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Application of Recombinant DNA Technologies on Sub-cloning of Transcriptional Co-factor

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Abstract:- The expression or repression of a wide variety of genes, which code for protectins, controls vital physiological processes like metabolism, development and immune responses. The transcription of genes started by activation of the glucocorticoid in both negative and positive manners like metabolism, immunization, and inflammation. The transcription process is complex and involves a large number of factors interconnected by a large number of co-factor factors. In response to stress TTC5 is an active factor because interaction and stabilization and regulation of transcriptional activity of GR depends on this co factor. There are many other factors are also present but TTC5 is the main co factor because regulation of GR in gene depends on it. The TTC5 control of GR is expected to contribute to glucose corticus and GR's physiological function. The TTC5 regulates the transcription of GR target genes, including inflammation, involved in various processes. Duo TC5 has novel potential targets for different compounds which enable better control over glucocorticoid-related therapies, considering the importance of glucocorticoids in treating inflammatory disorders and in general clinical practice. Wild type (2kb) is present on the agarose gel (but it is very blurry). Mutant type (2kb) is present on the agarose gel. PET28a (5kb) is present on the agarose gel.

I. INTRODUCTION

Glucocorticoid receptor belongs to superfamily receptor that is nuclear hormone .basically glucocorticoid are hormones known as lipophilic hormone adrenal cortex are the main component that releases this receptor at the time of stress inside cytoplasm adrenal cortex are attached with glucocorticoid in the cytoplasm . The transcription of genes started by activation of the glucocorticoid in both negative and positive manners like metabolism, immunization, and inflammation. In different steps like stability of protein, cofactor interaction, translational modification they are highly active because of different range of glucocorticoid action. During the transcriptional activity the stability of glucocorticoid is really very important. When the exposure of GR with hormone is very long then the regulation of GR is low during proteasome dependent process. Protein stability has great impact at the time of phosphorylation of receptor at the time of proteosomal degradation of GR the key role is played by ubiquitin ligases that are murine double minute w (Mdm2),CHIP (cterminus of hsp 70-interacting protein) and E6-AP. Other than regulation of GR proteasome machinery is responsible for of transcriptional activity of GR as well. For the rearrangement of GR at promoter the proteasome is necessary the transcriptional activity also has slight affection because of GR inhibition (Sanchez-Garcia et al., 2016). When hormone are not present then the binding of receptor is done by the coprocessor of GR that is NCoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoid and thyroid hormone receptor). It also repress GR dependent transcription by employing histone deacetylates to transcriptional complex NCoR and SMRT have CoRNR boxes or LXXI/HIXXXI/L helix motifs basically they are nuclear receptor that interacts domains. Helix motif form different helices which binds nuclear receptors in hydrophobic pocket along with C-terminal activating function domains. A major change occurs in this domain as the attachment of coprocessor are inhibited by ligand binding it also controls release of nuclear receptor for cytoplasmic complex. The interaction of co factor with GR is possible with AF1 or AF 2 domains. If it binds with AF2 domain then it is called hormone dependent the members of p160 family which are actually coactivators, p300/cAMP they activate against element binding protein BRG1 (Brahma-related gene 1), PCAF (p300 CBP associated factor), and vitamin receptor interacting protein 205 (DRIP205)/TRAP220 (thyroid receptor associated protein 220), interact with this domain. A surface is generated by hormones that has an ability of binding with LLXLL motif they are present in coactivators after this process changes occurs in GR. The interaction between NR and coactivators are possible by LXXLL motifs. On N terminus of receptor AF1 independent activation hormone is present. But it's not restricted to the structure among NR family. The main factor for the binding of AF1 has not been fully recognized yet. But on the other hand members of P160 family like PRIP150 and tumor gene 101 are recognize as interacting domain (Halimah, Rahmat and Redjeki, 2019). The linked between the two domains is possible because the members of p160 family and DRIP/TRAP complex both can interact with AF-1 and AF-2 domains. The 160 family is well known group which includes steroid receptor coactivator 1 (SRC-1), SRC-2, and SRC-3, which bind AF-2 of GR in a ligand-dependent manner. Proteins uses intrinsic histone acetyl transferase activity for co activation of GR. the GR can directly bind by the employment of other cofactors that are HATs p300/CBP (18). P300/CBP it can also act as co activators by acetylene histones and enrolling other HATs and RNA polymerase 2 to initiation point of transcription. For numerous transcription factor p300 is really very important p53 also includes in this family it contains transcriptional factor and serves as a component of multi protein factor PCAF, tetratricopeptide repeat domain 5

