Investigating the role of the Nus G protein in N mediated Anti-termination observed in Escherichia Coli

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Abstract

In E. coli, NusG is an essential regulator of RNA polymerase. Due to its two domain structure, NusG acts as flexible linker in the anti termination complex which promotes elonga- tion rates by suppressing transcriptional pausing in vitro. However the specific role of NusG in the process of N antitermination is still unclear as antitermination has been observed in the absence of NusG, in vivo. Here we attempt to resolve this issue by isolating a point mutation in NusG that blocks N antitermination. Doing so will reveal the structural interaction between the Nus G and N protein during phage Lambda infection.

Introduction

The N protein mediated antitermination process prevents the proper termination of transcription at the Pl and Pr operons of the Lambda phage(1,5,16) Typically, phages use anti termination to regulate progression from one phase of gene expression to the next. (2,7,16) However the Lambda gene N, specifically codes for an antitermination protein that allows RNA polymerase to read through the terminators located at the ends of the immediate early genes in Escherichia coli. (1,2,3) In the absence of the antitermination protein, RNA polymerase terminates at the terminator. When the antitermination protein is present, it continues past the terminator.

Incidentally, the activity of anti termination proves to be highly specific. It requires the protein N to attach to recognition sites upstream in the region of DNA being transcribed. These recognition sites, also known as nut or N utilization sites, are distinct from the termination sites and responsible for rightward or leftwards anti termination. Once protein N attaches to the recognition site, it forms an antitermination complex in conjunction with a number of E. coli host proteins. (5,16) These host proteins, Nus A,B, E and G, also known as Nus factors work with protein N to modify RNA polymerase to ignore the termination signal, and provide a mechanism in which one or more genes at the end of an operon either get switched on or off. However, It is worth noting that of all the classic E.coli Nus proteins, the role of NusG in the N antitermination remains unclear.

The 21kDa E. coli NusG protein is composed of two domains connected by a flexible linker and affects transcription elongation or antitermination through a variety of mechanisms. The NusG N-terminal domain (NTD) directly suppresses pausing and as a result enhances the overall rate of transcription elongation (9). Structural analysis of the archaeal NusG homologue, Spt5, suggest that the NusG-NTD enhances transcription elon- gation complex (TEC) processivity by com- pletely encircling the DNA binding channel of RNA polymerase (RNAP), effectively stabilizing the closed conformation of the RNAP clamp domain (14). Comparatively, the NusG carboxy-terminal domain (CTD) KOW do- main interacts with NusE/S10, linking TEC to the lead ribosome. Coupling of transcription to translation suppresses backtracking and possible clashes with the replisome (2). The NusG-CTD also binds to, and activates termi- nation factor Rho with the same interface with which it binds NusE/S10. Thus ribosome-associated NusG-CTD is not available to enhance Rho-dependent termination (1).

Futhermore, NusG has been indicated to bind directly and selectively to Rho (6) and more weakly to core RNA polymerase

in vitro (5). NusG also both stimulates and changes the pattern of Rho-dependent termination at the l tR1 and trp t6 terminators (6, 11). A model has been proposed in which NusG serves as a bridge between RNA polymerase and Rho, thus helping to recruit Rho into the termination complex (6). Indeed, recent evidence indicates that NusG stably associates with stalled elongation complexes only if Rho is bound to the nascent RNA and that the presence of NusG in the complex leads to a slower off-rate of Rho from the transcript (10).

In light of the recent findings about NusG's involvement in transcription elongation and certain Rho-dependent terminators, we hypothesize that NusG is necessary for anti termination. It is not only a component of the complete antitermination complex but also enhances N antitermination in vitro. However, alteration of Lambda BOXA to a variant called BOXA consensus allows NusB and NusE to assemble in the absence of NusG(15) Furthermore, depletion of NusG has no effect on Lambda N antitermination in vivo, and unlike nusA, nusB, and nusE, no point mutations in NusG that block N activity have been isolated. In order to rectify this discrepancy, we intend to isolate a NusG mutant that can replace the wild type NusG, resulting in the interference of N activity.

Experimental Procedure

Bacterial Strains and Plasmids

XL1-Red is an E. coli strain with a compromised DNA repair system. XL1-Red was obtained from Stratagene. pRM431 is a plasmid which encodes a pBAD-NusG(his6) sequence expressed from the IPTG-inducible trc promoter. The plasmid contains ptrc-ptac- NusG AmpicillinR which confers ampicillin resistance. Cell strain RSW472 comes direct- ly from the laboratory collection. The strain genotype MG165 Δrac::CamR contains a rac gene deletion and chloramphenicol resistance. Phage Lambda and H80 are clear phages that encode the protein N and bear different sur- face markers.

