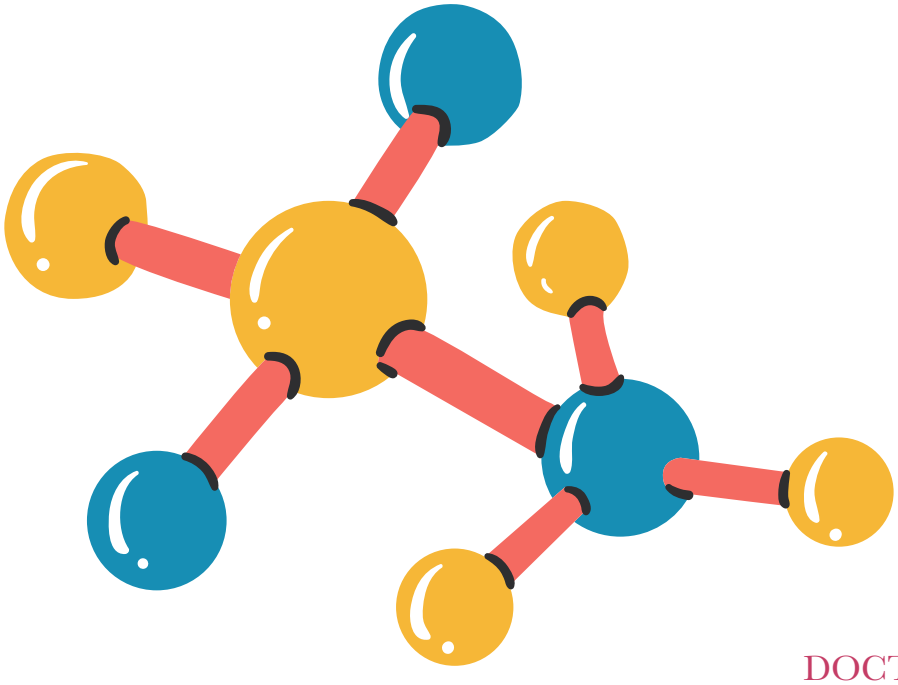
Sheet no.**10**

Biochemistry



DOCTOR SLIDES

DOCTOR NOTE

Summer 2022

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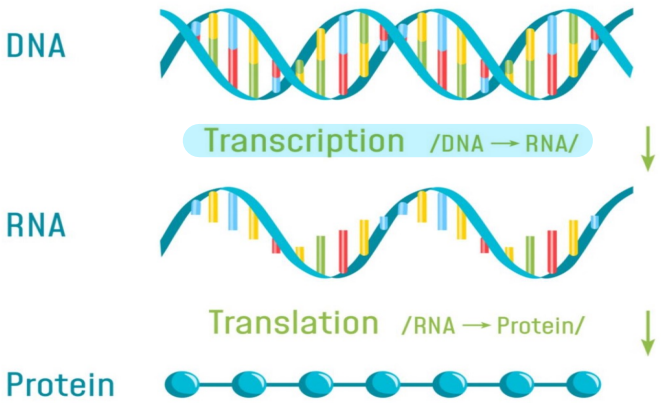
* the first concept of the lecture is about a technique that uses the Recombinant DNA to analyze the { transcriptional regulatory region }.

* some notes to remember :

- All the time , the DNA in our cells is transcripted to RNA , and from the RNA the proteins are made .

- the need for this process (DNA → RNA → Proteins) is variable , the cell sometimes requires one protein more than the others .

↳ so, the regulation of transcription and translation processes is important. { note : we are concerned with the regulation mechanism of **transcription** process , DNA → RNA }



- Regulation is controlled by :

cis-acting elements

** present in the DNA nearby the gene , such as :

1) the core promoter [TATA box] :

- is upstream the start site .
- is a binding site for transcriptional factor .

2) the promoter proximal :

- is upstream the core promoter .
- can bind activator or repressor proteins needed to regulate gene expression .

3) Enhancer :

- binds special transcription factor protein that increases the rate of transcription { positive regulation }

4) Silencer :

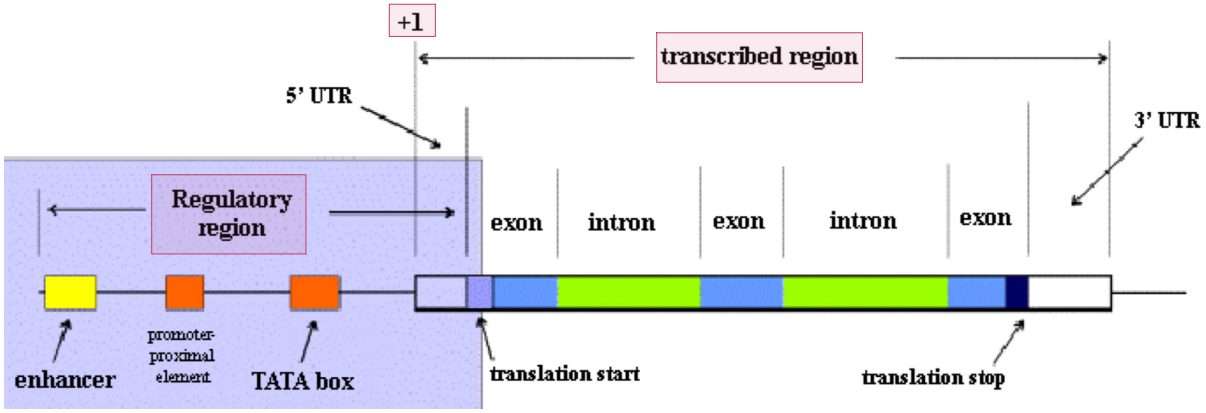
- binds special transcription factor protein that decreases the rate of transcription { negative regulation }

trans-acting factors

** based on the transcriptional regulatory proteins .

* note : we are concerned with studying the cis-acting elements.