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(TTC5), and junction mediating and regulatory protein (JMY) are includes in this factor. After phosphorylation by ataxia telangiectasia DNA is damage as a result TTC5 is stabilized. Composition of TTC5 is depend on six protein-protein interaction. In Yeast cell division motifs were first recognized and promotes anaphase complex. Motif present in tandem array are made up of 34 amino acids that have similarities in size, arrangement and stability (Qi et al., 2015). TPR motif are involves in many functions like transportation of protein. Folding of protein, degradation and transcriptional regulation. Many other motif proteins are also involved in regulation of GR.

II. MATERIALS AND METHODS

The expression or repression of a wide variety of genes, which code for protectins, controls vital physiological processes like metabolism, development and immune responses. The gene transcription system, which is regulated by transcription factors in effect, controls various cellular processes and environmental stimulus responses. The transcription process is complex and involves a large number of factors interconnected by a large number of co-factor factors (Chao, Yuan and Zhao, 2015). For eg, tumor suppressor p53 and glucocorticoid receptor are sequence defined transcription factors. To order to function efficiently, these variables must communicate withTTC5/STRAP co-factor, which can be evaluated in this practical manner. Demonacos et al. in 2001 described TTC5 as the p300 pressure sensitive activator (Demonacos, 2001; Calderwood, 2013) as motif 5 for tetratricopeptides. The protein of 440 amino acids was found to consist entirely of six TPR factors implicated in the development of multiprotein complexes and cancer (Wen, 2018).

Plasmids

Double-stranding circular DNA molecules found in bacterial cells are plasmids or vectors. These can be replicating and are used in molecular biology with multiple copies. They have a replication origin (ori) which allows them to replicate and produce large amounts of DNA in bacteria (World Health Organization, 2015). We have antibiotic markers that can live on plates that involve this antibiotic in bacteria used in selection process only. Furthermore, plasmids contain multiple cloning sites (MCS) which are DNA sequence clusters, which can be decreased by restrictive enzymes to incorporate the desired DNA (Puetz and Wurm, 2019).

PHATTC5 was developed as defined and permits temporary overexpriming of TTC5 in mammalian cells (Demonacos et al 2001). We cut TTC5's CDNA from this plasmid using the restrictive enzymes BamH1 and Xho1 and put it into the same-enzyme PET 28A plasmid cut, which enables bacteria to be exposed and the overproduction of TTC5 is easily purified and studied, for TTC5 to become overproduced. Remember that in-frame cloning without PCR, (classical cloning technique), is required in this strategy. If there is no feasible restriction areas, then the PCR-based approach should be used (Rasala and Mayfield, 2015).

Aim

The aim of this experiment was to subclone p53 and GR transcriptional cofactor TTC5 from mammalian expression vector into the bacterial expression vector to facilitate future studies of **TTC5 function**

Objectives

- Design strategy to make recombinant DNA
- Perform restriction digestion of the DNA
- Use electrophoretic techniques to separate different DNA fragments
- Isolate DNA fragments from the gel
- Learn how to ligate two different pieces of DNA
- Introduce plasmid DNA into bacteria

Steps

Step 1: Set up restriction digest to transfer TTC5 cDNA from mammalian expression vector (HAPCDNA3) to PET28a vector so that TTC5 can be expressed in bacteria
Step 2: Prepared agarose gel (this will be prepared for you)
Step 3: Run the gel, cut out and isolate fragment
Step 4: Ligate DNA overnight (you will prepare ligation mix, but one will be prepared for you previous day)
Step 5: Introduced DNA into bacteria (known as transformation of bacteria)

Step 1: Set up a restriction enzyme digest
Label 3x1.5 ml eppendorf tubes (very important in order not to mix up your samples). Pipette DNA, buffer, water and at the end add enzyme using p20 pipette according to the scheme below.

