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Mutations in Phd and Their Effects on Repression and Corepression

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MUTATIONS IN PHD AND THEIR EFFECTS ON REPRESSION AND COREPRESSION

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Abstract

Phd and DocH66Y are products of the P1 plasmid addiction operon. These products serve to negatively regulate the transcription of the operon, i.e. when more of the products are present, transcription of the operon is repressed (Lehnherr). The level of transcription may be measured by what is known as a β-galactosidase assay.

β-galactosidase is an enzyme encoded by the *lacZ* gene in *Escherichia coli*. In our test strains, the *lacZ* gene has been placed downstream from the P1 promoter. Consequently, the amount of β-galactosidase produced is proportional to the expression of the P1 operon. *In vivo*, β-galactosidase cleaves lactose into glucose and galactose, to make these products available to the cell. *In vitro*, O-nitrophenyl-β-D-galactoside (ONPG) also acts as a substrate, and will yield glucose and O-nitrophenol when cleaved. ONP is yellow in color, and can be quantified by means of a spectrophotometer. Using these values, the level of gene transcription can be measured; by this virtue, the amount of repression can also be determined.

DocH66Y and Phd form a complex that represses transcription of the P1 operon. This repression occurs because the complex binds to the promoter region of the operon and interferes with transcription by RNA polymerase (Magnuson). It was hypothesized that the deletions in Phd would affect the interaction between DocH66Y and Phd.

My hypothesis is that deletions and point mutations in the C-terminal of Phd will have a specific effect on corepression. My project will focus on these mutations and their effects on repression and corepression.

Introduction

It is known that the P1 plasmid addiction operon encodes two products, DocH66Y and Phd (Lehnherr). It is also known that Phd and DocH66Y form a complex which binds to the promoter region on the DNA; the binding of this complex serves to negatively regulate the transcription of the P1 operon (Magnuson). The purpose of the first part of this project was to create and test deletions in the C-terminal of Phd, and determine whether the deletions would have an effect on repression and corepression. These mutants were obtained from a colleague (Balasubramanian). The mutants were then tested for repression and corepression. The second part of the experiment was designed after obtaining the results for the first part, in order to obtain more precise information about the nature of the C-terminal of Phd. The mutants were created by preparing a plasmid miniprep from preexisting strains of bacteria, then transforming the plasmids into a strain without plasmids, DIT013.

Figure 1

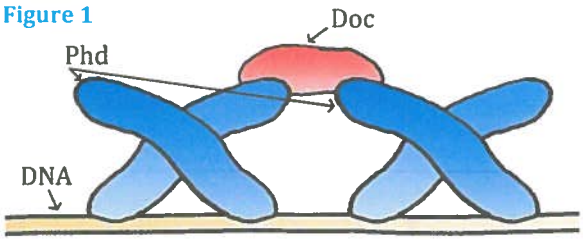

Figure 1 *shows a schematic representation of the Phd-Doc complex.*

Figure 2

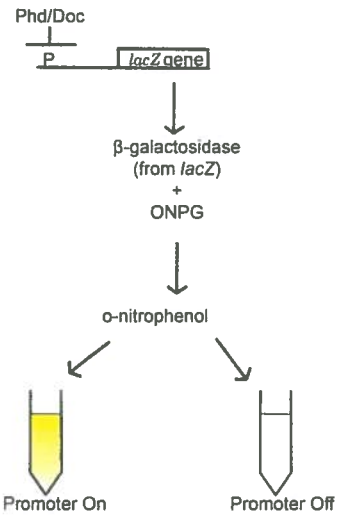

Figure 2 *shows how β-galactosidase specific activity may be measured.*

Central to this experiment was the β-galactosidase assay. This assay measures the amount of transcription taking place in the bacterial cell. Figure 2 elucidates the way in which β-galactosidase specific activity may be measured. In this figure, Phd and DocH66Y negatively regulate the

transcription of the operon. The β-galactosidase that is produced acts on ONPG, yielding yellow-colored ONP that may then be quantified spectrophotometrically.

This experiment, as noted above, was divided into two sections, the **First Experiment** and the **Second Experiment**. The First Experiment involved making two Phd mutants; one mutant had a 6-amino acid C-terminus deletion and the other mutant had a 10-amino acid C-terminus deletion. The Second Experiment was developed after interpreting the results of the First Experiment; because it was determined that the smaller deletion (the 6-amino acid deletion) did not appreciably affect repression or corepression, attention was focused on the amino acids absent in the larger deletion but present in the smaller deletion.

