UNIVERSITY OF CALIFORNIA, SAN DIEGO

Generation and molecular analysis of dominant negative alleles of anthrax Lethal Factor in *Drosophila*

A thesis submitted in partial satisfaction of the requirements
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in

Biology

by

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Professor Ethan Bier, Chair
Professor Victor Nizet
Professor Steven Wasserman

2008

The Thesis of Alexandra Kharazi is approved and it is acceptable in quality and form for publication:

Chair

University of California, San Diego

2008

DEDICATION

This thesis is dedicated to my parents, Alexander and Ludmila Kharazi, as well as my grandmother, Larissa Detinitch, who have given me love and support throughout my entire life.

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ABSTRACT OF THE THESIS

Generation and molecular analysis of dominant negative alleles of anthrax Lethal Factor in *Drosophila*

by

Alexandra Kharazi

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Professor Ethan Bier, Chair

Anthrax, a disease caused by the bacterium *Bacillus Anthracis*, poses a threat worldwide due to its debilitating symptoms and lethality. The toxins released by the bacteria (PA, LF, EF) enter cells, cause most symptoms and allow the bacterial growth. LF is a metalloprotease, which cleaves and inactivates MKK in humans, therefore inhibiting MAPK signaling in many cell types. EF is a potent adenylate cyclase and PA facilitates the entry of LF and EF into host cells. In addition to the classic antibiotic and vaccine therapies, one strategy to combat anthrax would be to introduce dominant

negative toxins, which could antagonize the wild type toxins, and potentially save lives in the later stages of the disease when antibiotics can no longer prevent death. Model systems such as *Drosophila Melanogaster*, can be used to study the activity of anthrax and other bacterial toxins, and provide a way to screen such Dominant-negative alleles. We used the Novel OVerexpression Allele screening method to generate several LF alleles which exhibit dominant negative activity as revealed by wing phenotypes in flies. We then attempted to analyze the molecular lesion causing the novel activity of these mutants.

Introduction

Anthrax disease is caused by *Bacillus anthracis*, a spore-forming, gram positive bacterium. Anthrax is more prevalent in developing countries, and is of vital concern world-wide due to its potential use as an agent of bioterrorism.

Anthrax can be inoculated cutaneously, orally, or by inhalation. In cutaneous anthrax, spores from bacteria enter a cut or scrape in the skin. The skin infection manifests itself as a papule which develops into a fluid-filled blister 1-2 days after infection. In about 7 to 10 days the blister has a black center filled with necrotic tissue. Subsequent symptoms include lymphodenopathy, fever and headache. In gastrointestinal anthrax, spores are acquired by eating meat contaminated with *bacillus anthracis*. Early symptoms include ulcers at the base of the tongue or tonsils, sore throat, loss of appetite, vomiting and fever. In later stages of the infection, symptoms include hemoptysis, bloody diarrhea, abdominal ascites and shock. Without treatment death occurs in 2-5 days. Inhalation anthrax is caused by breathing in the spores of *bacillis anthracis.* Early symptoms are similar to that of a common cold: sore throat, mild fever and muscle aches. Within a few days, symptoms may include severe breathing difficulty, shock, meningitis and death. Without immediate treatment this form of anthrax is usually fatal.

Bacillus anthracis releases there toxic factors, Protective Antigen (PA), Lethal Factor(LF) and Edema Factor(EF) which are critical for its infectivity. PA facilitates the entry into host cells of LF and EF which have catalytic activities. EF is a calcium/calmodulin adenylate cyclase which increases cAMP concentrations in the cell. LF is a zinc-dependent metalloprotease which cleaves MAPKK's, disrupting MAPK signaling. The full-length 83 kD PA binds to the ubiquitously expressed anthrax receptors (ATR), Tem8 and CMG2.

Upon binding, PA83 is cleaved into PA20 and PA63 by host furin-like proteins. PA 20 dissociates from the receptor and PA63 forms a heptameric complex known as a pre-pore which can bind up to three molecules of LF or EF. This complex undergoes receptor-mediated endocytosis and subsequent trafficking to a low-PH endosomal compartment. In this compartment, the PA pre-pore forms an ion-conductive channel by inserting into the membrane and allows LF and EF release into the cytosol.