** *What are transcriptional regulatory sequences*? … slide 3



* here we have an eukaryotic gene , we have the transcription start (+1 site) , then we have the transcription region (exons , introns) downstream

* but , what we are concerned with right now is the non-coding sequences that regulate the activity of the gene (cis-acting elements) :

the basic promotor { Core promoter ,TATA box }

the proximal promotor { like operons in prokaryote }

enhancer that positively regulates the gene expression

silencer that negatively regulates the gene expression

So,

How can we investigate these sequences (the cis-acting elements) ?
How can we pinpoint specific element that regulates gene expression ?
How do we know if it is a positive or a negative regulatory element ?

* we would talk about an Enzyme [**Firefly luciferase**] that we can use to investigate transcriptional regulatory sequences

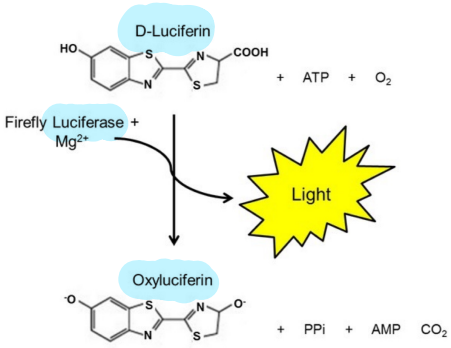
** *Firefly luciferase*… slide 4

* the scientists were fascinated by the firefly that produces light at night , and they investigated why the bottoms of these flies fluoresce (produce light)

* basically , there is a molecule called { Luciferin } that is converted into { OxyLuciferin } by an enzyme known as { lucifer**ase** }

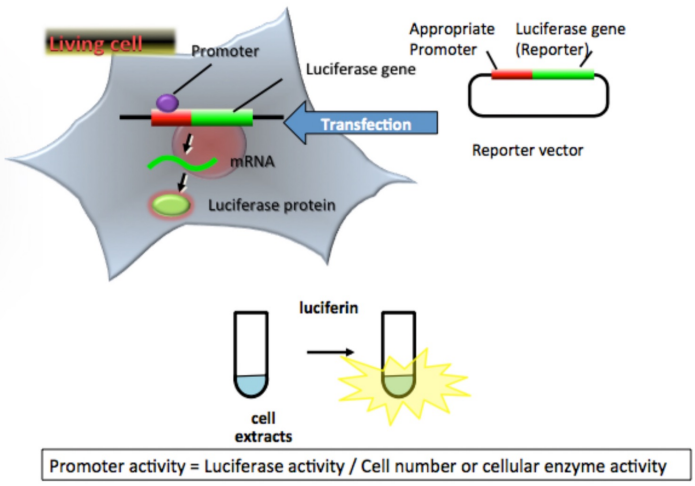
* OxyLuciferin is the molecule that produces the light

* the scientists took advantage of the Luciferase enzyme by using its gene to analyze the regulatory regions { رح تفهموا كيف استفادوا منه كمان شوي }



** *Luciferase reporter assay*… slide 5

* Purpose: study the activity of a gene at certain conditions or elucidate the function of certain regions of the promoter



* Reporter gene : is basically a gene that gives us an idea about something , here we want to know the activity of a certain region inside a cell under certain condition and the importance of several regions in terms of regulating gene expression

هو عبارة عن جين انا بضيفله regulatory region وبعمله expression عشان اختبر هاي ال regulatory region اللي ضفتها

* the reporter we would use in this technique is the gene that expresses the Luciferase enzym .

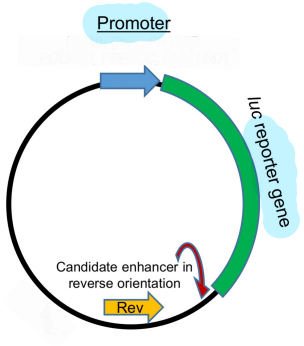
* so, that is what we do :

① we take the promoter region of any gene

② we make a DNA-Recombinant composes of :

- plasmid - the promoter region - the reporter

③ we put the promoter upstream (ahead) the reporter



* Only the regulatory region (e.g. promoter, PPE, etc.) of the gene is placed upstream of a "reporter gene" such as the luciferase gene in a plasmid.

* by doing this DNA-Recombinant , the reporter became under the control of the promoter chose to analyze it

* NOTE : we are not concerned with the reporter { which is the luciferase enzyme gene } , we are concerned with the regulatory elements .

④ The plasmid is transfected (inserted) into cells { cultured human cells) , and the expression level of luciferase {the reporter } (instead of the original gene itself) is measured.