Methods

Qiagen Miniprep

The Quiagen Miniprep removes plasmid DNA from cells containing plasmids of interest. This process was used to obtain the raw DNA necessary to perform transformations.

1. The strain containing the plasmid of interest was grown on an LB plate with antibiotics.
2. An overnight culture was prepared from these cells, allowing an incubation time of 18 hours at 30°C.
3. The overnight culture was centrifuged for 5 minutes.
4. The supernatant was aspirated off and the pellet was retained.
5. The pellet was resuspended in 250μL Buffer P1 and transferred to a 1.5mL microcentrifuge tube.

6. 250μL Buffer P2 were added and the tube was inverted 4 times to mix.
7. The tube was incubated at room temperature for a maximum of 5 minutes.
8. 300μL Buffer N3 were added and the tube was inverted 4 times to mix.
9. The solution was centrifuged for 10 minutes at 13,000 rpm.
10. The clear lysate was placed in a prelabeled QIAprep spin column + Collection Tube.
11. The tube was centrifuged for 1 minute at 13,000 rpm.
12. The flow through was poured out of the collection tube and the column was reinserted into the collection tube.
13. 750μL Buffer PE + EtOH were added to the Spin Column.
14. The column was spun for 1 minute at 13,000 rpm and the flow through was discarded.
15. The column was spun for an additional minute to remove excess buffer.
16. The column was transferred to a new prelabeled 1.5mL microcentrifuge tube and 50μL buffer EB was added to the Spin Column.
17. The column was then spun for 1 minute at 13,000 rpm and the column was discarded.
18. The plasmid DNA was stored at 4°C until it was needed.

Transformations

Transformation is the process of introducing foreign DNA plasmids into a competent strain of bacteria. Because this process was used frequently during this experiment, it will be outlined in detail here and simply referred to henceforth as a "transformation".

1. Competent (target) cells were streaked out on an LB plate with the appropriate antibiotics.
2. An overnight culture was prepared from these cells, allowing an incubation time of 18 hours at 30°C.
3. 100μL of the overnight culture was incubated in 5mL LB broth with appropriate antibiotics for 3 hours at 30°C.
4. The competent cells were spun at ¾ speed for 5 minutes, and the supernatant was poured off.
5. From this point forward, everything was kept on ice unless otherwise specified.
6. The pellet was resuspended with 1mL ice cold 100mM $CaCl_2$, and it was incubated on ice for 20 minutes.
7. The solution was transferred to a 1.5mL Eppendorf tube.
8. The tube was spun in the centrifuge at 13,000 rpm for 5 minutes at 4°C, and the supernatantwas aspirated away.
9. The pellet was resuspended by adding 500μL ice cold 100mM $CaCl_2$.
10. 50μL competent cells and 1μL plasmid DNA were mixed and placed on ice for 20 minutes.

11. The cells were then heat-shocked at 42°C for exactly 90 seconds.
12. The cells were then placed on ice for 2 minutes.
13. 250μL LB broth (no antibiotics) was added and the tube was incubated for 40 minutes at 30°C.
14. 100μL of transformed cells were then plated on the appropriate plate and incubated at 37°C, until the cells grew enough to pick out an isolated colony (Ferguson).

Creation of Controls

First, it was necessary to create positive controls that had no mutations in the wild type Phd product. This was necessary because the controls serve as a reference point against which the mutants may be compared. The creation of the controls proceeded as follows:

1. Obtained strain containingλRDM12, which was renamed (and henceforth known as) DIT013.
2. Transformed pLacI (from strain BEJ010) into DIT013; this strain was known as DIT014.
3. Transformed pGB2 (from strain BR7059) into DIT014; this strain was known as DIT015.
4. Transformed pGB2-Ptac-docH66y (from strain BR7063) into DIT014; this strain was known as DIT016.
5. Transformed pkk223-3 (from BR7028)into DIT015; this strain was known as DIT017.

6. Transformed Ptac-Phd (from BR7030) into DIT015; this strain was known as DIT018.
7. Transformed pkk223-3 (from BR7028) into DIT016; this strain was known as DIT019.
8. Transformed Ptac-Phd (from BR7030) into DIT016; this strain was known as DIT020.