The type of anthrax currently of greatest concern is inhalation anthrax because it is the most lethal form of anthrax and therefore can be used as an effective biological weapon. Fortunately, the mechanisms of this type of infection have been well-characterized. *Bacillus anthracis* spores are inhaled and captured by pulmonary phagocytes. The main cell populations involved in phagocytosis of *bacillus anthracis* are alveolar macrophages and lung dendritic cells. Inside the phagosome, the spores germinate and secrete three toxins: Protective Antigen, Lethal Factor and Factor, which are critical virulence factors. Mutants lacking either LF or EF have decreased virulence (1). Essentially, the phagocytes act as a vehicle to transport toxins across the alveo-capillary space and ensure their arrival in the mediastinal lymph nodes which are located in the central part of the chest in between the two lungs. During this transport phase, the toxins suppress the immune response. Lethal Factor inhibits macrophage secretion of pro-inflammatory cytokines, blocks recruitment of polymorphonuclear cells (PMN's), and prevents monocyte differentiation into macrophages. EF cooperates with LF during this phase to inhibit PMN and monocyte recruitment, and blocks the secretion of pro-inflammatory cytokines. During the second phase of inhalation anthrax, anthrax toxins escape from the phagosome upon arrival in the lymph nodes and disrupt the immune response by several mechanisms. LF blocks expression of co-stimulatory molecules by dendritic cells, inhibiting antigen production. LF also negatively affects the humoral

response, most-likely by disrupting MKK-dependent B-cell proliferation and IgM production. Furthermore, LF and EF disrupt T-cell activation during this phase. During the terminal phase of inhalation anthrax, bacilli are released into the bloodstream where they proliferate. They also accumulate and proliferate in preferential sites, such as the brain. LF causes endothelial barrier disruption and apoptosis, resulting in vascular leakage and collapse.

Currently, there are various strategies to combat anthrax infection. In the early stages of anthrax, antibiotics can be used effectively to combat bacterial growth. However, antibiotics only provide protection against bacterial proliferation, and do not inhibit the toxins which are already in the system, which can kill the patient at a later stage of infection. Also, early anthrax infection is either asymptomatic or is manifested through symptoms that are not specific to the disease. Therefore, other strategies employed against the toxins themselves are of vital interest to treat of the infection at late stages.

Firstly, there are antibody-based anti-toxin strategies. For example, antibodies raised against PA are currently used to block receptor binding. Antibodies raised against LF and CMG2 receptor are also under investigation. These strategies, although promising, are not effective against toxins that have already entered host cells. Secondly, an anti-anthrax vaccine (Anthrax Vaccine Adsorbed) is available. ADA is composed mainly of cultures of avirulent, non-encapsulated *B. anthracis*. However, costs and side effects of this treatment (fever, chills, nausea, general body aches, edema and others) make routine administration impractical. A third effective method in anthrax therapeutics is the use of receptor decoys. Essentially, these decoys are soluble ATR/TEM8 and CMG2 receptor proteins which bind the toxins, preventing them from binding to endogenous receptors. Another method to stop toxin processing is through Hexa-d-

arginine, a furin inhibitor which stops full-length PA from being cleaved into PA20 and PA63. This stops further processing of toxins. Moreover, PVI, a peptide inhibitor stops LF and EF association with the pre-pore complex. Other strategies are aimed to block the activity of EF and LF after they have entered host cells. For example, some synthetic substrates were identified on the basis of MKK consensus sequences. These substrates bind LF, but are not cleaved and remain bound to LF. Some of these substrates are synthesized with groups which chelate zinc, which make them more potent inhibitors of LF. In addition, there are small-molecule inhibitors such as DS988 and GM6001 which subdue LF activity by binding to its active site. However, some of these strategies can cause side-effects. Dominant negative inhibitors may provide a treatment option that may not cause as many side-effects. For example, dominant negative forms of PA have been identified (2). This DNI has mutated residues on a domain crucial for toxin translocation. Similarly, dominant negative forms of LF and EF may also serve as potential therapeutic measures by competing with actual toxins that have already entered host cells. The development of dominant negative LF and EF development is a potential inexpensive and effective approach for the treatment of anthrax in conjunction with antibiotics.