* the transcriptional factor proteins (اللي هي في الوضع الطبيعي كانت رح تعمل expression للجين الاصلي) in the cell , would control the expression of the reporter { luciferase gene }

↳ so , we would have the reporter { luciferaese gene } expressed instead of expressing the original gene

⑤ we add the **luciferin** molecules to the cell

⑥ we measure the amount of light produced by the cells to give us an indication about the amount of Luciferase that is expressed

↳ so , if the luciferase is highly expressed → that means the gene of interest is also highly expressed in the same conditions (and vice versa)

_ we said that DNA → RNA → Proteins

↳ so, the number of proteins gives an indication about the rate of the transcription process , which is regulated by the transcriptional regulatory elements in the DNA

يعني انا رح اقدر احلل ال regulatory elements من خلال كمية ال proteins

↳ also , we said that the firefly produces light → this light is produced by the Oxyluciferin molecule → and this molecule is produced by the Luciferase enzyme , remember that most enzymes are proteins

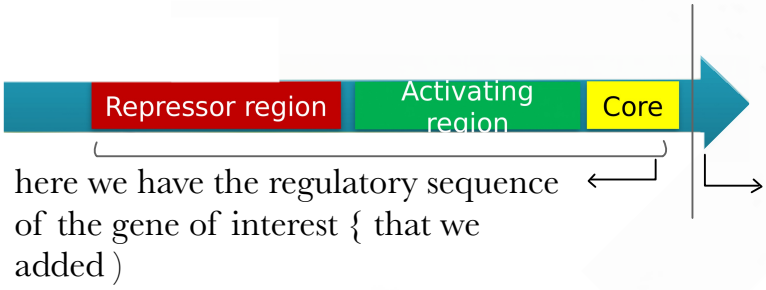
يعني كمية الضوء رح تعطي انطباع عن كمية البروتينات ، وكمية البروتينات بتعطي انطباع عن ا ل regulatory region

* The conclusion :

if the regulatory region activates the transcription → more enzymes → more light

if the regulatory region represses the transcription → less enzymes → less light

** ***Example*** … slide 6



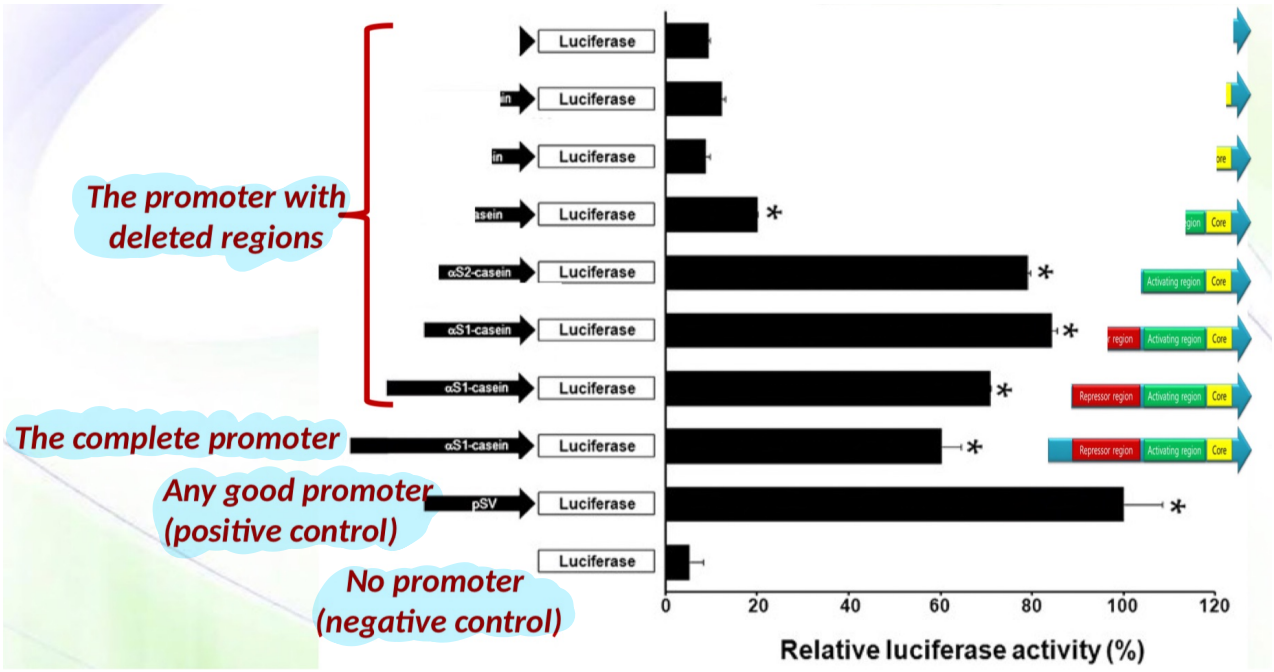
here we have the regulatory sequence of the gene of interest { that we added)

here we have the transcription start site of the luciferase gene { reporter }

* note : the core promoter region: the RNA polymerase binding site that produces the basal [minimum] expression of a certain gene

* we do an experiment by doing multi samples , for each sample we add different part of the regulatory region to the plasmid (note that the luciferase gene is exist in the plasmid in all samples)
and then we measure the luciferase activity in each sample

* this diagram shows the results for the different sample



- in the first sample : we add a plasmid without any promoter
  - ↪ this means that there should be no expression of the luciferase gene (zero or minimal)
  - ↪ as a result zero or minimal light
  - ↪ this is called the negative control , when we add a plasmid without any promoter inside
  - ↪ there is a little signal because there is a leakage of expression (back ground color or white)