Preparing XL1-Red pRM431 transformants

10mL of XL1-Red cells in log phase were centrifuged for 10 mins at 5000rpm in order to spin down and concentrate cells. After the initial centrifugation, the supernatant was removed to reveal a pellet of XL1-Red cells. The pellet was them resuspended in 1mL of 80:20mmol MgCl2 and centrifuged again for 5 mins at 5000rpm. After the 2nd centrifuge, pellet streaks could be observed along the sides of the centrifuge tube. The supernatant was removed and the pellet was resuspended in 200 µL of 100 mmol CaCl2 to be centrifuged for the 3rd time. 100 µL sam- ples of the cells were then transferred into 2 1.5ml microcentrifuge tubes labeled A and B. In the following step, already prepared pRM431 plasmids were added tomicrocentrifuge tubes and then placed into an ice bucket for 20 mins as transformation progressed. The samples were then streaked onto 2 labeled 100mg ampicillin plates and placed into 37 0C incubator overnight. The contents of tubes A containing the cells with pRM431 plasmid added was used as the experimental colony while 1.5ml microcentrifuge tubes B served as a control plate.

Purification of Colonies with NusG plasmid

3 different spots from the experimental plate containing colonies were selected for purification to make sure cells carried the NusG plasmid. A new ampicillin plate was divided into 3 sections. Each section was la- beled 1-3 to be designated to each of the 3 colonies selected. Each individual colony was then selected and rinsed in spot 1 of TMG buffer solution. The tip of the loop was then flamed and used to pick up cells from

TMG spot and streaked in one continuous zig zag line onto position A of section 1 in ampicillin plate. The tip of the loop was then flamed again and used to collect sample from position A. The sample on the loop was then used to create two more continuos zig zag line within section 1 called position B & C. The same steps were repeated for colonies 2 and 3 which were streaked onto corresponding section of the ampicillin plate. The plate was then placed in the 37 °C incubator overnight.

Inoculating colonies containing plasmid with carbenicillin

10 mL of liquid LB and 50mg/mL of carbenicillin were placed into flasks labeled A & B. Using a loop to pick up spots from section 1 & 2, single colonies were transferred from plates into flasks. The flasks were then placed into 37 °C shakers and cells were al- lowed to grow in order to create as many NusG mutations as possible with each generation. Inoculation procedure was carried out 3 more times for the same intent of creating as many mutations as possible.

Retrieving Cells with Plasmids

Following the procedures listed in QIAprep Spin Miniprep Kit, DNA from the XL1-Red strain was isolated. The 1.5ml microcentrifuge tubes labeled A & B were filled with 1.5mL of culture from flask A & B respectively and centrifuged at 14000rpm for 5mins. The supernant was removed and replaced with another 1.5mL of culture from flask A & B. The 1.5ml microcentrifuge tubes were then centrifuged again for 5 mins. after supernant was removed, The pelleted cells in the 1.5ml microcentrifuge tubes were resuspended in 250 µL of p1 buffer, 250 µL of P2 buffer and 350 µL of N3 buffer. The aforementioned buffers contained ribonuclease A which digests any RNA that might interfere with DNA analysis. The subsequent solution was then centrifuged for 10 mins and placed into columns with membranes. The columns were then centrifuged for 1 min, allowing DNA to attach to membranes while remaining supernant was removed. 500 µL of PB buffer solution was added to the columns which were then centrifuged for 1 min. Subsequently, the remaining supernant was removed and the column was refilled with 750 µL of PE buffer solution. The column was centrifuged twice after and the filter portions containing the attached DNA were placed into new 1.5ml microcentrifuge tubes. The 1.5ml microcentrifuge tubes filters were then filled with 750 µL of RNA- free water and centrifuged for 1 min. The resulting supernant contained pRM431 mutagenized DNA from flask A and B.

Transformation of recipient RSW472 strain

The plasmids were then prepped and used to transform the super-competent RSW472 recipient culture via a transformation mix technique. The wild type RSW472 was streaked out at 37 °C and allowed to grow overnight in liquid LB medium. 10mL of super competent recipient culture RSW472 was centrifuged to concentrated and create a pellet.The pellet was then resuspended in 1mL of 80:20 mmol MgCl2/ CaCl2. The mix was also centrifuged and resuspended in 400 µL of CaCl2. 200 µL of resuspended cells was then added to 2 separate microcentrifuge tubes.

In tubes A containing recipient cells, 5µL of mutagenized pRM431 plasmids, from the previous step, was added to the recipient culture. The transformants in Tube B were used as a control culture. The contents of both tubes were allowed to sit in an ice bucket for 1 hour before 100µL of solution from each was plated onto an EMB AMP plates. The experimental plate was prepared beforehand, contained by spreading both phages

Lambda and H80 unto the plate containing IPTG and ampicillin as our plasmid marker. Meanwhile the control plate contained no phages, but all the other components of the first. After plat- ing, the transformants were then allowed to grow overnight at 37°C. The results are shown in Figure 1 and Table 1.