Drosophila melanogaster is an excellent model organism for human disease and determination of vital signaling pathways. In fact, seventy-five percent of human disease genes have fly homologues, indicating there is remarkable pathway conservation, and that Drosophila can be used as a tool for investigating molecular networks involved in pathological processes. Because of this conservation, the fly is also a powerful instrument for the analysis of bacterial toxin activity. Although it is not a host for *B. anthracis,* most likely because it does not have the ATR/TEM8 or CMG receptors, it provides a good model for toxin action after bacterial release because the signaling pathways (MAPKK and cAMP) targeted by virulence factors are conserved.

The purpose of my research is use genetic screens in Drosophila to generate dominant negative alleles of LF and analyze them at the molecular level. This research was initially encouraged by the discovery of dominant negative versions of PA. However, while dominant negative versions of PA can stop toxins from entering cells, a dominant negative version of LF would target toxins which have already entered the cells. The Novel OVerexpression Activity (NOVA) screening method is a good approach for creating dominant negative toxin activity. Our group has developed a method to generate dominant negative versions of potentially any gene. This method was first employed in screens for dominant mutations applied to components in the Drosophila EGF-R pathway, and involves the GAL-4/UAS mis-expression system. Here, I used a modified version of N1.OVA screening, in which a UAS-toxin transgene is exposed to mutagenesis and expressed in the F1 progeny using a GAL4 driver, along with a wild-type copy of the toxin. The progeny is screened for suppression of the phenotype induced by the wild-type toxin. In this case, a UAS-LF transgene was subjected to mutagenesis, and tested for the suppression of a visible wing phenotype induced by LF.

Materials and Methods

Fly Genomic DNA extraction

10 flies were collected in an Eppendorf tube. 200 ml homogenizing buffer was added (0.1M Tris-HCL of pH 9, 0.1 M EDTA, 1% SDS). Flies were homogenized with glass rod. Tube was heated at 60-65 degrees Celsius for 30 minutes to lyse cells. 28 ul 8M potassium acetate was added and tube was chilled on ice for 30 minutes to precipitate proteins. Protein precipitate and cuticular debris were removed by centrifugation at 4 degrees Celsius for 10 minutes and supernatant containing nucleic acids was transferred to new tube. 0.5 volumes of isopropanol was added and left at room temperature for 5 minutes to precipitate DNA Tube was centrifuged at room temperature for 5 minutes. Precipitate was washed with 70% ethanol, dried and resuspended in 100 ul buffer TE. DNA was stored at 4 degrees Celsius

Primer Sequence and Design

Primers were designed as 20mers with a 50% GC content when possible.

The location of the primers we used on the LF coding sequence is indicated below:

Cgaaataaaacacaggaagagcatttaaa**ggaaatcatgaaacacattgt**aaaaatagaa LFS5UPAS1 ⟵

Gtaaaaggggaggaagctgttaaaaaa**gaggcagcagaaaagctacttgag**aaagtacca LFS7 ⟶

Tatgggagaacaaatgaagcggaattt**ttgcagaagcctttaggttaa**tgcattctacg LFCTAS2 ⟵

Gaccatgctgaacgtttaaaagttcaaaaaaatgctccgaaaactttccaatttattaac LFCTS1 ⟶

Figure 1. Location of N and C Terminal Primers on LF sequence.