HAPCDNA3TTC5wt(tube1)	**HAPCDNA3TTC5**	**A243G mutant (tube 2)**	**Pet28a vector (tube 3)**
DNA	14µl	14µl	14µl
Buffer	2µl	2µl	2µl
BamH1	1µl	1µl	1µl
Xho1	1µl	1µl	1µl
Water	2µl	2µl	2µl
Total	20µl	20µl	20µl

Mix the liquid and then spin briefly in the microcentrifuge. Incubate samples for 1 hr in a 37^0C water bath

Step 2: Prepare agarose gel (this will be done by technical staff for you)
Agarose gel is prepared by technical staff for students

Step 3: Run the gel and isolate fragment
1. Removed the tubes from the water bath, add 5 µl of loading buffer, mix and pulse spin
2. Removed the comb, placed the gel in electrophoresis tank filled with the 1x TAE, with the wells next to negative electrode.
3. In the first lane load 10 µl of the Hyperladder 1 (DNA size marker) and in the rest of lanes load the entire samples into respective wells.
4. Placed the lid on top of the tank, switched the power pack and set it to 100V. Checked if the current is flowing. After 20min stopped the electrophoresis (make sure blue dye does not run of.
5. Took the gel out and placed it in the UV transiluminator to visualise the DNA.
6. Noted that only linear DNA will run in line with the marker and therefore uncut circular plasmid size cannot be estimated
7. Cut out the bands: lane 2 and 3 (HAPCDNA3TTC5) around 2kb-this is your insert; cut out the PET28a plasmid band (around 5kb)-this is the plasmid you are inserting the insert to.
8. Isolate the DNA using QIAquick gel extraction kit (or if no time purified fragments will be provided for you)
9. Weigh the excised fragment and add 3 volumes of QG buffer
10. Vortex every 2 min and incubate at 50^0C for 10 min to dissolve the gel
11. Add one volume of isopropanol and mix
12. Add this to the column that is in its 2 ml tube, and spin 1min 13000 rpm
13. Discard flow through add 750 µl of PE buffer to wash the column, spin again 1min 13000 rpm
14. Discard flow through and place the column in the clean 1.5 ml tube
15. Elute the DNA by adding 50 µl of the EB buffer and spinning again 1min 13000 RPM
16. Use eluted DNA for ligation

Step 4: Ligate DNA
1. Label three 1.5 ml tubes and pipette into all of them 1 µl of 10X ligation buffer, then 4 µl of distilled water into the first tube and 2 µl of distilled water into tubes 2 and 3
2. Pipette 2 µl of the cut PET28A vector into tube number 1
3. Pipette 2 µl of the cut PET28A vector and 2 µl of the insert 1 (WT) into tube number 2
4. Pipette 2 µl of the cut PET28A vector and 2 µl of the insert 1 (mut) into tube number 3
5. Add 1 µl of the enzyme ligase into all tubes, mix pulse spin and incubate on the room temperature for few hours.

Step 5: Introduce DNA into bacteria (known as transformation of bacteria)
Using sterile tubes containing 50 µl DH5α E.coli from Thermo Fisher Scientific chemically competent host cells.
Key:
W/type = 10a
M/type =10b
pHSG298 = 10c
pET28a = 10d

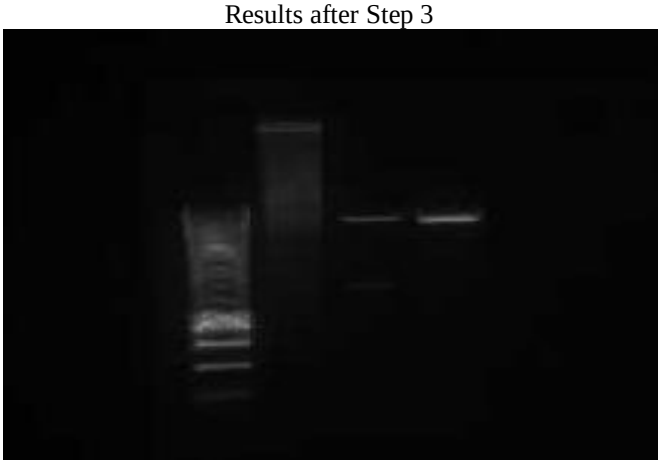
Before plating out, labelled each LB plate appropriately with name.

