Developing tools for biochemical studies of *Saccharomyces cerevisiae* nuclear envelope proteins Brr6 and Brl1

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INTRODUCTION

There are many different strains of yeast that have been genetically studied for centuries, and specifically, *Saccharomyces cerevisiae*, commonly referred to as bakers yeast, has been widely characterized. *S. cerevisiae* is a eukaryotic microorganisms that is classified under the kingdom fungi. In 1680 when yeast was first looked at microscopically, it was seen as a non-living, round, globular structure. Louis Pasteur later proved that in fact, these unicellular microorganisms were living organisms that played a crucial role in fermentation. The growth of bioinformatics has made *S. cerevisiae* a common model in the study of genetics.
The reason for the popular use of *S. cerevisiae* in scientific research is due to its homology with human cells. Processes such as the cell cycle, DNA replication, and recombination are carried out very similarly between both humans and yeasts. Much is known about yeast and human processes on a DNA level, however not much has been documented on the protein level. There are many different proteins found within the yeast genome that are only characterized on the functional level.

The nuclear envelope of eukaryotic cells compartmentalizes the nuclear material and separates it from the cytoplasm (Lone *et al*. year). Nuclear pore complexes facilitate all transport between nucleus and cytoplasm. BRR6 and BRL1 are essential nuclear envelope integral membrane proteins that function in lipid homeostasis (yeastgenome). Outside of that, very little is known about these specific genes on a protein level. Aspects unknown include localization, potential interactions and conditional mutations. Due to the critical role of BRR6 and BRL1 in the formation of the nuclear pore complexes, we wished to develop tools to biochemically study BRR6 and BRL1 more extensively; however, this required these proteins to be fused with tags such that the cloning and transformation into vectors, allowed the ability to regulate expression.
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Principal Approaches

Liquid Culture Preparation and DNA Extraction

5ml of liquid YPD media was added to sterile test tube. Scrapings from frozen stocks were put into separate sterile test tubes and incubated in a 37°C shaker. DNA was subsequently extracted following 24-hour overnight culture incubation period. DNA was extracted using QIAprep Spin MiniPrep Kit. Samples were then ran on a BioTek Epoch Spectrophotometer and analyzed using Gen 5 software to determine concentration.After 24-hour incubation period of overnight liquid culture of FY86, DNA was extracted using YeaStar Genomic DNA Kit.

Primer Construction

Plasmids (PDDGFP_LEUD, PYc46 and PYc48) were chosen using addgene software to find vectors that the genes of interest could be ligated into. Primers were constructed using Gene Construction Kit Software. PDDGFP_LEUD plasmid used Sma1 as a restriction site for the plasmid. PYc46 and Pyc48 both used Sac1 as the forward primer and BAMH1 as the reverse primer. 1kb were added to both sides of BRR6 and BRL1 for the PYc46 and PYc48 plasmid to accommodate to promoter.

PCR Sample Preparation

10ul of 2x Master mix was combined with 3ul of sterile water. Added to that mixture was 1ul of FY86 yeast DNA that was previously extracted. Finally, 3ul of both forward and reverse primers were added into their respective PCR sample, creating a total of 12 samples. PCR samples were run overnight using a Bio Rad T100 Thermal Cycler. PCR conditions were as follows: 95°C for 4 hours for denaturation, 50°C for 1 hour for annealing, 72°C for 6 ½ hours for extension and then consequentially held at 12°C.

Gel Electrophoresis/ DNA Extraction of BRR6 and BRL1

PCR samples were then run on a 1% agarose gel at 093amps for 45 minutes. After gel analysis, DNA was extracted from the gel using QIAquick Gel Extraction Kit.

Overnight Plasmid Culture

Plasmids were grown out on LB +ampicillin agar plates overnight and incubated at 37°C. Plasmid cultures were then scraped from plate following 24 hour incubation period and grown in liquid LB +ampicillin media.

DNA Extraction and Digestion

Following 24 hours incubation of liquid plasmid culture, the DNA was extracted using QIAprep Spin MiniPrep Kit. Following extraction2 digestions were run for each sample. To the plasmid sample, Cutsmart buffer was added to a mix of vector DNA and its corresponding restriction enzyme.

Samples were then filled with sterile water to create a total reaction amount of 20ul. As with plasmids, 2 digestions were run for each insert sample. Cutsmart buffer was mixed with the inserts corresponding restriction enzyme. Instead of water insert DNA was added to produce a total volume reaction of 20ul. All PDDGFP_LEUD related digestions were run at 25°C overnight due to Sma1 restriction enzyme protocol. Other digestions were incubated overnight in a 37°C water bath.

