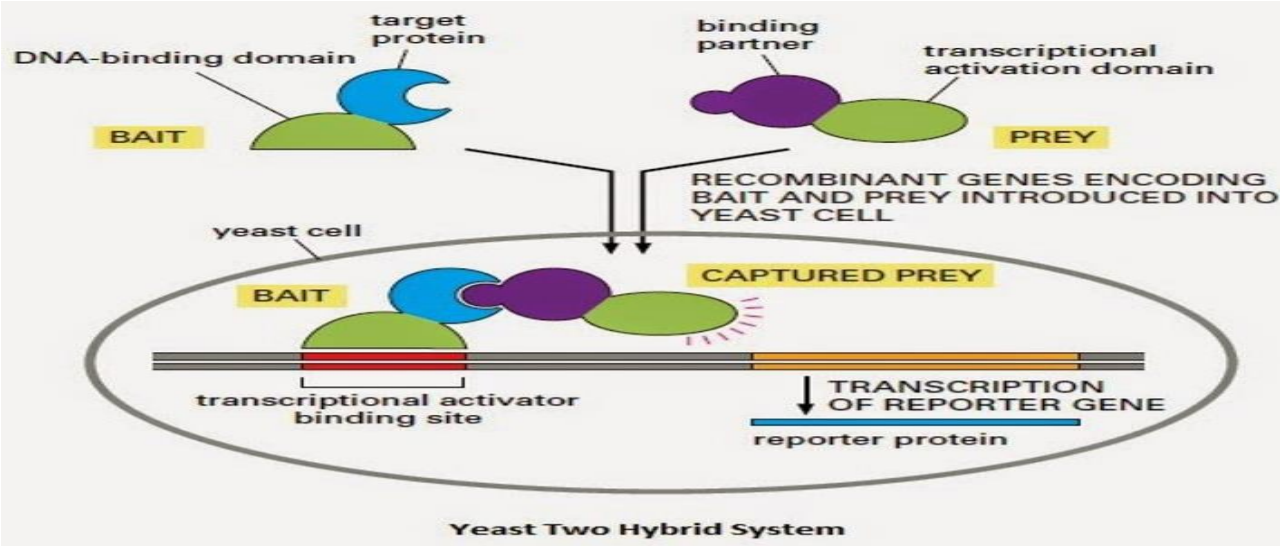
Remember what we talk yesterday about the recombinant proteins (attention … protein)



(from the previous lec: we have gene A which is translated and transcribed to produce a protein with to domains, and also we have gene B which produce a different protein with two different domains, I can create a recombinant gene to produce a new DNA with two domains each comes from a different protein)

Warning!!!!!

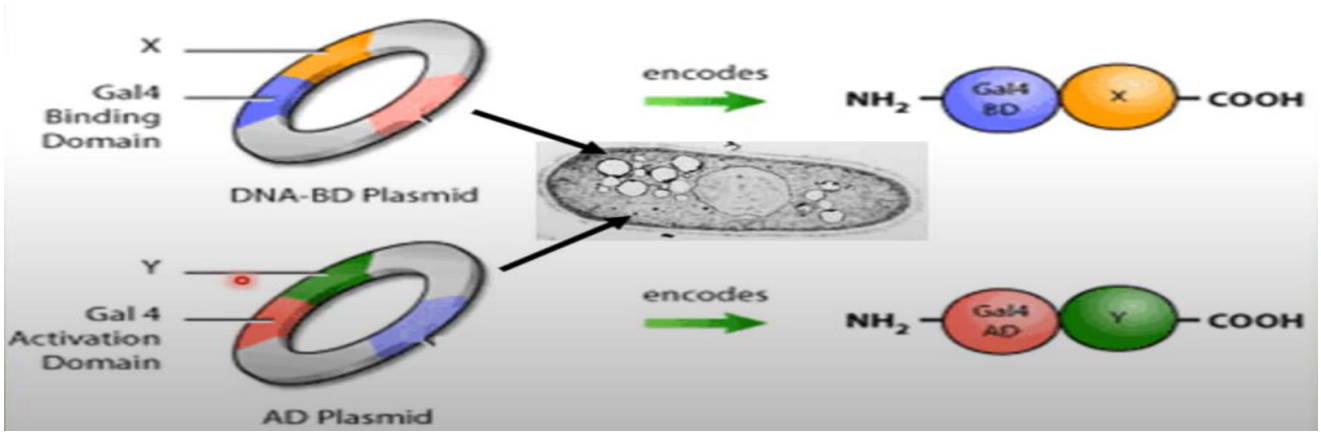
Be sure that you understand the following picture very well