Thaw competent bacteria cells on ice (50 µl per ligation mix)
1. Add 5 µl of the ligation mix to 50 µl of competent bacteria mix by flicking.
2. Place cells on ice for 1 min
3. Heat shock in a water bath at 42^0C for 40 seconds
4. Place back on ice for 1 min
5. Add 500 µl of LB
6. Place on horizontal shaker for 30 min at 37^0C
7. Plate 250 µl of each mix onto LB-kan plates
8. Allow 5 min for absorption then place plates upside down in a 37^oC incubator for 24 hrs
9. Next day pictures will be taken of a plate with -this will be figure 2 in your report

III. RESULTS

Results are obtained after **step 3 and step 5** respectively.

Results after Step 3



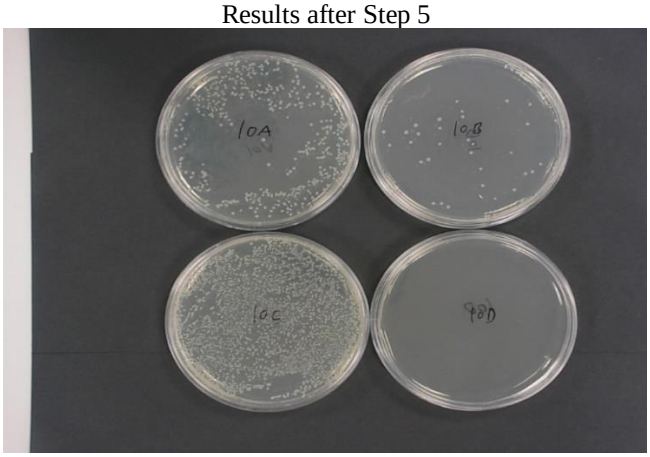
Agarose gel electrophoresis of wild type, mutant type and PET28a

Column 1: Hyperladder 1 (DNA size marker)
Column 2: Wild type
Column 3: Mutant type
Column 4: PET28a

Results interpretation: Wild type (2kb) is present on the agarose gel (but it is very blurry). Mutant type (2kb) is present on the agarose gel. PET28a (5kb) is present on the agarose gel.

It is provided that the DNA is extracted from organism as per the study which is being cut into small fragments of a size that will be suitable for the procedure of cloning. In most of the cases the cleaving the DNA is being performed to achieve this with a restriction enzyme (Jia and Jeon, 2016). Whereas different types of strains and species of bacteria are used to extract restriction enzymes, in which they act as defense mechanisms against viruses.

Results after Step 5



10A – wild type
10B – Mutant type
10C – PHSG298 (positive control): pUC-type bacterial cloning vector with kanamycin resistance gene,
10D – cut pET28a plasmid (negative control)

It is analyzed that staggered cuts are caused by most useful restriction enzymes as they leave a single-stranded overhang at the site of cleavage. If the donor DNA as well as the vector DNA are both cut with the similar enzyme. In addition to this it is also proven that glucocorticoid receptor are related to superfamily receptor which originally is a nuclear hormone (Stehr, 2015). Basically glucocorticoid are hormones which are called lipophilic hormone adrenal cortex which are the key component releasing this receptor in stressing conditions inside cytoplasm (Yun and Yoon, 2017).

The results mentioned that wild type (2kb) is present on the agarose gel. While on the other hand mutant type (2kb) is present on the agarose gel. Moreover, it is also identified in this report that when 2 bands are present there are chances of the fact that there is no cut on plasmid.

Potential explanations for different results

Gel

Lane 1 Hyperladder is used to determine molecular weight, note depending how long the gel is run the patter between provided example and your data may differ

Lane 2/3 no bands or smear-cause:
1. Didn't cut the DNA
2. Pipetting error
3. Not high enough concentration of DNA to start with or degraded DNA

Lane 4 Pet only

If there are 2 bands it is possible the plasmid was not cut-perhaps enzymes were not added due to pipetting error or enzymes were inactivated by being left at room temperature too long – uncut plasmid can be supercoiled DNA that can give rise to several bands o a gel

Plates

No colonies can be seen if:
- wrong plates-wrong antibiotic-but unlikely as there are colonies in other plates
- ligation not done properly, but positive ctrl should have colonies transformation not done properly-pipetting error, or competent cells left on room temperature too long, of spreading not done properly etc.