Molecular lesion in dominant negative Lethal Factor analysis using inverse pcr

Fly genomic DNA was digested with restriction enzymes (DNA of 2 flies in a 20 ul digestion), and enzyme was heat inactivated. After dilution, the digestion was submitted to ligation. The ligation reaction was precipitated, resuspended, and used as a template for subsequent PCR. Sense and anti-sense primers used for the PCR: N-Terminal primers: Sense: LFS7: gaggcagcagaaaagctacttgag, Anti-sense: LFSFUPAS1: taaatgctcttcctgtgttt

C-Terminal primers: Sense: LFCTS1: gaccatgctgaacgtttaaaag, Anti-Sense: LFCTAS2: cttttaaacgttcagcatggtc.

PCR

All PCR was done according to protocol: Expand Long Range dNTP-Pack by Roche, Catalog No. 04829034001

Table 1. PCR Conditions used to analyze LF molecular lesion.

Step	Temperature (Celsius)	Time	Cycles
Denaturation	92	2 min	1X
Denaturation	92	10 sec	10X
Annealing	45-65	15 sec	
Elongation	68	4 min	
Denaturation	92	10 sec	25X
Annealing	45-65	15 sec	
Elongation	68	4 min	
Final Elongation	68	7 min	1X

Results

Approach

To search for Dominant-negative forms of LF, we expose a chromosome carrying three UAS-LF insertions to the Δ2-3 transposase as a mutagen. Males carrying UAS-LF3X and Δ2-3 were then crossed to females expressing LF under the control of a strong wing-specific GAL4 driver named MS-1096. In the progeny of this cross (performed at medium scale), I searched for the reversion of a curly wing phenotype caused by the expression of wt-LF.

The screen was done with about 16,000 total fly progeny. As a mutagen, we used a Δ 2-3 insertion, which encodes a transposase which specifically acts on P-elements. Most of the time, the transposase only promotes excision and re-insertion events, but in some cases the repair process following excision events is followed by an incomplete or erroneous repair of the initial insertion. This can generate deletions, inversions, duplications, or combinations thereof, specifically affecting the initial P(UAS-LF) insertion. The advantage of using Δ2-3 as opposed to a chemical mutagen such as EMS is the high frequency of recombination events affecting specifically the UAS transgene. On the other hand, no point mutations are known to be induced when Δ 2-3 is the mutagen. The MS-1096 is a strong Gal4 driver which is expressed on the wing pouch of the wing disc. It is expressed on the dorsal side more than on the ventral side. The LF insertion causes cell death and inhibition of growth by disrupting the Dsor/MAPK pathway. Thus, the curly wing phenotype presumably derives from higher levels of LF expression in the developing wing pouch.

Original stock has two copies of LF

As a starting point for our screen, we had a UAS-LF stock (on the X chromosome). We tried to determine if this original stock had two copies or just one copy of LF by doing the following cross and analyzing the eye color of the progeny:

$$F\ \frac{LF1or2X}{+}\ \times\ \frac{Xw\text{-}}{Y}$$

Out of 210 flies, most had orange eyes, but 1 male had pale eyes, suggesting that 2 rather than one UAS-LF copies were present in this stock. When crossed to MS1096, the male with paler eyes induced a curly wing phenotype which was weaker than that of the initial stock. We concluded that the insertion did in fact have 2 copies separated by a very short genetic distance. A second stock was generated from this first one by exposing UAS-LF2X to the transposase, and searching for darker eyes. This stock has at least three copies of UAS-LF (UAS-LF3X).

NOVA screen for dominant negative activity:

