ISSN: 2755-0168

Journal of Biotechnology & Bioinformatics Research

SCIENTIFIC Research and Community

Research Article

Open Access

Chromosome Gene, Hsl7 Gene in Vitro Studies

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ABSTRACT

Chromosome gene can expression many functions in human body. Eukaryotic cells are presence in yeast and human, in order to more understanding human gene, we use yeast as a model to research. The ORF (open reading frame) position of the Hsl7 gene is in yeast Saccharomyces YBR133C between 501804bp~504287bp of Chromosome II, with a total length of 2483bp, and the protein size is 95 kDa. Hsl7 gene is a methyltransferase. Protein-arginine N-methyltransferase involved in protein catabolism and regulation of the G2/M transition of mitosis; localizes to the septin collar of the bud neck, and the outer plaque of the spindle pole body in the G1 phase of the cell cycle (Saccharomyces Genome Database), Hsl7 (histone synthetic lethal 7) represent protein methylation, protein methylation is a type of post-translational modification that involves the addition of methyl groups to proteins, Protein methylation, including histone methylation and non-histone protein methylation, is one of the most important forms of posttranscriptional and epigenetic modifications.

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Received: August 03, 2024; **Accepted:**August 05, 2024; **Published:** August 19, 2024

Keywords: Arginine, Methyltransferease, Histone, Epigenetic

Introduction

Hsl7 gene is a methyltransferase, it's has a protein methylation function, Protein methylation is a type of post-translational modification featuring the addition of methyl groups to proteins. It can occur on the nitrogen-containing side-chains of arginine and lysine, but also at the amino- and carboxy-termini of a number of different proteins. Methylation can occur on multiple sites of proteins. For instance, arginine can be methylated once (monomethylated arginine) or twice (dimethylated arginine). Methylation of arginine residues is catalyzed by three different classes of protein arginine methyltransferases (PRMTs). Type I and II PRMTs both generate N G-monomethylarginine intermediates; PRMT7, the only known type III PRMT, produces only monomethylated arginine. Hsl7 (histone synthetic lethal 7) means histone methyltransferase, it's occurring on Histone H3 and Histone H4, our experiment goal is to defined Hsl7 function and it methylated on Histone.

Material and Method

PCR Amplified

Design primer, infront of 5'-terminal add NdeI restrict site CATATG and sense primer is CATATGCATAGCAACGTATTTGTTG, the primer at the 3 -end is TCACAGGCAGGGGAAAGGC, The Tm value determined by the 5 -terminal is 53 ° C, and the Tm value of the 3 end is 51°C. After determining the primer at both ends, you can prepare a polymerase chain reaction. First of all, you must first add the raw materials required for the polymerase chain reaction. There are Template, dNTP, Taq polymerase [TaKaRa Bio], The amount you need to add is ddH2O 18.5 μL, 10xbuffer 2.5 μl, primer (R) 1 μL, primer (L) 1μl, template 1μl, dNTP 0.5μl, Taq polymerase 0.5μl, total 25 μl. The primer synthesis was purchased from GENOMICS, the product was dry powder inside tube and need to dissolved in ddH2O and adjusted suitable concentration. The temperature set by the PCR [1]. 94°C 7 minute [2]. 94°C 30 second [3]. 55°C 45 second [4]. 72 °C 3 minute [5]. 72°C 10 minute, the step [4] back to step [2] and repeat, total 31 cycle. After running the PCR, the size of the electrophoresis confirmation fragment is 2484 BP, after running the second PCR, this time the first temperature was 55°C, and it was ready to increase the three degrees, and the number of cycles was used at 58°C. After running the PCR, run the electrophoresis to confirm whether the size of the fragment is 2484 BP. After confirming, change then change Taq polymerase to Taq+Pfu. After running PCR, run the electrophoresis again to confirm whether the size of the fragment is 2484 BP. [Figure 1]. Confirm the fragment correct then slice the fragment of the gel and read for DNA purify, we use kit for purified, the kit purchased by bio company, DNA purify also can use traditional method to do, it just kit spent less time, DNA recovery is 50 percent. Then purified product connect A on both ends, the material is 10 x buffer 3μl, (DNA) Sample 25μl, dNTP1 μl, Taq1μl, then 72°C reacts for 30 minutes, after connect A then purify the sample, the method is same as DNA purify, then run electrophoresis to confirm. The concentration needs 360ng to next step, one purified amount maybe not enough, collect many tubes amount to reach enough concentration.