DNA Digestion Gel Electrophoresis/ DNA Extraction

Before samples were run on 1% agarose gel, all plasmid digestion samples were treated with CIP enzyme. After CIP treatment, 3ul of loading dye and EZ-Vis were added to each sample to prepare reactions for gel electrophoresis. Gel ran at amplitude of 093 for 45 minutes. Once gel was complete and analyzed, DNA was extracted using QIAquick Gel Extraction Kit.

DNA Ligations and transformation

DNA ligations were setup using the NEBio labs ligation calculator software. A ratio of 5:1 insert to vector was used. To reach a total volume of 20ul, 4ul of 10x DNA ligase buffer and 1ul of T4 DNA ligase were added to a mixture of insert and vector DNA. Final volume was reached by adding sterile water. Ligations for PDDGFP_LEUD were run at 25°C for 2 hours, due to the blunt cutting of the restriction enzyme. All other ligations were run at 25°C for 10 minutes.

After completed ligations, One Shot Top10 Competent Cells protocol was used to for transformations.

Present Knowledge

In order to determine if products of PCR, DNA extraction and DNA digestions were what we expected, gel electrophoresis was run after each step. These gels were then analyzed under a UV light to determine if necessary bands were in fact present.

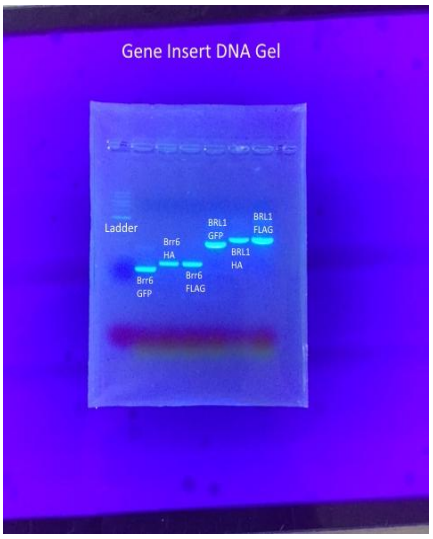

Figure 1. Gel of PCR Products

Figure 1 displays the result of the gel that was run using the amplified PCR insert products. On the far left in lane 1, the standard of a 1kb ladder is shown to be present. In lane 2, the BRR6 GFP band is present around 570bp. Lane 3, in the BRR6 HA insert with a band roughly around 710bp. In lane 4 we have BRR6 FLAG insert, with a band at roughly 730bp. Lane 5 BRL1 GFP insert has a band present at roughly 1390bp. Lane 6 has a band present at around 1630bp to represent BRL1 HA insert. In the last lane, lane 7, a band at 1670bp represent the BRL1 FLAG insert.

Sample	ng/ul
GFP	528
HA	273
FLAG	395
BRR6 GFP	12
BRR6 HA	11

Sample	ng/ul
BRR6 FLAG	17
BRL1 GFP	21
BRL1 HA	10
BRL1 FLAG	21

Figure 2. PCR Concentration Values

In figure 2 presented above, the tables display the concentrations of the PCR products. The GFP plasmid had the highest concentration with a value of 528ng/ul. The HA plasmid displayed a value of 273ng/ul, while the FLAG plasmid displayed the second highest concentration with a values of 395ng/ul. Following the plasmids were the BRR6 and BRL1 insert values respectively. BRR6 GFP had a value of 12ng/ul. BRR6 HA displayed the lowest value of the BRR6 inserts with a value of 11ng/ul and lastly BRR6 FLAG displayed the highest concentration reading with a value of 17ng/ul. BRL1 GFP and BRL1 FLAG both had the highest concentration value with a value of 21ng/ul. BRL1 HA had the lowest with a value at only 10ng/ul.