... اول إشي بدي domainsراح اشرحها بالعربي بداية للتبسيط ... الفكرة من هاي العملية إني أستفيد من مبدأ ال ريكومباينتت بروتين تحديدا ال
والثاني بنشط DNA منفصلين عن بعض واحد منهم برتبط بال domains وهدول ال 2 domains اللي مكون من GAL 4أصنع البروتين
يكون مرتبط ببروتين معين آخر بسميه DNA binding domainعملية النسخ ... راح أصنع هاد البروتين بطريقة مختلفة شوي بحيث انه ال
راح يكون مرتبط باشي بشبه المستقبلات بسميه شريك الإرتباط (حكيت قبل إنه عشان يصير activation domainالبروتين الهدف أما ال
يكونوا قريبين من بعض) لما يكونوا قريبين من بعض راح يصير ارتباط بين البروتين 2 domainsنسخ وبالتالي ترجمة وانتاج بروتين لازم ال

So we create the GAL4 protein which contains 2 separated domains, so you have DNA binding domain separated from the activation domain, and we create **the DNA binding domain to be bound with another certain protein which is called the target protein (Target proteins control the action)**, and **the activation domain is generated to bound with another domain or protein with is called binding partner**, if these two proteins interact with each other, these two domains will be close to each other (AD & BD domains) an the transcription will be going on and the protein (galactosidase) will be produced, on other hand, if these proteins (target protein and binding partner) don't interact with each other (that means that two domains are far away from each other), as a result no signal comes out from that cell

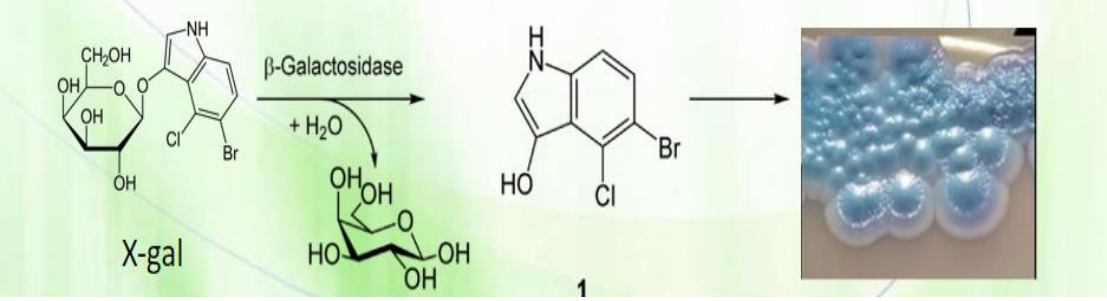
What signal are we talking about here?!