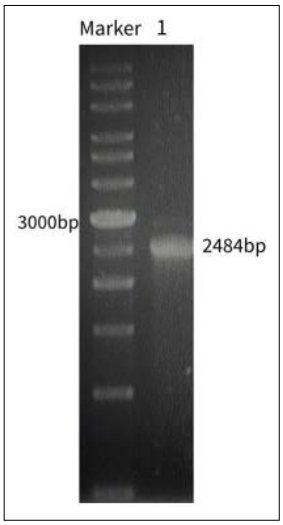

Figure 1: PCR Amplified Hsl7 gene, Lane 1 is Targeting gene, it also DNA Purified Photo

TA Cloning and Ligation

The next step is TA Cloning. The materials are10xbuffer1µl, T4 DNA Ligase 1 µl [TaKaRa Bio], pGEMT-easy vector2µl, DNA (insert) 6 µl [360ng], the materials are added, and placed on 4°C refrigerator 16-18 hours. Take Ta Cloning product transformation to DH5α competent cell, the operation needs on ice, take plasmid droplet on competent cell, put the centrifugal tube on the ice for 30 minutes, 42°C water bath Heat Shock for 45 seconds, put it on ice for 2 minutes, add 1 mL LB to mix and mix well, 90 minutes in the 37°C incubator, the speed is adjusted to 150-200rpm. prepare the IPTG 100 µL (100mM), X-Gal 50µg/ml 20 µl, inject the two into eppendorf, prepare to coating plate, and then pour the balls into the plate in the middle, inject mixed IPTG and X-Gals into the plate, shake the plate, so that the roller can evenly spread throughout the plate, then place the plate in aseptic laboratory table let it dry, prepare two LB culture plate containing Ampicillin, one is low concentration bacteria solution, and the other is high concentration bacteria solution. Take 150 µl of bacterial solution from the incubator 37°C, add the bacteria liquid on the culture plate, and apply it with a roller ball (this is a low concentration bacteria solution). Operation centrifugal tube (3000-4000 rpm, 10 minutes), remove 700 µl of clear liquid, and then mix the remaining upper liquid and liquid with precipitated cells. This is a high concentration bacteria solution. Put the plate at Incubator for 16-18 hours at 37°C. After the transformation was screened, the blue and white colonies will be produced on the plate. It is ready to choose the white bacteria. If it is not successful, prepare Ampicillin (10 mg/ml), LB, add 50 µl ampicillin to the test tube, add 5 ml LB to the test tube, then select the white bacteria, add it in the test tube and place it in the Incubator 16-18 hours, 37°C. After the transformation, the blue and white colonies will be produced on the plate. It is ready to choose the white bacteria. White colonie is successful insert into vector, blue colonie is failure, prepare Ampicillin (10 mg/ml), LB, add take 50 µl ampicillin to the tube, add 5 ml LB to the tube, then select the white bacteria, add it in the tube and place it in the incubator 16-18 hours, 37°C. Then prepare 30% Glycerol and sterilized eppendorf, 30% Glycerol and 200 µl of bacterial liquid, add the volume ratio of 1: 1 to the eppendorf and mix well. The total of bacteria and Glycerol is 400 µL. After mixing, the eppendorf is stored in -80°C. Ready for plasmis extraction, prepare Solution I, Solution II, Solution III. Solution I: C6H12O6 50 Mm, Tris-CL (PH 8.0) 25 mm, EDTA (pH 8.0) 10 mm, RNase. Solution II: NaOH 0.2 N, SDS 1%. Solution III: 5M KAC 60 ml, 11.5 ml of ice acetic acid, add ddH2O to 100 ml. Then take 1.5 mL bacteria solution, centrifuge at 5000 rpm for 10 minutes in centrifuge, and then remove the upper liquid to leave the precipitated cells. 2 add ice-cold Solution I 200 µl (including RNase 40 µg/ml), and vortex, then remove the liquid and place into eppendorf. Add Solution II 200 µL and rotate eppendorf five times to mix it. Add ice-cold Solution III 300 µL, flip slightly five times, insert eppendorf into the ice for 3-5 minutes. 5 minutes of centrifugation in 13500 rpm, take out the liquid to the new eppendorf. Add 700 µl phenol: chloroform (1: 1, v/v) vortex. Then put in centrifugal 13500 rpm 2 minutes. Take out 400 µl of clear liquid to the new eppendorf, take 2x volume 95 % ETOH (ice-cold, -20°C), and vortex, and place it for 2 minutes. Centrifuged 13500 rpm for 5 minutes and remove the upper liquid. Add 1 ml 70% EtOH (ice-cold, -20°C), centrifuged 13500 rpm for 2 minutes (this step is to remove the upper clearing liquid, then add 70% EtOH, and then centrifuged 13500 rpm for 2 minutes. Remove the upper liquid and dry the vacuum for 10 minutes. Add 50 µl ddH2O (including RNase 40 µg/ml) into tube. Ready for check plasmid, run electrophorisis to confirm DNA then we use restrict enzyme [TaKaRa Bio] EcoRI to cut plasmid, prepare ddH2O, EcoRI Buffer, EcoRI, plasmid, materials are added in eppendorf, put it in 37°C water bath for 2 hours. Then run electrophorisis, confirm the sequence correct. DNA sequence correct then ready for ligation to vector. Use restrict enzyme EcoRI [TaKaRa Bio] and NdeI [TaKaRa Bio] to cut pET28c vector [Novagen], also cut the TA clone with EcoRI and Nde I, and the segment of the HSL7 gene cut from pGEMTesy vector to ligation with pET28c vector. Before checked pET28c vector, we use restric enzyme EcoRI and ClaI [TaKaRa Bio] to check pET28c vector, the materials are ddH2O, EcorI buffer, EcorI enzyme, Plasmid (pET28C Vector), ClaI enzyme, ClaI buffer. All material added then put on water bath an hour and a half. Run electrophoresis to confirm the sequence correct. After the examination is correct, then can cut the pET28c vector with the enzyme EcoRI and NdeI, materials are EcorI Buffer, EcorI enzyme, plasmid (pET28c vector), NdeI enzyme, NdeI buffer, all materials added then put on water bath three hours 37°C, then use DNA purified method to purified pET28c vector, then we need to cut the end of 5' terminal phosphate, use CIAP (Calf Intestinal Alkaline Phosphatase) and CIAP buffer, buffer and enzyme added into eppendorf, After that, put on the dry bath 37°C for 15 minutes, and the water bath 50°C for 15 minutes. Then use DNA purified method to plasmid. Before ligation, we need to know quentity of Hsl7 gene [insert] and pET28c vector, run electrophoresis and check, we need insert 1µl is 9 ng, vector 1µl is 23.25ng. Ligation materials are 10x T4 DNA Ligase Buffer, vector (pET28c), T4 DNA ligase, insert (Hsl7 gene), ddH2O. After all the materials are added, the pipettman suction is uniformly mixed and placed in 4°C, 12-16 hours. After the ligation is finished, perform transformation, culture 5ml LB, Kanamycine (50 mg/ml) of bacterial liquid, extrac plasmid and check is Hsl7 gene ligation to vector successfully. pET28c vector had two cut sites are EcoRV and EcoRI, use these two-cut site to check fragment. then expression Hsl7 protein with E.Coli. the ligation product transform to BL21(DE3), after transformation to BL21(DE3), then culture 5ml LB (kanamycine 50 mg/ml) and extract plasmid, and then use EcoRV to confirm it success sent into BL21(DE3), then stored in -80°C refrigerators.