- the second sample : we add a plasmid has a good promoter (not the promoter of interest gene)
  - ↪ the sample gives the maximal expression of the luciferase gene
  - ↪ the luciferase activity like 100% → much light

- the third sample : we add a plasmid has the complete promoter of the interest gene
  - ↪ we get a specfic activity like 80%
  - ↪ the signal is less than the one that is produced by the good prompter

* then we do a genetic engineering , we chop off parts of the promoter region

- the fourth sample : we remove this part from the promoter

  - ↪ the expression sort of similar to the third sample because we haven't really removed any regulator part
  - ↪ there are some differences but not that significant

- Then, we remove part of the repressor region
  - ↳ we have an increase in the sample compared to the complete promoter
  - ↳ this tells us that the region we just removed contains a repressing element.

- then , we remove the activating region
  - ↳ we have a huge drop in the expression of the luciferase gene
  - ↳ This tells us that there is an activating region , an element that positively regulates the expression of gene of interest

-the last sample : we remove the core promoter
  - ↳ there is no expression
  - ↳ we can compare this condition to the negative control

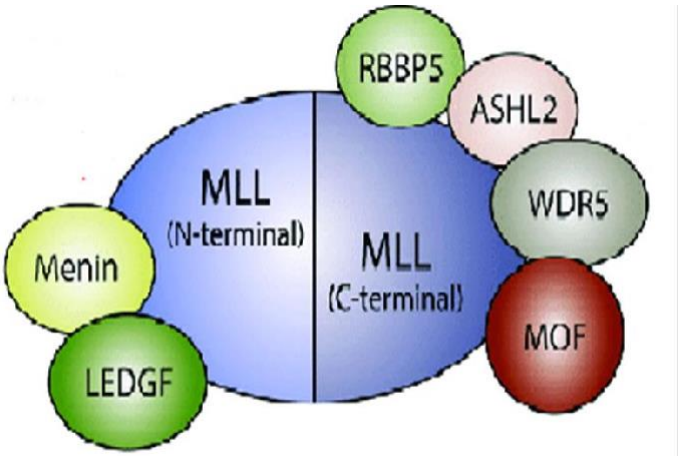
* this experiment tells us what certain region in the promoter are represent , if they activating or repressing region , enhancers or silencers , or proximal promoter elements , etc .

Protein-protein interaction:

It is known that proteins don't act by themselves, they mainly interact with other proteins in order to produce effect on cells:

See the figure bellow to understand:

Proteins form complexes



This blue big protein has 2 domains N- terminus and C-terminus, the N-terminus interact with 2 proteins that interact with each other also, the C-terminus interacts with 4 proteins as shown the adjacent proteins interact together directly, but the red and green ones don't!

The interaction of proteins is COMPLEX!

So, we will talk about 2 techniques in Protein-protein interaction:

1)Co-immunoprecipitation 2)Yeast- two hybrid system

***CO-immunoprecipitation:**

We talked previously about immunoprecipitation, where antibodies bind to protein of interest and pulls it down then we remove the other soluble proteins and then study the protein of interest by immunoblotting or SDS-page…

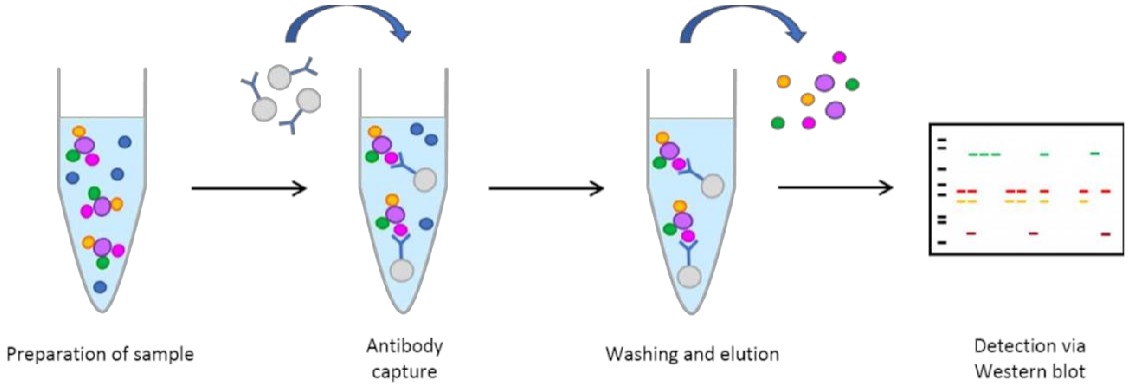
*BUT here we are talking about CO-immunoprecipitation: so we don't pull down the protein of interest only but also all other proteins that interact with it.

*the steps are:

*Antibody molecules that target a specific protein are conjugated to special beads.

*A mixture of cell proteins are added to the beads.

*Only the protein of interest is precipitated as well as other proteins bound to it (co-precipitated).



1)we have cells, we open up these cells and releasing their contents of proteins

2)we add antibodies to bind with the protein of interest

3) it pulls down with its CO-proteins

4) we then take the sample and study it by immunoprecipitation, SDS-page,…

**southern blotting is for DNA

**western for protein=immunoprecipitation

**northern blotting for RNA

Before talking about the second technique, we have to understand the following concept:

What is a DNA library?

A library can be created for DNA fragments just like book libraries.