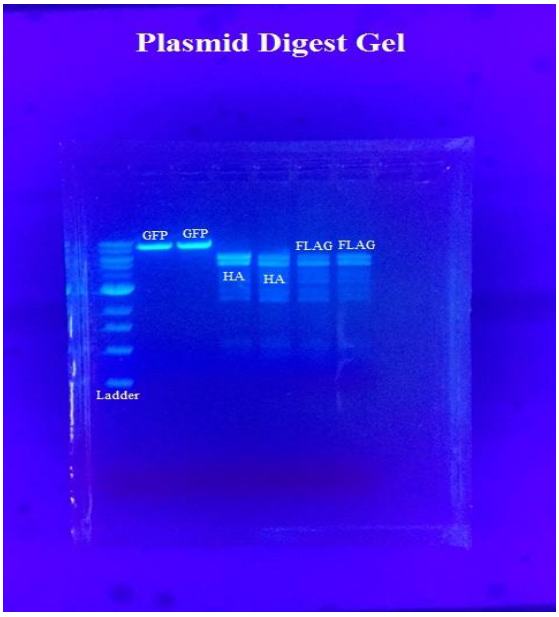

Figure 3 displays the result of the gel that was run using the digested plasmid products. On the far left in lane 1, the standard of a 1kb ladder is shown to be present. In lane 2, the GFP band is present around 8693bp. Lane 3, the second GFP digest sample, has a band at 8693bp, which is the same as the GFP sample in lane 2. In lane 4 we have the HA digest product, with a band at roughly 5915bp. Lane 5 has a band at 5915bp just like the first HA digest sample in lane 4. Lane 6 has a band present at around 5915bp to represent FLAG digest products. In the last lane, lane 7, a band at 5915bp the second FLAG digest sample.

Figure 3--Plasmid DNA digest gel

Sample	ng/ul
GFP	10.27
GFP (lane 3)	15.03
HA	6.91
Sample	ng/ul
HA (lane 5)	9.13
FLAG	9.50
FLAG (lane 7)	7.69

Figure 4--Plasmid concentrations

In figure 4 presented above, the tables display the concentration on the digested plasmid products. The GFP plasmid in lane 2 displayed the second highest value of 10.27 ng/ul. The second GFP in lane 3 displayed the highest concentration value of 15.03 ng/ul. The HA plasmid in lane 4 displayed a value of 6.91ng/ul, while the second HA digested product displayed a higher value of 9.13ng/ul. Lastly, the FLAG plasmid products in lanes 6 and lane 7, displayed concentration values of 9.50ng/ul and 7.69ng/ul respectively.

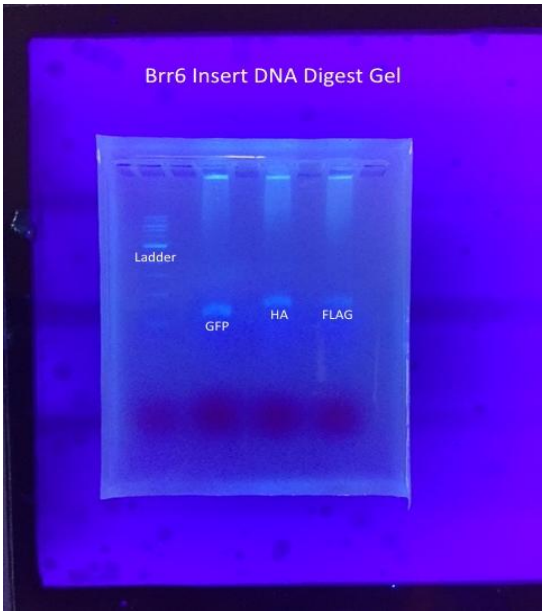

Figure 5. BRR6 Insert Gels

Figure 5 displays the result of the gel that was run using the digested BRR6 plasmid products. On the far left in lane 1, the standard of a 1kb ladder is shown to be present. In lane 3, the BRR6 GFP insert band is present around 570bp. Lane 5, the BRR6 HA insert digest sample, has a band at 710bp. In lane 7, the band for the BRR6 FLAG, has a band at roughly 730bp.

Sample	ng/ul
BRR6 GFP	11.59
BRR6 HA	5.32
BRR6 FLAG	11.52

Figure 6. BRR6 Insert Digest Concentrations

In figure 6 presented above, the tables display the concentration on the BRR6 digested insert products. The BRR6 GFP sample displayed the highest concentration of the BRR6 inserts with a value of 11.59ng/ul. The BRR6 HA insert product with a value of 5.32ng/ul displayed the lowest concentration value. BRR6 FLAG insert digest displayed a value of 11.52ng/ul.