Protein Expression

Ready for Escherichia coli BL21(DE3) protein expression, at first culture 5 ml LB (ampicillin 10mg/ml), and then put in incubator 37°C shaking until OD600. Then take LB dilute to OD600=0.05,

final volume 100ml, then put in incubator culture to D600=0.6, add lactose 0.5% harvest at 17,18 hour. but in this condition didn't find protein expression, so change the solution LB, we add sorbitol, sorbitol materials are tryptone 10g, Yeast- Extract 5g, NaCl 10g, D-Sorbitol (molecular weight 182.2) 91.1g ddH2O 1000ml, (John R. Blackwell and Roger Horgan 1991), Knowing that Sorbitol can improve the soluble of the recombinant protein and help the protein manifestation, we take BL21 (DE3) out from refrigerator -80°C and culture in 5ml LB for 22 hours, O.D600=2.41,so take the bacteria 4.3ml, LB+Sorbitol 95.7ml, amp(10mg/ml), and finally the volume of 100 ml, O.D600= 0.6,add lactose 0.5%,temperature 18°C in incubator [150-200 rpm], harvest at 48 hour. [Figure.2] This step is use pET28c vector to protein expression, we also want to know is another vector can have the same quantity, so we choose pMAL-c2x vector, use restrict enzyme SalI [TaKaRa Bio] and EcoRV [TaKaRa Bio] to cut vector and insert Hsl7 gene into vector to ligation, then transform to Escherichia coli tRNA380 and Escherichia coli Rosetta, use IPTG 0.8 mM, 30°C.we found the pET28c vector in BL21(DE3) has better protein expression than pMAL-c2x vector in tRNA380 and Rosetta, so next step we use pET28c vector in BL21(DE3) to experiment.