In general, library is a place where collection of books are found, you can get a catalogue and search about the specific place of the book(the floor, the roof and etc…)

According to that, you would expect that DNA library is a collection of DNA fragments saved in freezer and with its catalogue you could find its place accurately (its tube, roof, floor…)

You can have clones of bacteria each containing a specific piece of DNA.

You can save these clones in the freezer and take whichever clone you want to study.

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

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types of DNA library:

there are 2 types :

genomic DNA library & cDNA library

**Genomic DNA library :

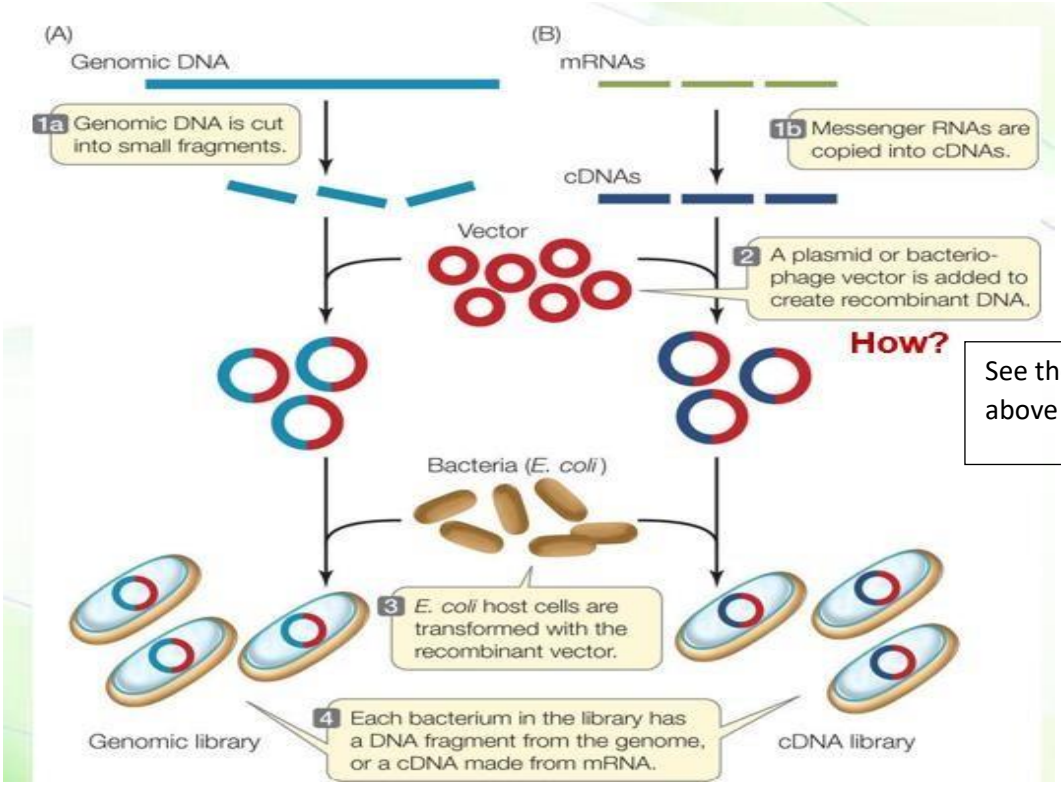
We bring the whole genome (with all its parts from exons, introns, PPE, promoter, silencer,coding and noncoding regions…)from a group of cells then we cut it into small fragments using restriction endonuclease, inserting the fragments into vectors like plasmids(each fragment is inserted into a plasmid) , theses recombinant plasmids will be inserted into bacteria, forming colonies(each colony contains a copy of plasmid with specific fragment), so, when we want a specific fragment we can find it in these colonies.

**cDNA library(complementary DNA library):

It is cleaner and simpler comparing with genomic library, cause we extract only the gene(the part that is transcribed and may be translated) from the cell,

We bring an mRNA(the mature one that contains exons only with translated and untranslated region : 3` UTR which represents the starting point and the 5` UTR).

This mRNA will be converted into cDNA using reverse transcriptase, then insert cDNA inside the plasmid, and insert the recombinant plasmid in bacteria to produce bacteria colonies as a library.



See the explanation above

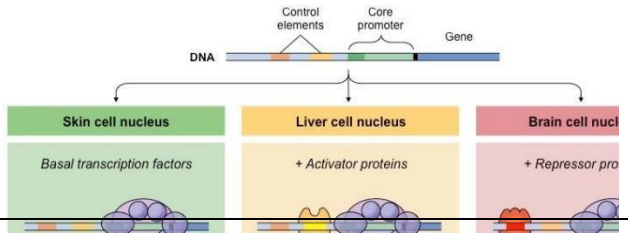
*if we take a human skin cell and neurons libraries and compare between them:

We will notice that the genomic libraries for both are identical cause all the genome(genome is the collection of genes with their promoters, introns, other sequences… الجين وكل اللي معه)in all cells are the same.