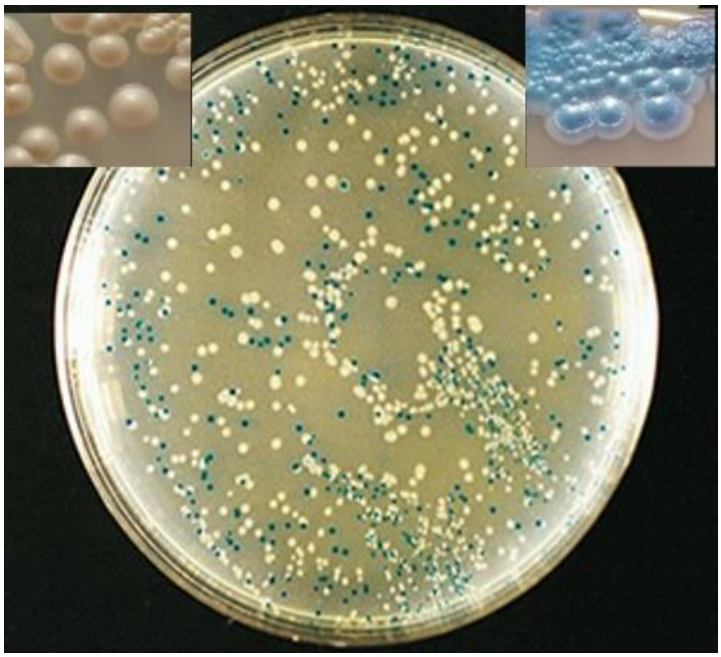
So again, we have a plasmid with a gene of GAL4 BD and a gene for protein X (target protein) and we have another plasmid with the gene of GAL4 AD and gene of protein Y (binding partner), **I can have a library of that plasmid (a library of Y) I can have different types of Y gene associated with the plasmid (expression vector)**, so we transfect these two plasmids in the yeast cells, so we would have production of X and Y, if Y interacts with X it means that the two domains are close to each other and the transcription will take place, but how can I know if there is transcription, what is the signal?!

We use a special molecule (lactose analog) called X-gal (it looks like a lactose but when it is cleaved by a galactosidase it produces a molecule that turns blue).