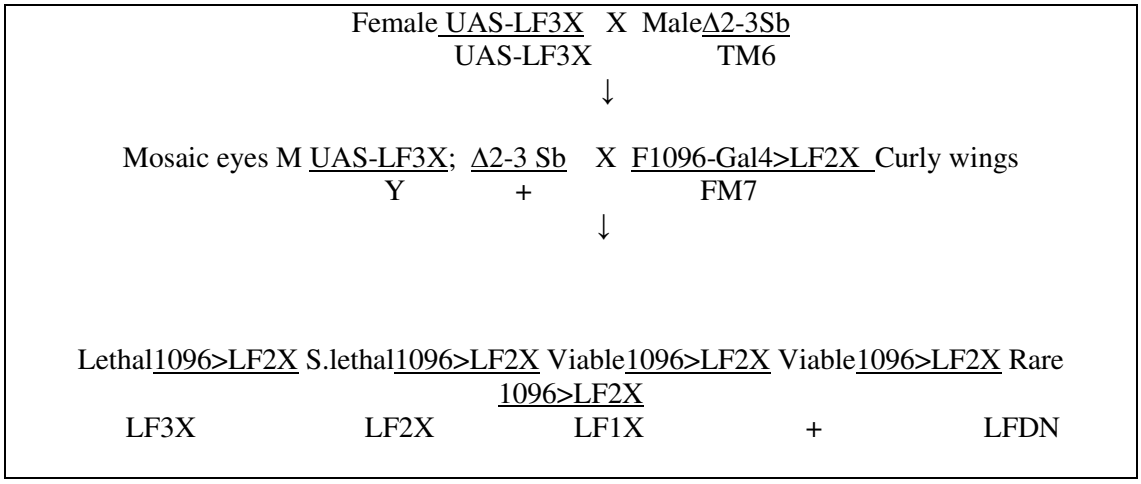


Figure 2. Generation of flies with suppression phenotype using the NOVA screen method.

We only looked among the non-Sb progeny because we did not want flies which had inherited the transposase, which would cause further mutation. We knew that any flies which inherited bar eyes encoded by the FM7 mutation did not have LF-wt expression. We then looked at the phenotypes of non-FM7 flies, most of which had curly wings. However, the rare event we were looking for was a fly which had normal eyes (non-FM7) and flat wings. This meant we had an LF-phenotype which was suppressed.

We generated 10 F $\frac{1096>LF2X}{LF2X(+/-)}$; $\frac{+}{LFDN}$ +

From these crosses, 9 male progeny from each female with no LF phenotype were selected and crossed individually. Because of recombination events, we had to find which male progeny inherited the DN activity

$$\frac{1096?\ LFwt?\ LFDN?}{Y} \times \frac{1096>LF2X}{FM7}$$

↓

Establish a stock: $\frac{1096?\ LFwt?\ LFDN?}{FM7}$

When we found that the DN activity had been transmitted (non FM7 females had flat wings), we could establish a balanced stock. Each of these mutant stocks has the LF-DN activity, but may also have kept the GAL4 driver (MS1096), and some copies of UAS-LFwt.

It is desirable to eliminate LF wild- type from each mutant stock because during molecular analysis by PCR or Southern Blotting, UAS_LF insertions will generate an unwanted signal which can mask the LFDN signal and confound the molecular analysis:

M UAS-LFDN*UAS-LFwt X Xw-
Y Xw-

↓

F UAS-LFDN*UASLFwt X Any male
Xw-

↓

White eyes pale eyes orange eyes red eyes
y y y y

Cross each of these males to F 1096>LF2X

FM7

We looked for the palest possible phenotype which retained the suppression of the LF phenotype, to establish a "purified" stock. No male with white eyes showed the suppression of the LF curly wing phenotype, suggesting that each LF-DN insertion has conserved its white + marker, or had retained a copy of UAS-LF-wt. We established the following stocks:

LFDN5B* LFDN1A* LFDN6B*

FM7 FM7 FM7

1096>LF2X	1096>LF2X	1096>LF2X	1096>LF2X
FM7	LFDN5b*	FM7	LFDN5b*

Figure 3. Generation of LFDN mutant stocks.