Meanwhile, the cDNA libraries will be different cause they represent the genes only, and the activity of genes` expression differs according to cell type and function

Remember from molecular course:

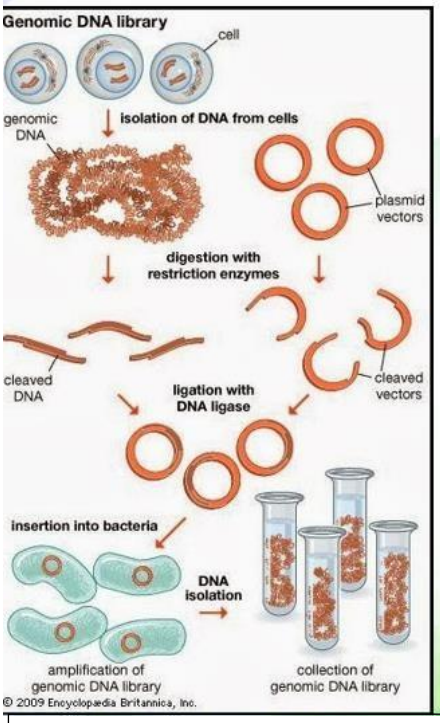
كل الخلايا لها نفس الجينوم ولكن يتم نسخ الجين حسب الحاجة تبعًا لنوع الخلية

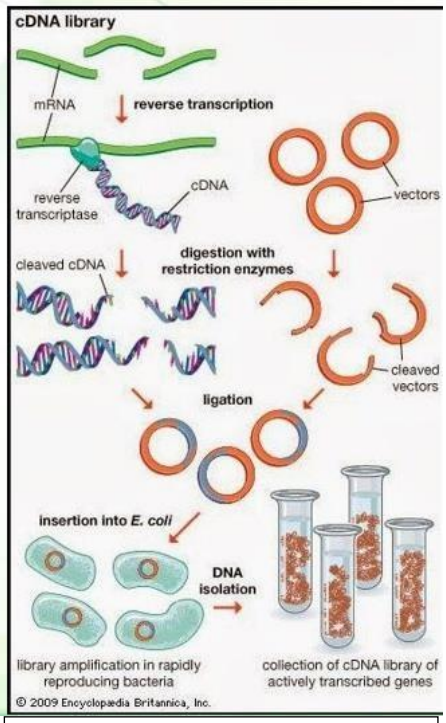


The differences between types of DNA library:

Genomic	cDNA
Present all DNA in a single cell	Present active genes only in a single cell
Same among different cells of the body	Different among different cells in the body
Contain introns	No introns
Need restriction endonuclease	Need reverse transcriptase

Another revision about the topic and the last revision!! 😇





Genomic library:

1) extract the whole genome from a Cell
2) fragmentising it using restriction Endonuclease
3) insert it into plasmid
4) insert the recombinant plasmid into Bacteria, they will make colonies

**so you will get a library with a lot Of DNA fragments each in a plasmid

cDNA library:

1) extract mature mRNA
2) convert it to cDNA using reverse transcriptase
3) insert it in plasmid
4) insert the recombinant plasmid into bacteria

**the cDNA library differs from cell to another!

Yeast two-hybrid system
Taking advantage of domains:

It is an example of how scientists take advantage of biological system in their discoveries:

First of all Lets deal with some terminologies:

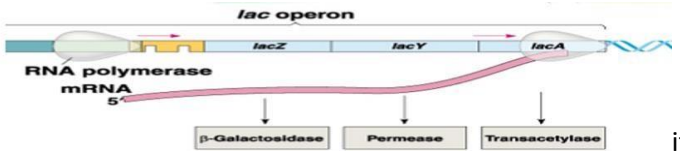
*UAS: a regulatory element in yeast means upstream activating sequence, it is the sequence that induces transcription to its downstream gene if a certain transcription factor (TF) binds to (like GAL4):

*GAL4 : is a transcription factor binds on AUS allow transcription to start, it has 2 domains:

1) BD: DNA binding domain, it binds to AUS

2) AD: activating domain, that induces RNA pol to start transcription.

*reporter gene: it is under control of UAS and GAL4, a gene that its transcription gives indication if it exists and how much of it exists in a cell, eg:
*LacZ: a reporter gene that produces B-galactosidase an enzyme that cleaves lactose to Glu and Gal.



if you feel lac Z is familiar to you, yes it is 😉

**now, lets see the mechanism of yeast two hybrid system:

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 activation 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.

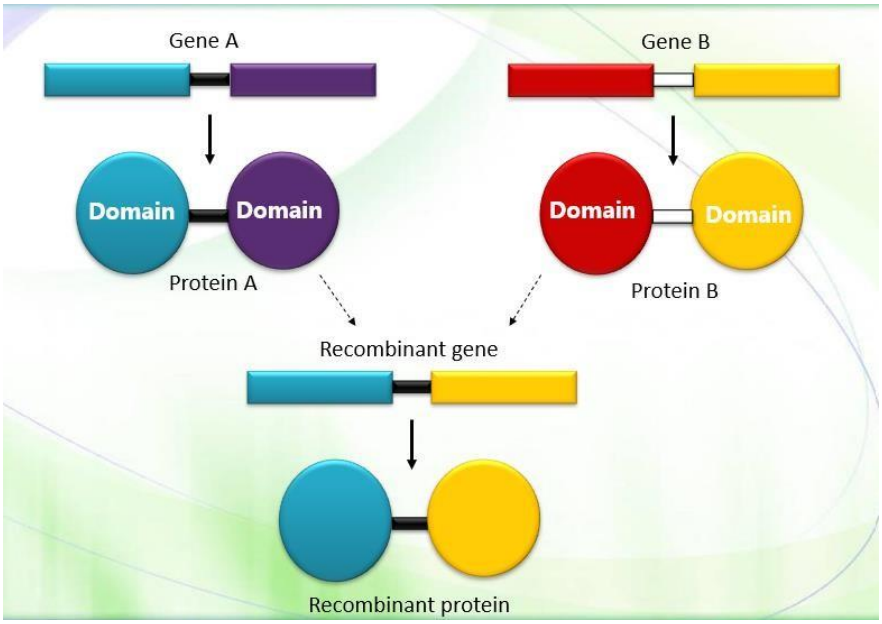
A. Regular transcription of the reporter gene