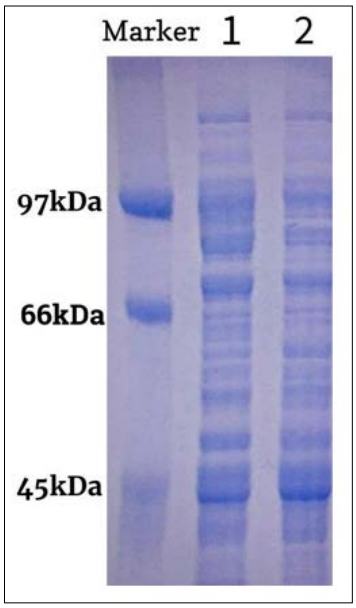

Figure 2: Before Purified, Cell Extract and Running SDS-PAGE, Hsl7 Protein. Lane 1 is Hsl7 gene Expression to Hsl7p is 95kDa, Lane 2 is E. Coli Strain without Hsl7 gene

Protein Purification

Then we purified protein, prepare materials are Lysis buffer [Tris-base 3.0285g, 6mM 2- mercaptoethanol, ddH2O 500ml, glycerol 100% 50ml,final pH7.4], then use ultrasonic processor to break cell, this experiment is design for His-tag [BD TALON™ Metal Affinity Resins], so we need prepare solution, equilibration/ washing buffer(50mM PB, 300 mM NaCl), pH7.0, elution buffer(50 mM PB, 300 mM NaCl, 150 mM imidazole), pH7.0, 20 mM 2-(Nmorpholine)-ethanesulfonic acid(MES), pH 5.0, 20% EtOH(contain1% azide), ddH2O. We use medical syringe, remove the needle, use it as a column without needle, then put the resin inside bottom, the gel was amylose [2ml], purification steps (1) pour into 10-20 mL Equilibration/Washing Buffer. (2) pour into break cell liquid 9ml. (3) Add about 10-20ml equilibration/washing buffer. (4) 2ml elution buffer. (5) 10ml 20mM MES through the column. (6) 10-20 ml ddH2O washing. Then run SDS-PAGE to check protein quantity, we found protein quantity not much, so we want to get more protein, we use 200ml cell liquid to purified [Figure.3].

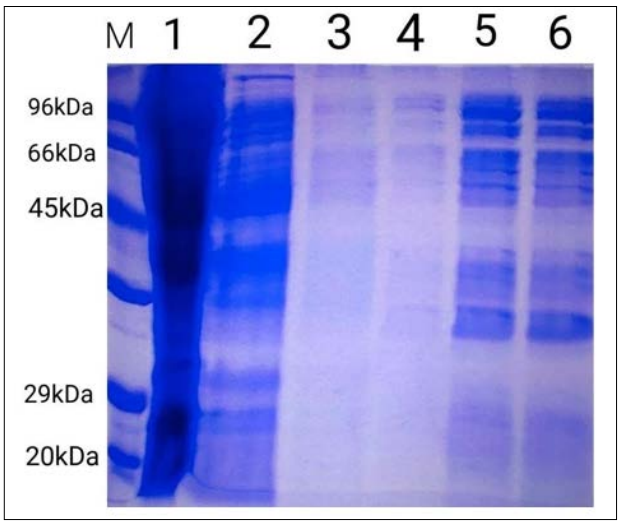

Figure 3: Purified 200ml Cell Extract Liquid and Running SDS-PAGE figure, M: Marker, Lane 1 is total Cell Extract Liquid, Lane 2 is added Wash Buffer, Lane 3 also added Wash Buffer, Lane 4 and Lane 5 use Elution Buffer, Lane 6 use MES