IV. DISCUSSION

In response to stress TTC5 is an active factor because interaction and stabilization and regulation of transcriptional activity of GR depends on this co factor. There are many other factors are also present but TTC5 is the main co factor because regulation of GR in gene depends on it (Kurachi and Kinoshita, 2019).

It was observed that in GR and ER ttc5 is hormone inducible factor where as in AR it non hormone inducible factor. In GR interaction different motifs are included for the binding of GR the LXLLIS the most important factor because modern mutation occurred in this arrangement which can be altered later with the receptor (Withers III et al., 2017). On the other hand many other motifs are also present like TRP motifs 2,3 and most important is 6 because most important mutation minimize in this motif. Whether hormone are present or not bing of GR is possible in both possibilities buy the most activity and affinity was observed in the presence of hormone. TTC5 might be necessary as molecular scaffold protein and can employ other cofactor during transcriptional process along with numerous enzymatic activity. In protein - protein interaction TPR motif plays the key role and total six motif are recognized in this structure. Hsps and p300 are also associated with TTC5 and GR the most difficult structure can be easier by the involvement of numerous motifs in GR and TTCF interaction many other substance can employ in context dependent manner (Kaddour et al., 2019). The most important factor for regulating the stability of P53 is TTCF same act is performed in GR regulation. The stability of GR is increased by TTC5 and important role is played in stabilization of endogenous GR. For stabilization impact interaction of TTC5 and GR is really very important. Because their no interaction between the TTCF mutant 1380 but able to stabilize GR when hormone is present (Marshall et al., 2018). Our observation indicates that TTC5 protects the receptor from the action of ubiquitin ligases like MDm2, which produce in minimization proteasome mediated degradation of GR. Stability of NRs and transcriptional factor are associated with transcriptional factor (Timm and Niemeyer, 2015). Whenever transcription is inhibited ubiquitination of Est-bound is delayed for cycling of promoter degradation of ubquinated ER is very important

for efficient transcription. GR promoter cycle is also known as proteasome dependent which clearly shows that ubiquitination is also a part of transcriptional regulation. Some of ubiquitin ligase like CHIP,Mdm2 and E6-ap are the part of of ubiquitin pathway and it shows that the effect on the function of GR. P300 shows the ubiquitin ligase performance and p53 work a substrate. Moreover many other substrate need to be recognized (Heather and Chain, 2016). For transcriptional performance ubiquition sometimes permit factor and co factor and also variate stability of GR. Transcriptional activity of GR may be affected by TTC5.variation in different factor may occurs according to the environment and sensitivity to cycling GR. For example TTC5 has great impact on transcription in genetic specific manner.by the stabilization of GR TTC5 can trigger the transcriptional activity but it is very rare because it is the only mechanism because the treatment of MG132 cell increases the transcriptional activity but it is not possible on GR protein level. It results that that inhibition of the proteasome associated with the TTC5 conscription to the GILZ promoter suggests an intricate association between the proteasome, TTC5, and regulation of GR transcriptional activity (Heather and Chain, 2016). For the regulation of transcriptional activity of GR it shows that TTC5 have to perform various mechanism and performance. In the regulation of GR the major function of TTC5 is controlled by stress. The activity and performance of numerous post transitionally modified version of TTC5, which could vary structure, habitation, arrangement and surface for the attachment of GR or other cofactor or TTC5 protein levels. That is the reason, different stress other than hormone could command the cellular response to glucocorticoids during stress condition. The transcriptional activity of GR is regulated by employing the multiple mechanisms by the TTC5.

TTC5's function in GR control seems to be motivated by stress. Stress. This could represent the results of translationally altered TTC5 variants, which could affect the transcription, composition, binding sites accessible for GR or other GR or protein levels of TTC5. Consequently, the cellular reaction to glucocorticoids can primarily be calculated under stressful conditions by cortisol rather than hormone (Skraly and Li, 2016). The TTC5 control of GR is expected to contribute to glucose corticus and GR's physiological function. The TTC5 regulates the transcription of GR target genes, including inflammation, involved in various processes. Duo TC5 has novel potential targets for different compounds which enable better control over glucocorticoid-related therapies, considering the importance of glucocorticoids in treating inflammatory disorders and in general clinical practice (Omidfar and Daneshpour, 2015).

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