GAL4 BD binds on UAS activates and induces reporter gene (LAC Z) through Gal4AD to produce B- galactosidase that cleaves Lac, if they are not, B-galactosidase wont be produced.
**important note🤓:
Gal4 AD and BD must be close to each other to induce transcription!

Production of a recombinant protein

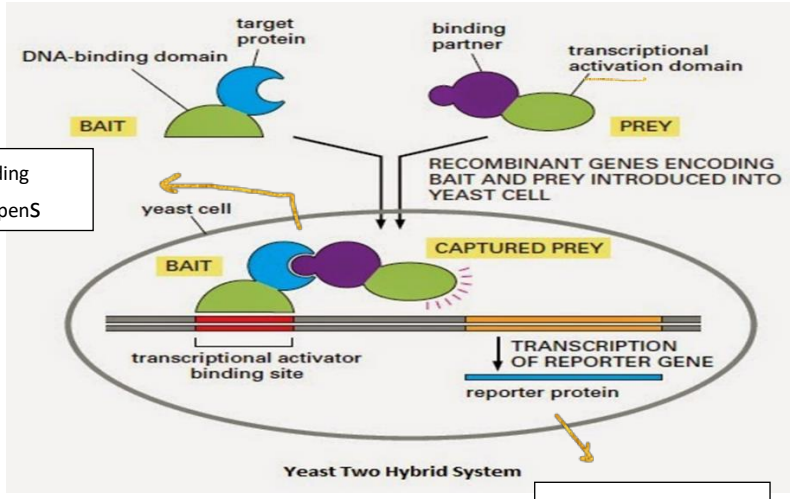
To remind you about the concept of domain:

It is a 3D structure, a region within a protein that folds independently of the rest of the protein, it is self-stabilising, if we cut it from the protein it will still function.It still interacts with other molecules (DNA, protein,…)



So we can use recombinant DNA techniques to bind 2 different domains from 2 different proteins, and they will maintain their function.

Quick illustration



Binding happenS

Transcription starts

The purpose of the yeast two hybrid system is to investigate if 2 proteins interact with each other or not.

Gal4 AD and BD could bind directly to each other or via proteins

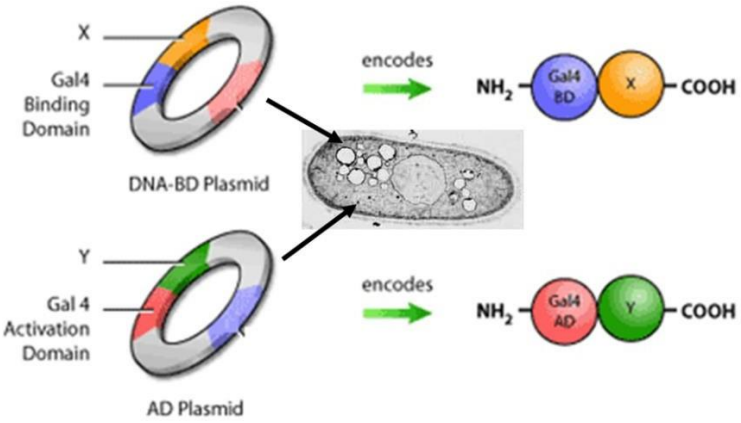
By recombinant DNA technology, lets suppose there is X protein binds with Gal4 BD and Y protein binds with AD if proteins X & Y interacts together, the AD and BD will be close together so the transcription will start. 😊

If X& Y don't interact, AD &BD won't be close and the transcription wont happen ☹

Cloning of hybrid proteins

In order to discover unknown proteins (Y's) that interact with a known protein (X), the X gene is cloned so it is produced recombined with the DB domain and the unknown Y gene (or genes) are separately cloned so that they are produced recombined with AD.

Both recombinant plasmids are transferred into yeast cells so all of them express the known X gene-BD hybrid, but each one expresses a different unknown Y gene-AD hybrid.

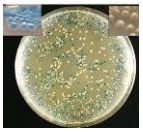


suppose we made a recombinant plasmid of known gene X and BD gene of GAL4,

They will be encoded to protein X and BD since transfection to a yeast cell, and we create a library DNA contains many many many plasmids of unknown genes with AD genes,we pick one of these and put it in the yeast, then a Y1 protein produce with AD, after getting both recombinant proteins of (X, BD)

and (Y1, AD) from 1st cell and (Y2 with AD) from 2nd cell we can test to which X will bind so transcription starts or not.

**we have used many yeast cells, each contains BD with X protein recombinant plasmid and Y1(unknown) with BD plasmid from the library.



Why is the LacZ gene used? What is X-gal?