Isotope Labelling

Then we test the methyltransferase activity, prepare SIGMA H 9250 Histone Type II-A, SIGMA H 5505 Histone Type III-S, SIGMA MYELIN BASIC PROTEIN M1891, Hot SAM (S-adenosyl methionine), PB(pH7.0), we also prepare Yeast Histone, cultured YBR133C [ΔHsl7] 100ml to O.D600=0.648,25°C,6500rpm added 1ml YPD, 10000rpm, added 200µl PB, 200µl glass bead, 4°C High speed extract cell, 4°C 10000rpm, 10 minute, upper liquid is lysate. We use isotope labelling, use 3H to label, dry bath 30°C, the substrate and Hsl7p added into eppendorf, we need added benzonase, benzonase could decompose nucleic acid, then run 18% SDS PAGE, the film put in -80°C for a week.

Figure 4: Before Film Expose, Isotope Labelling Film. It can see the Dark Band

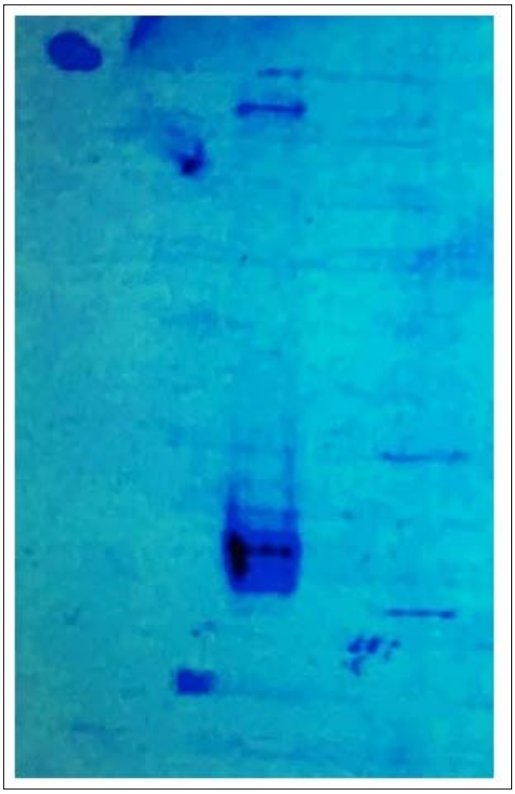

Figure 5: Isotope Film Exposed to Light

Result

To realized protein methylation, we need to expression Hsl7 gene and produced Hsl7p, this protein activity defined methylation process, at this experiment, we saw the methylation reaction.

Discussion

In this experiment, we success simulation the cell work, from DNA to protein, in His-tag protein expression. We first use BL21 (DE3) to induce at 30°C, but there is no protein expression. Then we change the temperature, use 18°C to induce it. But found the problem of inclusion body, in order to solve this problem, we add sorbitol in LB, hoping to stabilize the growth of E. Coli so that the cells can be more stable to produced protein, in purified protein, our column is roughly, it could change a better column to get more pure protein, In isotope label, we use Histone, Sbp1, Myelin basic protein (MBP), Calf thymus histone as a substrate, the yeast Histone extract, we got fifty percent Histone, it still can improve it, the band in picture is not easy to see, so we use computer to made the film expose to light and we can see clearly band, Histne molecular weight is 20 kDa to 29 kDa, and sbp1 molecular weight is 56kDa, it closely to picture band. The Hsl7 gene expression slowly, its expression in older cell stage, about G2/M stage, most protein expression in young cell stage, why Hsl7 gene expression in older stage have large expression, it still has a disscussion space [6-20].

Conclusion

In this paper we use yeast as a model for studying human genes, this is because yeast and human cells are both eukaryotic cells, but yeast is more convenient to use. This paper uses genes on chromosomes for research, the reason for choosing the HSL7 gene is that it was found in past reports that the HSL7 gene plays a methylation function. In the past, reproduction could only be observed through budding. Now we can use protein expression methods and understand the function of proteins. There are several important points in this article, one of them is temperature and the other is time, this gene has complete protein expression at 48 hours, but at this time, the cells have aged, and the reactions within the cells are very complicated, what causes methylation is still unclear. The method used in this paper can provide scientists with a new research method, it is hoped that in the future we will better understand the function of methylation.

Acknowledge

Thanks for Ming kai chern and classmate, also thanks National Taiwan University College of Medicine offer an isotope room to experiment, thanks Tam Ming F Professor.

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