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Investigation of a Mycobacteriophage Transcription Repressor

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Abstract
Mycobacteriophage HelDan is a lysogenic, or temperate, phage (virus) of the bacterium *Mycobacterium smegmatis*, which is a fast-growing, close relative of *Mycobacterium tuberculosis*, the causative agent of tuberculosis. The phage replication cycle is dependent on time-coordinated gene expression events. In order to study the regulation of gene expression during phage replication, HelDan protein gp73, a putative transcriptional repressor, was studied. Characterization of gp73 structure and function, such as DNA binding activity and affinity, was done using both bioinformatics and biochemical analyses. To this end, the gene encoding gp73 was cloned and the ability of the recombinant gp73 protein to bind to HelDan genomic DNA was examined. This work contributes not only to the understanding of mycobacteriophage repressor function, but also has implications in human health.

Introduction

Temperate phages can pursue one of two lifestyles at one time: lysogeny, in which the phage does not actively reproduce within its host, or lytic growth, in which the phage actively reproduces and then kills its host through the release of new phage molecules. Mycobacteriophage HelDan is one example of a temperate phage.

The lifestyle of HelDan is of particular interest: plates inoculated with a mixture of *Mycobacterium smegmatis* and HelDan produce both small, clear plaques (typical of lytic lifestyle) and larger plaques with indistinct edges (typical of temperate lifestyle). These results are reproducible despite repeated purification of HelDan.

Parallel to the phenomenon of two distinct plaque morphologies, genome sequencing data of HelDan returns two distinct genomic sequences. It appears as though a specific 4.1 kb section of DNA is frequently deleted from the HelDan genome. One of the gene products deleted in this 4.1 kb section is gp73, a putative repressor of lytic lifestyle gene transcription.

HelDan is a cluster A (subcluster A3) phage. Two other lysogenic cluster A phages, L5 (A2) and Bxb1 (A1), have been well-studied, especially in regard to their lytic lifestyle transcriptional repressor genes (gp71 and gp69, respectively). Because there is much similarity among clusters of phages, even across subclusters, HelDan's lytic lifestyle transcriptional repressor can be studied in conjunction and comparison to those of L5 and Bxb1.

The purpose of the following experiments was to determine the function of HelDan gp73, namely if this gene product

is responsible for inhibition of transcription of lytic lifestyle genes.

Materials and Methods

Extraction and Purification of GST-gp73-His6.2

E. coli cells containing a previously-prepared lactose-inducible recombinant plasmid pGEX-2T with tagged gp73 (GST-gp73-His6.2) were cultured in 500ml LB liquid media containing 10% lactose overnight, until OD600 reached 1.5. Crude protein extract was prepared via sonication, in which the liquid culture was centrifuged at 5000 rpm to produce a pellet, which was resuspended in 1 ml of RSB-100/10% glycerol per every gram of pellet weight. Lysozyme was added to a final concentration of 50 µg/ml, and the resulting solution was incubated at 37°C for 1 hour. 100X PI was added to a final concentration of 1X PI. The solution was then sonicated in 10 second pulses for a total of 200 seconds. The lysate was centrifuged at 13,000 rpm, and the supernatant was retained. Through quantification with a Bradford assay, protein concentration of the crude extract was determined. To produce a less crude extract, column chromatography was performed. The crude extract was equilibrated for 90 minutes in a 10 ml column containing 1 ml GSH resin, and volume was standardized to 8.5 ml using a solution of 1X PBS/.05% NP-40. Initial liquid was allowed to flow through the column, which was then washed thrice with 5 ml 1X PBS/.05% NP-40. Gp73 was eluted from the column using 3 ml 100 mM GSH in 1X PBS/.05% NP-40. Bradford assay was utilized to determine protein concentration in the extract. The same methodology was used to extract and purify GST.

DNA Binding Assays

To test the DNA-binding ability of gp73, reactions with increasing amounts of gp73 and .5 pmoles of HelDan gDNA were established. Controls for both protein and DNA were established by mixing gp73 and pGEX-2T DNA, by mixing GST and HelDan gDNA, and by combining GST and pGEX-2T DNA. Amounts of gp73 increased by reaction, as follows: 0 nM, .5 nM, 1 nM, 5 nM, 10 nM, 50 nM, 100 nM, 500 nM, and 1 µM. Once combined with HelDan gDNA, reaction volume was standardized to 50 µl with RSB-150. Reactions were incubated at 37°C for 30 minutes, then run on a dot-spotter through nitrocellulose and nylon membranes. DNA was then cross-linked to the membranes to prevent dissociation of DNA from the membrane.