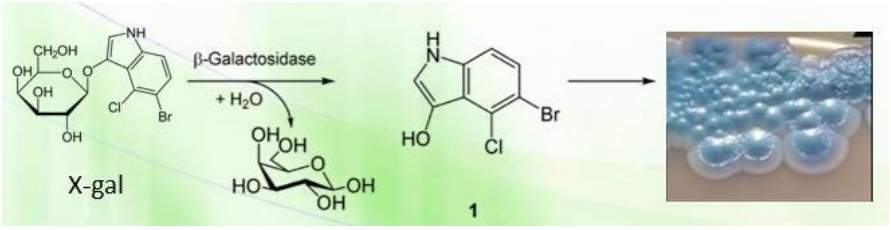
**Againnn: yeast cells are like bacterial cells grow in colonies they produce 2 proteins (X & Y) , if they interact with each other , they will stimulate transcription, producing B-galactosidase, which cleaves Lactose.

But here we wont use Lac we will use X gal : a substrate that is similar to Lac but with its specific structure if it is cleaved it would produce blue colour. 

BUT if interaction doesn't happen, transcription wont start and B galactosidase wont cleave x gal so the colony will be white. 

Yeast cells are grown in the presence of a lactose analog called X-gal, which generates a blue product when cleaved.

When the LacZ gene is activated, beta-galactosidase is produced, which cleaves X-gal generating blue colonies.



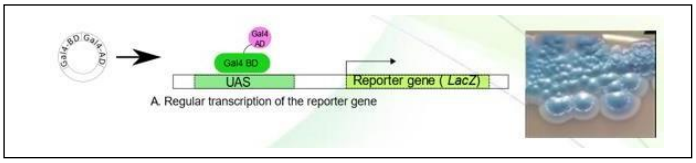
The possibilities and outcomes

The following 4 possibilities are random, we add the plasmid of X and BD in the old yeast cells and select a yeast cell by using antibiotics for example, we take the Y gene from the DNA library and express it in the yeast via plasmid

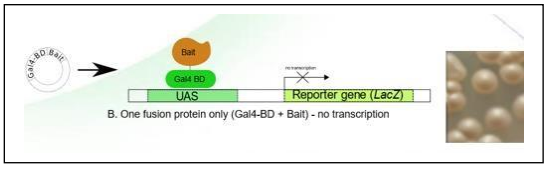
*if they interact, the colony is blue

*if they don't interact, the colony is white.

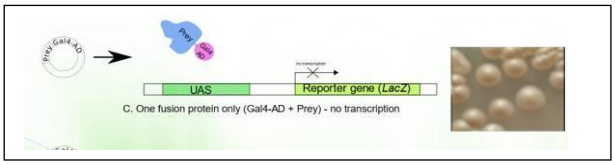
1) A normal Gal4 domains(AD & BD) interact directly to each other, so transcription starts and the colony is blue



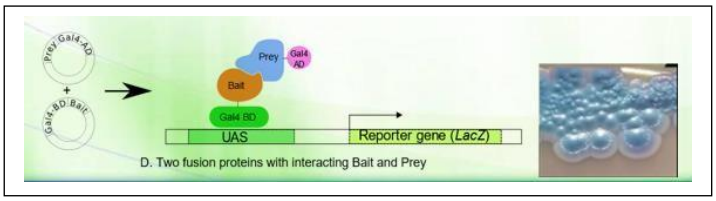
2) the yeast cell expresses only the X protein and BD, so transcription wont happen and the colony stays white.



3) only the Y protein and AD are expresses so transcription wont happen and the colony is white.It is a negative control and these cells wont produce a blue metabolite.



4) our experimental sample, AD & BD are close via protein X & Y so transcription starts and the colony appears blue.



*WHAT NEXT?

White Colonies : Express X&Y but don't interact

BLUE COLONIES:

Express X & Y and they interact together

Then, Blue yeast colonies are picked and plasmids are isolated to identify the unknown genes/proteins that interact with the known gene/protein***.(pick these colonies, growing them to expand them then open up these cells releasing the 2 plasmids, take the Y plasmid to identify this unknown gene by PCR, sequencing, immunoblotting,…).***

PAST PAPER:

1)A blue colony generated in yeast two-hybrid system indicates

A. The enzyme beta-glactosidase is inactive

B. The recombinant plasmid are successfully inserted into yeast

C. No expression of LacZ gene

D. A confirmation of protein -protein interaction

E. Lactose is metabolized

2) The luciferase reporter assay is used to

A. Identify transcription start sites

B. Identify introns and exons within eukaryotic genes

C. Identify termination sequences of genes

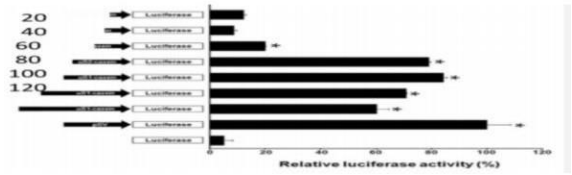
D. Identify genes

E. Identify regulatory sequences within promoters

*3)*The promoter of a specific gene only is placed upstream of a "reporter gene"

luciferase gene in a plasmid, the plasmid is transfected (inserted) into the cells, and

the expression level of luciferase is measured, what can you tell?



A. There is inhibitor region within 80 and 100.

B. There is repressor region within 100 and 120.

C. Gene transcripted at best when there is no promoter.

D. Promoter does NOT affect the transcription.

D

E

B

All the best

الله معاكم