Results

Mutagenesis of NusG

Over the course of the experiment, we successfully passaged the pBAD-NusG plasmid pRM431 through an E.coli mutator strain XL1-Red. The transformed strain XL1-Red/pRM431 along with an XL1-Red control were then plated onto an LB AMP plates. Afterward, we reinoculated the XL1-Red/pRM431 cultures multiple times in order to mutagenize the NusG plasmid in preparation for transformation into the wild type recipient. In doing so, we were able to generate a diverse library of random NusG mutants. The host strain could then be screened for plasmid presence using the antibiotic ampicillin. Without the plasmid, the host would be unable to the survive in the presence of the antibiotic. After identifying survivors, the plas- mids were isolated and prepared for transformation of the RSW472 strain.

Transformation of RSW472 E.Coli strain

The mutated pRM431 plasmids retrieved using the QIAprep Spin Miniprep procedure listed above, was used to transform the RSW472, our recipient cell. After conducting the transformation process, the tubes containing the transformants were plated at both 37°C, with IPTG (Results shown in Figure 1 and Table 1) in order to induce the promoter on the plasmid. The plate without the phages displayed abundant growth of healthy transformants. The colonies appear reflective green on the plate surface indicating E.coli growth in the presence of the ampicillin marker. This growth simply indicates the efficiency of transformation and provides a comparison for survival rates.

Figure 1. Transformation of RSW472 with XL1-Red/pRM431 A or N10976/pRM431 B and plating onto IPTG/AMP/EMB Agar. The reflective green colonies in plates with transformants A indicate transformation efficiency at 37°C, while also providing information about survival rates. (a) RSW472 & XL1-Red/pRM431 A transformants. (b) RSW472 & XL1-Red/pRM431 B transformants. Note that these transformants are grown in the presence of both phage Lambda and H80. Also that plates from other transformations are not included in the figure.

Table 1

Plate	Temp °C	IPTG	Plasmid	Phage	Observation
a) N10976/pRM431 A	37	Y	Y	Y	Abundant growth with full coverage of plate
b) N10976/prm431 B	37	Y	Y	N	Sparse growth with individual colonies

On the other hand, cells grown in the presence of both clear phages showed significantly less growth than the plate without phages. As expected, most E.coli cells succumb to the bacteriophage infection and eventually lyse in the presence of the protein N. However, we identified several mutants on the plate that survived the phage infection, indicating plasmid pRM431 encoding the mutagenized NusG was able to interfere with the assembly of the N anti-termination complex and inhibit cell lysis from occurring. In response to these findings, we intend to perform multiple round of further analysis of the plasmid in order to ascertain the location of the point mutation, if present, in the NusG mutants.

Discussion

Ultimately, the role of NusG appears to be rooted in its necessity for the maintenance of the antitermination complex. In the absence

of NusG, the protein N is unable to function and therefore incapable of triggering the anti termination mechanism. The result is that RNA polymerase properly terminates at termination sites.

The experiment further provides evidence that NusG is necessary for effective N- mediated transcription antitermination. Earlier iterations of the experiment involved iden- tifying temperate sensitive mutants in an E.coli strain containing a Protein N fusion that activated a toxic gene at 42°C. However the inability to identify any survivors at 42°C lead to the reevaluation of the dominance issue. Experimentally, the design simply relied on trying to overpower the wild type NusG by inundating the host with mutant NusG DNA and using IPTG to hyper express the gene. In the process, the design had overlooked the consideration of the relative dominance of the NusG mutant in relation to the wild type. In order to resolve this dilemma, an attempt to transform the mutagenized NusG pRM431 plasmid into the (MDS42 □nusG) strain was made. This strain,deleted for NusG was intended to address the issue of dominance and simultaneously screen for survivors that block N activity. Constructing a NusG deletion with the protein N fusion proved to be initially detrimental to the cells, however secondary findings point to the blocking of N activity.

The final iteration of the project attempts to clarify the role of NusG in the anti termination process by identifying mutants that are resistant to phage infection. The find- ings show that the mutated plasmid pRM431 which is transformed into the new recipient RSW472 did not only confer ampicillin resistance but also interferes with the protein N during infection by the phage. In the experiment, both the phage Lambda and H80 were used in order to account for variations in receptor sites of E.coli cells. Phage Lambda and H80 both carry the gene for protein N and therefore the propensity for antitermination, although each phage attaches to a phenotypically different receptor site. Accounting for variants in receptor sites eliminates the occurrence of survivors as a result of mutations at the receptor sites, in which the phage is unable to infect the cell because of the inability to attach.

Conclusion

As evidenced by the result, the NusG mutants appear to have an effect on the growth of the phage lambda. However it remains to be demonstrated that the mutation actually inhibits N activity. The next step would be to purify our survivors and verify that resistance to phage infection is truly a result of the mutated NusG plasmid and not spontaneous mutations at the recipient sites. After that, we would sequence the mutant plasmid in order to qualify the location of the particular mutation of interest and surmise how it interacts with the protein N. Doing so would finally specify the role of NusG in the formation of the antitermination complex.

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