Detection of DNA on each of the membranes was performed using DIG Oligonucleotide 3'-End Probe Labeling (2^{nd} Generation Roche) kit. To produce probes for both HelDan gDNA and pGEX-2T, 100 pmoles of appropriate primer (CRU 133 and Bla Rev, respectively) were standardized with sterile double distilled water to final volume of 10 µl; the primers were mixed with 4 µl 5x reaction buffer, 4 µl $CoCl_2$ solution, 1 µl DIG-ddUTP solution, and 1 µl terminal transferase. The reactions were incubated at 37°C for 15 minutes, placed on ice for 2 minutes, and were stopped by adding 2 µl .5 M EDTA (pH 8).

Both blots were prehybridized at 42°C for 30 minutes in 15 ml Hybridization buffer. Once complete, the buffer was removed from the blots, and 10 ml Hybridization buffer with 10 pmoles (2.2 µl) of each probe were added to the blots. The blots were hybridized at 42°C overnight.

Probe detection was done using CDP-Star. Both the nylon and nitrocellulose membranes were washed briefly in Wash Buffer, and then incubated in 100 ml 1X dry

milk in Maleic Acid Buffer for 30 minutes. The membranes were then incubated in a solution of Anti-Digoxigenin-AP diluted 1:20,000 in 20 ml blocking solution for 30 minutes. The membranes were washed in 100 ml Wash Buffer for 15 minutes twice, then briefly equilibrated in 20 ml detection buffer. CDP-Star was diluted 1:100 in detection buffer to a final volume of 2 ml; 1 ml of this CDP-Star working solution was applied to the DNA side of each membrane. The membranes were immediately covered by plastic wrap and allowed to incubate for 5 minutes at room temperature. Images were captured using Versa-Doc software with 5 minute exposure time.

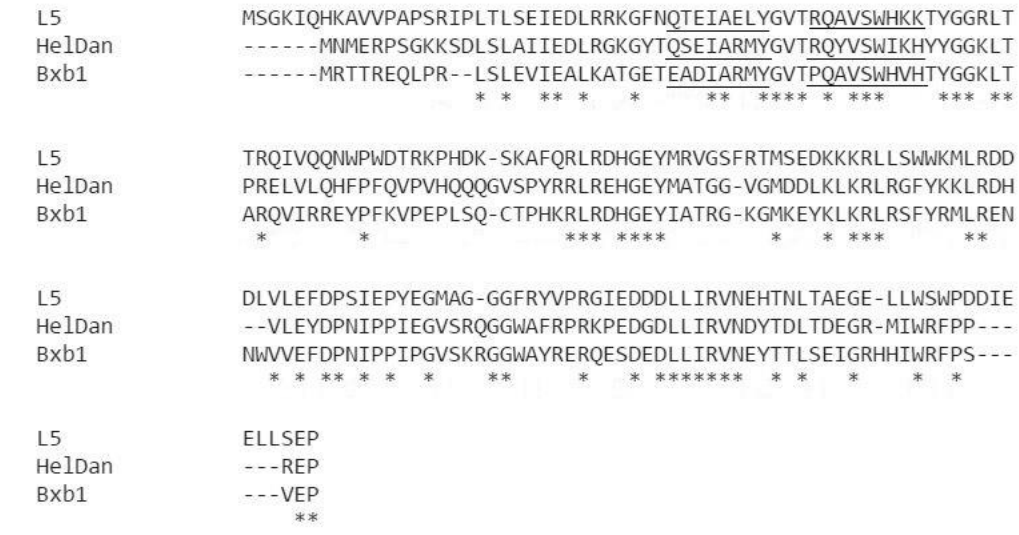
Results

Genomic analysis of HelDan revealed 58% identity between the polypeptide sequences of gp73 and Bxb1 repressor gp69. This analysis also revealed 49% identity between the polypeptide sequences of gp73 and L5 repressor gp71, an analogous repressor to Bxb1 gp69.

The respective lytic lifestyle gene transcriptional repressors of HelDan, Bxb1, and L5 were aligned using MUSCLE multiple sequence alignment tool (**figure 1**). This analysis revealed 36% identity among the polypeptide sequences of HelDan gp73, Bxb1 gp69, and L5 gp71. Additionally, putative helix-turn-helix domains, which function in DNA binding activity of a protein, of all three genes have high identity (50% among all three genes).