The results of these transformations are noted here:

DIT017 λRDM12(Pr92-lacZYA) Lac BR7028 into DIT015
PlacI Cm
pGB2 Spec
pkk223-3 Amp

DIT018 λRDM12(Pr92-lacZYA) Lac BR7030 into DIT015
PlacI Cm
pGB2 Spec
ptac-Phd Amp

DIT019 λRDM12(Pr92-lacZYA) Lac BR7028 into DIT016
PlacI Cm
pGB2-ptac-docH66Y Spec
pkk223-3 Amp

DIT020 λRDM12(Pr92-lacZYA) Lac BR7030 into DIT016
PlacI Cm
pGB2-ptac-docH66Y Spec
ptac-Phd Amp

The Beta-Galactosidase Assay

Core to this experiment was the Beta-Galactosidase assay, which measures the amount of transcription taking place in the cell. The procedure for the Beta-Galactosidase assay is as follows (Miller):

1. The strains to be tested were streaked out on an LB plate with appropriate antibiotics.
2. An isolated colony was chosen and restreaked onto a new plate.
3. An overnight culture was prepared and incubated for 18 hours.
4. 25mL LB broth with the appropriate antibiotics were added to a pre-labeled baffled flask.
5. 25μL of the overnight culture were added to the flask.
6. The flask was placed in a shaking water bath and incubated for three hours, until the OD_{600} was between 0.05 and 0.50.
7. The OD_{600} was measured over several time points, at 0, 30, 60, and 90 minutes.
8. 400μL were removed from the cuvette, put in a 2.0mL microcentrifuge tube, and placed on ice.
9. 10μL toluene were added to the tube. The tube was vortexed for 20 seconds and placed on ice.
10. Z-Buffer was prepared via the following instructions:
    a. The appropriate amount of Z-Buffer was measured out and put in an autoclaved bottle
    b. 2.7mL β-mercaptoethanol (BME) was added for every liter of Z-Buffer.

c. 1mg ONPG was added for every mL Z-Buffer.

d. The bottle was wrapped in aluminum foil.

11. The permeabilized cells were incubated in a 30°C water bath for 5 minutes.
12. 800μL Z-Buffer solution was added to the tube and the time was recorded as the "start time".
13. After yellow color developed, 500μL 1M Na_2CO_3 was added and the time was recorded as the "stop time".
14. The tube was inverted and placed on ice.
15. The cells were centrifuged for 5 minutes at 13,000 rpm.
16. 800μL of the yellow liquid was removed and the OD_{420} was measured.
17. The β-galactosidase specific activity for each sample were determined by using the following formula:

$$\frac{1000 * OD_{420}}{(Time\ of\ Incubation\ in\ Minutes)(Volume\ of\ Cells\ in\ mL)(OD_{420})}$$

Results

The First Experiment

Description	β-gal specific activity	Ratio	Interpretation
(DIT017) Vector + Vector	2500 ±350		
(DIT019) Vector + DocH66Y	3300 ±120		
(DIT018) Phd + Vector	250 ±25		
(DIT020) Phd + DocH66Y	16 ±5	~15.6x	
(DIT009) Phd Δ(63-73) + Vector	540 ±70		
(DIT010) Phd Δ(63-73) + DocH66Y	420 ±20	~1.3x	
(DIT011) Phd Δ(68-73) + Vector	220 ±25		~wild type
(DIT012) Phd Δ(68-73) + DocH66Y	31 ±5	~7.1x	~wild type

The Second Experiment

Description	β-gal specific activity	Ratio	Interpretation
(DIT017) Vector + Vector	2500 ±350		
(DIT019) Vector + DocH66Y	3300 ±120		
(DIT018) Phd + Vector	250 ±25		
(DIT020) Phd + DocH66Y	16 ±5	~15.6x	
(DIT045) Phd L63A + Vector	238 ±27		Rep^+
(DIT061) Phd L63A + DocH66Y	**122 ±20**	**~2x**	Cor^-
(DIT046) Phd D64A + Vector	230 ±24		Rep^+
(DIT062) Phd D64A + DocH66Y	**160 ±62**	**~1.4x**	Cor^-
(DIT049) Phd S65A + Vector	229 ±17		Rep^+
(DIT065) Phd S65A + DocH66Y	31 ±7	~7.4x	Cor^+
(DIT047) Phd T66A + Vector	241 ±34		Rep^+
(DIT063) Phd T66A + DocH66Y	13 ±5	~18.5	Cor^+
(DIT048) Phd N67A + Vector	684 ±84		$Rep^{+/-}$
(DIT064) Phd N67A + DocH66Y	56 ±12	~12.2	Cor^+

Discussion

The First Experiment

The first experiment involved making two deletions, a 6-amino acid deletion and a 10-amino acid deletion. The deletions were then tested for their repression and corepression using the Miller assay.