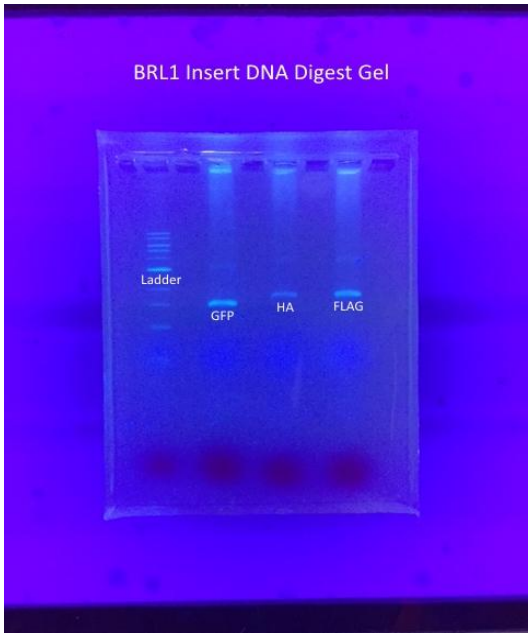

Figure 7 above displays the result of the gel that was run using the digested BRL1 insert products. In Lane 1, the standard 1kb ladder band is present. In Lane 3, The GFP BRL1 digest insert product band was present around the 1390kb mark. Lane 5 was loaded with the BRL1 HA insert digest product. This sample displayed a band around the 1630kb area. Lastly in Lane 7, there is a band located in the 1670kb region of the gel. This band is representative of the BRL1 FLAG digest product.

Sample	ng/ul
BRL1 GFP	12.04
BRL1 HA	5.87
BRL1 FLAG	4.00

Figure 8. BRL1 Insert Digestion Concentrations

In figure 8 presented above, the tables display the concentration on the BRL1 digested insert products. The BRL1 GFP sample displayed the highest concentration of the BRR6 inserts with a value of 12.04ng/ul. The BRL1 HA insert product with a value of 5.87ng/ul displayed the lowest concentration value. BRL1 FLAG insert digest displayed a value of 4.00ng/ul.

DISCUSSION/ CONCLUSION

The DNA concentrations of the plasmids were determined after extraction using the QIAprep Spin MiniPrep Kit. The concentrations of GFP, HA and FLAG displayed values of 528ng/ul, 273ng/ul and 395ng/ul respectively. This indicates that the DNA extractions were successfuly. The PCR samples were setup and prepared without error due to the bright bands being present at each appropriate region of the agarose gel (figure 1). There was expected to be a band around the 570bp region to indicate the successful amplification of the BRR6 GFP inserts. In fact there was a band present within that region based on the placement of the band compared to the 1kb ladder. For BRR6 HA, the band on the gel was found to be present at 710bp as expected. Lastly for the BRR6 inserts, the band in lane 4 is represented around the 730bp region and displaying the amplification of the BRR6 FLAG insert. To confirm the successful amplification of the BRR6 inserts, their DNA concentrations were read on a plate reader. Their concentrations were read on using a BioTek Epoch Spectrophotometer and analyzed using Gen 5 software. The concentrations were as follows, BRR6 GFP 12.00ng/ul, 11.00ng/ul for BRR6 HA and 17.00ng/ul for BRR6 FLAG. With these values displaying double digits >10ng/ul, this further confirmed that the insert genes were amplified and DNA was extracted successfully. The BRL1 gene inserts were also simultaneously run through the PCR. These inserts were also run on the same gel as the BRR6 inserts mentioned above. The BRL1 inserts were expected to display bands in a higher region on the gel than the BRR6 inserts. After analysis of the gel, the bands are in fact present and in the proper region of the gel. The BRL1 GFP insert is displayed at roughly the 1390bp region. The BRL1 HA and the BRL1 FLAG inserts are very close together ranging within only 40bp of each other. The BRL1 HA insert is determined to be at the 1630bp position on the gel, while the BRL1 FLAG insert is displayed in the same region but at the 1670bp position. As with the BRR6 inserts, further confirmation was needed in order to confirm the successful amplification of the BRL1 inserts. These samples were also read using a BioTek Epoch Spectrophotometer and analyzed using Gen 5 software. The BRL1 GFP, BRL1 HA and BRL1 FLAG samples displayed DNA concentration values of 21.00ng/ul, 10.00ng/ul and 21.00ng/ul respectively. With the concentration values reading >10ng/ul, for all samples, it can be concluded that the PCR reaction and DNA extraction of the BRL1 inserts was successful.