Non-consensus repressor-binding sites have been identified in both Bxb1 and L5 just downstream of their lytic lifestyle transcription repressor genes, indicating self-regulation activity of these repressors (Jain and Hatfull, 2000). A similar, assumedly non-consensus repressor-binding site was putatively identified in HelDan, just downstream of gp73. The sequences in each phage genome were aligned using MUSCLE multiple sequence alignment tool, revealing 69% identity between HelDan and L5 sequences, 69% identity between HelDan and Bxb1 sequences, and 46% identity among all three sequences (**figure 2**).

Figure 1



HelDan gp73 was aligned against L5 gp71 and Bxb1 gp69. Asterisks denote sequence identity among the three proteins. Underlined portions denote putative helix-turn-helix DNA-binding domains. Dashes denote spaces inserted for optimal alignment

Figure 2

A:

```
HelDan          GTTATATGTCAAG
L5              GGTGCCTGTCAAG
Bxb1            GTTACAGCGCAAG
                * *      ****
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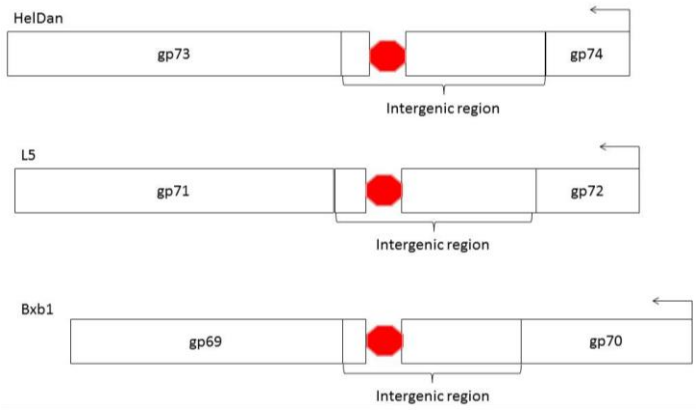
B:



A) HelDan gp73-binding sequence upstream of gp73 was aligned against binding sequences of L5 gp71 and Bxb1 gp69 upstream of each respective repressor gene. Asterisks denote sequence homogeny among the three binding sequences.

B) Configuration of the negative-strand repressor proteins followed closely by a positive-strand repressor-binding site. L5 gp71 and its binding site are separated by 53 nucleotides; Bxb1 gp69 and its binding site are separated by 40 nucleotides; HelDan gp73 and its binding site are separated by 42 nucleotides.

Discussion

Procedural development took longer than expected, as the progression of experiments was consistently stunted. Culturing the transformed *E. coli* cells to the point of sufficient gp73-GST expression took much tinkering, and only after discovering (by way of culture PCR) even lower plasmid uptake than expected. Producing a crude protein extract also took several tries, using several different buffers and cell-shearing methods, before determining the combination of 1X PBS/.05% NP-40 and sonication was optimal. Producing a purer protein extract also went through a trial-and-error phase before ultimate success with affinity column chromatography. DNA binding assays have yet to produce results, potentially due to use of such small quantities of DNA (pmoles), but also potentially because of blocking with nonfat milk instead of kit blocking reagent. DNA binding assay success is necessary to identify binding ability of gp73 to HelDan gDNA.

The proposed mechanism of transcription repression of L5 gp71 and Bxb1 gp69 is via stoperator activity – the repressor binds its binding sequence during transcription elongation and physically prevents RNA polymerase from continuing, thus promoting termination (Brown et. al 1997). Size of intergenic region is indicative of stoperator activity (Brown et. al 1997). The size of the intergenic region between HelDan gp73 and gp74 is relatively small (268 bp), approximately the same size as those following the homologous repressor proteins in Bxb1 and L5, indicating potential stoperator activity at that site. Furthermore, the organization of the forward gp73-

binding site downstream of reversely transcribed gp73, which is similar to the organization of L5 gp71 and Bxb1 gp69, suggests that HelDan gp73 also functions via stoperator activity, but that has yet to be confirmed experimentally. Whether gp73 functions as a stoperator would also be indicated by the binding sites of the adjacent genes to gp73; opposite directions imply stoperator activity (Brown et. al 1997).

The computational analysis has ultimately identified a putative helix-turn-helix domain in HelDan gp73 with high identity to helix-turn-helix domains in two homologous genes. It has also identified putative repressor binding sites with high homology between 40 and 53 nucleotides upstream of homologous repressor protein start codons in three cluster A phages.

References

Brown, K.L., Sarkis, G.J., Wadsworth, C., Hatfull, G.F. (1997). Transcriptional silencing by the mycobacteriophage L5 repressor. *The EMBO Journal*, *16*(19), 5914-5921. doi:10.1093/emboj/16.19.5914.

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