It was noted that the β -galactosidase specific activities for DIT018 (Phd + Vector) and DIT011 (Phd Δ(68-73) + Vector) were about the same, at 250 ±25 and 220 ±25, respectively. Given that these values are approximately equal, it can be inferred that

mutations in the last six amino acids do not affect repression in any appreciable manner.

It was noted that the β -galactosidase specific activities for DIT020 (Phd + DocH66Y) and DIT012 (Phd Δ(68-73) + DocH66Y) were about the same, at 16 ±5 and 31 ±5, respectively. Given that these values are approximately equal, it can be inferred that the mutations in the last six amino acids do not affect corepression in any appreciable manner.

It was noted that the β -galactosidase specific activity for DIT018 (Phd + Vector) was 250 ±25 and the β -galactosidase specific activity for DIT009 (Phd Δ(63-73) + Vector) was 540 ±70. Because the B-galactosidase specific activity was higher for the mutant, it was determined that the mutation had a slight defect in repression activity.

It was noted that the β -galactosidase specific activity for DIT020 (Phd +DocH66Y) was 16 ±5 and the β -galactosidase specific activity for DIT010 (Phd Δ(63-73) + DocH66Y) was 420 ±20. Because the β -galactosidase specific activity was higher for the mutant, it was determined that the mutation had a profound defect in corepression activity.

The Second Experiment

For the second experiment, corepression was the factor being tested. To compare the results for this part of the experiment, the difference between Mutant + Vector and Mutant + DocH66Y was noted and compared against the appropriate controls. For the corepression comparison, the mutant strains were compared against the

difference between DIT018 (Phd + Vector) and DIT020 (Phd + DocH66Y). The β -galactosidase specific activities for these strains were 250±25 and 16±5, respectively. The drop in measured corepression from DIT018 to DIT020 was approximately 15.6 fold.

It was noted that the β -galactosidase specific activity for DIT045 (L63A + vector) was 238 ±27 and the β -galactosidase specific activity for DIT061 (L63A + DocH66Y) was 122±20. The drop in measured corepression from DIT045 to DIT061 was approximately 2 fold. Comparing this value to the drop observed in controls, it appears that there is a profound defect in corepression.

It was noted that the β -galactosidase specific activity for DIT046 (D64A + Vector) was 230±24 and the β -galactosidase specific activity for DIT062 (D64A + Doc H66Y) was 160±62. The drop in measured corepression from DIT046 to DIT062 was approximately 1.4 fold. Comparing this value to the drop observed in the controls, it appears that there is a profound defect in corepression.

It was noted that the β -galactosidase specific activity for DIT049 (S65A + Vector) was 229±17 and the β -galactosidase specific activity for DIT065 (S65A + DocH66Y) was 30±7. The drop in measured corepression from DIT049 to DIT065 was approximately 7.8 fold. Comparing this value to the drop observed in the controls, it appears that there is not much defect in corepression.

It was noted that the β -galactosidase specific activity for DIT047 (T66A + Vector) was 241±34 and the β -galactosidase specific activity for DIT063 (T66A + DocH66Y) was 13±5. The drop in measured corepression from DIT047 to DIT063 was

approximately 18.5 fold. Comparing this to the drop observed in the controls, it appears that there is not much defect in corepression.

It was noted that the β -galactosidase specific activity for DIT048 (N67A + Vector) was 684±84 and the β -galactosidase specific activity for DIT064 (N67A + DocH66Y) was 56±12. The drop in measured corepression from DIT048 to DIT064 was approximately 12.2 fold. Comparing this to the drop observed in the controls, it appears that there is not much defect in corepression.

Because mutations in amino acids L63A, D64A, and S65A show defects in corepression, it may be reasoned that these amino acids are key in the Phd-DocH66Y interface. Furthermore, because mutations in the amino acids L63A and D64A show profound defects in corepression (as compared to the slight defect found in mutations in S65A), it may be reasoned that these amino acids are somehow more important in the Phd-DocH66Y interface.

My initial hypothesis was that C-terminal deletions and mutations would somehow affect repression and corepression. The results and analysis support this hypothesis. It was shown that a deletion in the 63-73 amino acid range in Phd caused a slight defect in repression. In addition, the deletion in the 63-73 amino acid range caused a profound defect in corepression. After this experiment, a further experiment was planned in which specific amino acids were tested for corepression defects. It was found that amino acids L63A, D64A, and S65A show defects in corepression (with particularly profound defects found in L63A and D64A.

Acknowledgement

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