Digestions were then performed on all the samples after the PCR process and DNA extractions. The digestions were run without error in the preparation. This can be concluded due to the analysis of the agarose gels (figures 3,5 &7). Figure 3 displays the gel on which the plasmid digests were run on. Each plasmid had two samples run to ensure validity. Analysis of the gel in lane 2 and lane 3 shows the expected single bands at roughly the 8693bp region this would correlate to the presence of the properly digested pDDGFP_LEU2D plasmid. The next two plasmids, pYc46 (FLAG) and pYc48 (HA), were expected to have bands presents at regions below the pDDGFP_LEU2D plasmid. Analysis of the gel (figure 3), further confirmed the successful digestion of the pYc46 and pYc48 plasmids. Lane 4 and lane 5 were loaded with the pYc48 plasmid digest and displays bands around the 5915bp region. Instead of a single band being present, there is double bands/non-specific cutting present in the lane. This could be due to the two different restriction enzymes used to cut the plasmid; leaving a trail of impurities below the pure digested plasmid product. During DNA excision of the gel, the two brighter bands were excised from the gel, while the trailing bands were discarded. Lane 6 and lane 7 displayed the same pattern as mentioned above for lanes 4 & 5. There seemed to be double bands present along with non-specific cutting with trailing impurities at the 5915bp region. Lanes 6 and 7 were, however, loaded with pYc48 plasmid. This plasmid also used two different restriction enzymes in the digestion process. This is believed to be the cause for the double banding present. Like lanes 4 & 5, during DNA excision, the two brightest bands were excised, while the rest was discarded. These samples were also read using a BioTek Epoch Spectrophotometer and analyzed using Gen 5 software. The pDDGFP_LEU2D, pYc48 (HA) and pYc46 (FLAG) samples displayed DNA concentration values of 10.27ng/ul (lane 2), 15.03ng/ul (lane 3), 6.91ng/ul (lane 4), 9.13ng/ul (lane 5), 9.50ng/ul (lane 6) and 7.69ng/ul (lane 7) respectively. The values of lanes 2 and 3 are great values meaning that there is a high concentration of the digested GFP plasmid DNA. Values for the other lanes were all <10.00ng/ul, while not what we would have liked to see, this reading is compensated for in the ligation protocol. After digestion of the plasmids, the pure products were all treated with Calf Intestinal Alkaline Phosphatase (CIP). The use of this enzyme was to ensure that the ends of the excised DNA would not reattach to itself.

Subsequently after the plasmid digestions, the insert genes were also run through the digestion process. Figure 5 displays the agarose gel that the BRR6 inserts were run on. Lane 3 was loaded with the BRR6 GFP insert. This band was present at roughly the 570bp region, which is what was expected. The next two bands for the BRR6 HA and BRR6 FLAG inserts were appropriately present around the 710bp and 730bp regions on the gel. Due

to the presence of only single bands, the bands were easily excised for DNA extraction. To determine the concentrations of the of the excised DNA, the samples were read using a BioTek Epoch Spectrophotometer and analyzed using Gen 5 software. The BRR6 GFP sample contained a concentration of 11.59ng/ul, the BRR6 HA concentration was determined to be 5.32ng/ul and lastly, the BRR6 FLAG concentration was 11.52ng/ul. While a concentration of >10ng/ul would have been best, any samples that read less than that was compensated for in the ligation protocol.

The next sets of inserts digested were the BRL1 inserts. Figure 7 above displays the results of the electro-gelphoresis for these inserts. Lane 3 correlates to the BRL1 GFP insert. This lane displays a band at the 1390bp region, which was expected. Lane 5 was loaded with the BRL1 HA insert and displayed a band around the 1630bp region, which what was expected. Lastly, the BRL1 FLAG digested product was loaded into lane 7. This lane contained a band located at the 1670bp region, which was again the expected result. The samples DNA concentrations were then to be determined using the BioTek Epoch Spectrophotometer and analyzed using Gen 5 software. The concentrations of the BRL1 GFP, BRL1 HA and BRL1 FLAG inserts were determined to be 12.04ng/ul, 5.87ng/ul and 4.00ng/ul respectively. The value for the BRL1 GFP insert was the highest and was around the value of what we wanted. However, the BRL1 HA and the BRLA FLAG inserts displayed values of >10.00ng/ul meaning that it was to be compensated for in the ligation protocol.

After DNA excision from the gels of the digested insert and plasmid products, the digested products underwent a ligation reaction. The ligation reactions were calculated out using New England BioLabs ligation calculator. The ratio of digested insert to digested plasmid product was 5:1. The ligation reaction seemed to be prepared without error. However, here in the research is where there could have been a compromising error. The ligated products after appropriate incubation time were transformed using Invitrogen's One Shot TOP10 Competent Cells. The cells were then smeared on LB +ampicillin agar plates for the growth of the colonies. After designated incubation time, the agar plates appeared to have no growth at all. This means that there was an error in the experimentation sometime between the ligation phase and transformation. After no growth the first time, new restriction enzymes were ordered and new TOP10 Competent Cells were ordered to see if that would in fact reverse the error. Unfortunately, that did not occur and trials two and three ended with the same result of no growth. There is a possibility of the insert digest products not lining up properly so that they insert into the vector correctly causing a malformed non-functioning ligated product. The error has yet to be identified; however, further studies will be conducted to identify the error. The ultimate future of this research is to properly transform the inserts of interest into the vectors and from there manipulate different aspects of the gene and determine its ability to function properly.

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