Look deeply to the photo bellow

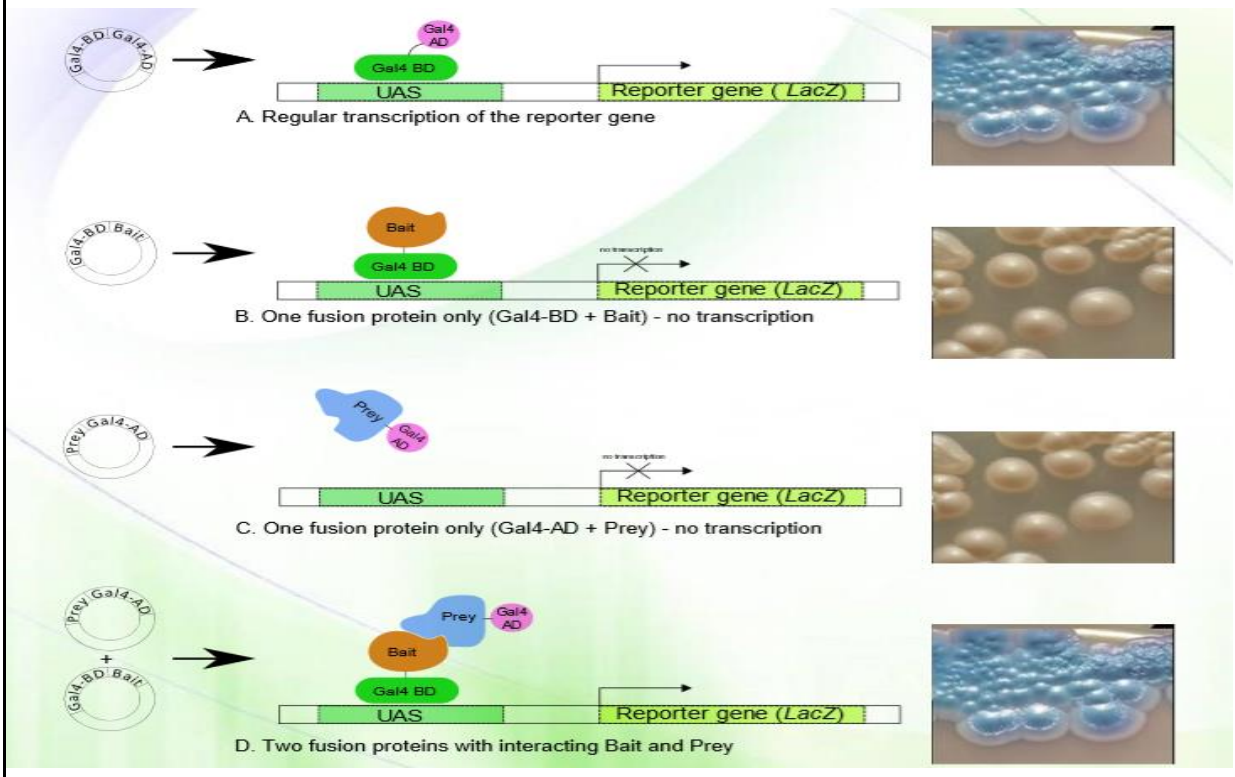


So if a yeast cell has an active galactosidase (the galactosidase was produced) that means that will be able to metabolize X-gal and the yeast cell will turn blue like the photo bellow.



If there is no production of galactosidase that means that there will be no metabolism of X-gal and the yeas t cell will look white.

Notice that the gene of galactosidase is the reporter gene that tell us what is going on.



These are the possibilities:

In the first case labeled with green star we use a plasmid that contains the whole GAL4 system, of course there will be a production of LacZ (galactosidase) and the yeast will appear blue

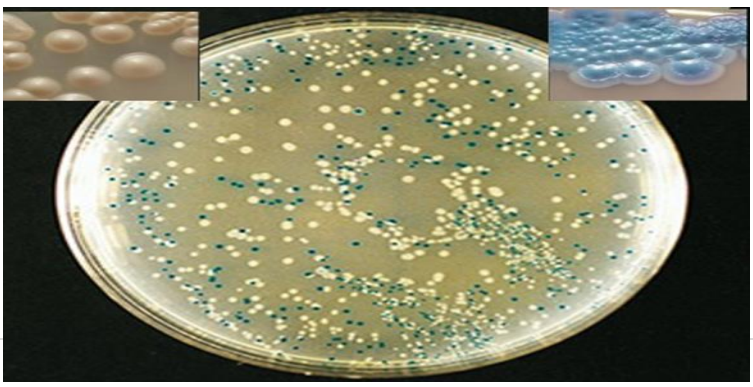
In the second case labeled with red star we use a plasmid with GAL4 BD that is attached to a target protein (we don't have GAL4 AD), so eventually we will get a white yeast because we don't have GAL4 AD and its associated protein.

In the third case labeled with yellow star, we have the same story as the previous one, but here we have GAL4 AD with a binding partner without GAL4 BD (there is no interaction to happen and the two domains will not be close to each other and the transcription of galactosidase won't take place and eventually is white.

And finally the last case labeled with pinky star, here we have two plasmids each contain a domain of GAL4 with its associated protein, when the transcription happens the associated proteins will bind and the domain will be close to each other, and as a result the transcription of galactosidase will take place and the X-gal will be cleaved and the yeast will appear blue.

Generally, what's the difference between the last and first case?!

In the first one we use one plasmid that contain the genes of both domains so the result was a whole GAL4 protein with two domains attached the each other directly (we didn't use associated protein), but in the last one we use two separated plasmids expression vectors each with a domain and its associated protein so the final result is two associated proteins connected with each other and the domains are not directly attached with each other (like what happened in the first case ... direct attachment)



صل على الرسول

What do we have here?!

Look at the previous plate, each one of the blue colonies means that there is interaction between X & Y, and each colony contains different Y (Y library) but they are all contain the same X, **because I want to know protein X interact with what Y protein**, I am not interested about the white colonies because there is no interaction between X & Y, in the blue colonies where the galactosidase was produced and X-gal was cleaved and the color was turned, I have interaction between X & Y, so I can take the blue colony and isolate the plasmid inside them and we can determine which Y protein inside this plasmid which inside the blue yeast (by sequencing or other techniques… we will talk about them later), **this is how we can determine protein-protein interaction.**

The end

لا تنسونا من دعائكم