I. Molecular Analysis

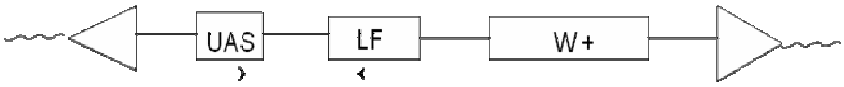
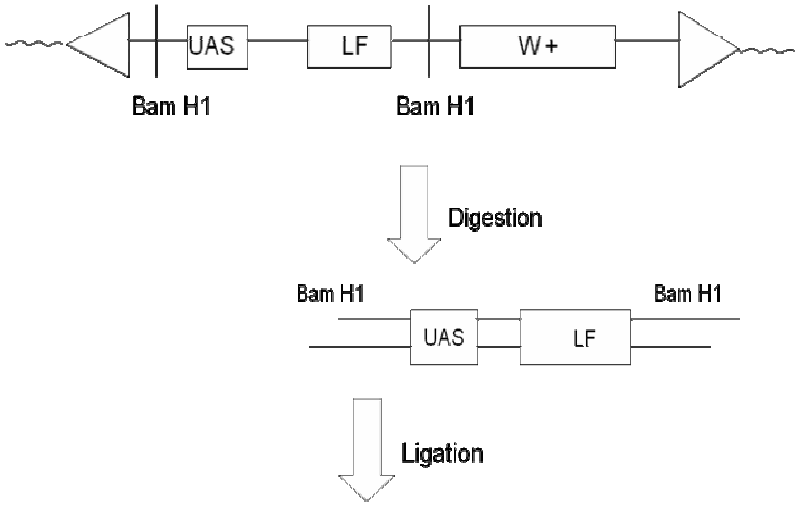


Figure 4. Schematic depiction of regular PCR with primer locations on UAS-LF



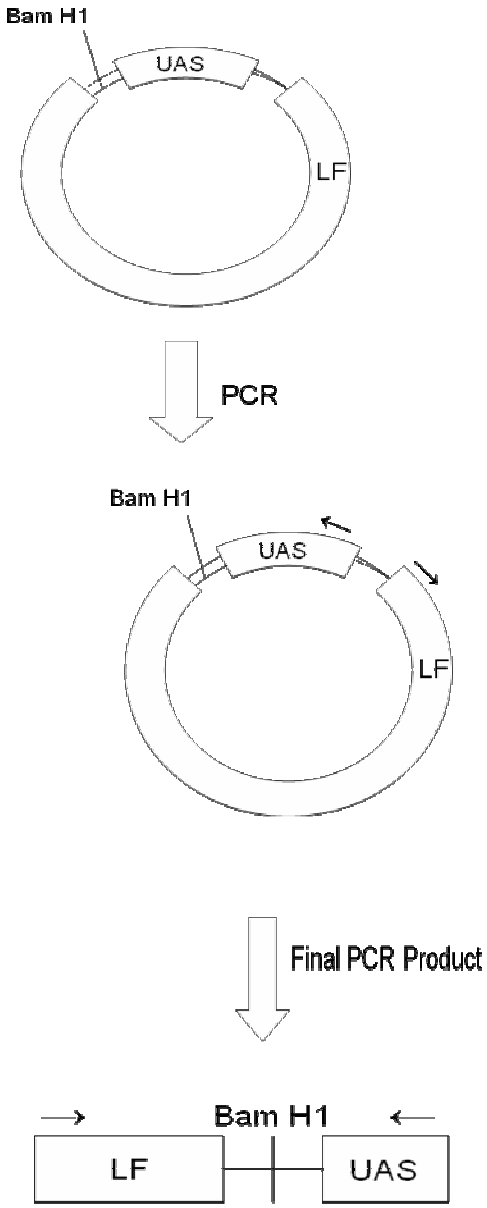


Figure 5. Schematic depiction of inverse PCR reaction

We first used genomic DNA preparations from the different mutant stocks and control stocks (wt, UAS-LF2X, UAS-LF3X) in regular PCR reactions to amplify the LF sequences and determine whether there was a truncation of LF DNA in the LF-DN mutant stocks. We also did not restrict our analysis to the LF sequence, used primers outside the sequence and amplified the biggest product possible. Primers specific for genomic sequences surrounding two of the UAS-LF insertions were used and failed to amplify the UAS-LF sequence, generating no signal or high background. We concluded that primers specific to the LF sequence, rather than for surrounding genomic sequences or vector sequences were necessary to amplify LF.

Each DN stock contains at least one copy of wild-type UAS-LF. Upon sequencing, no point mutation or small truncation that could result in the observed DN activity was found.

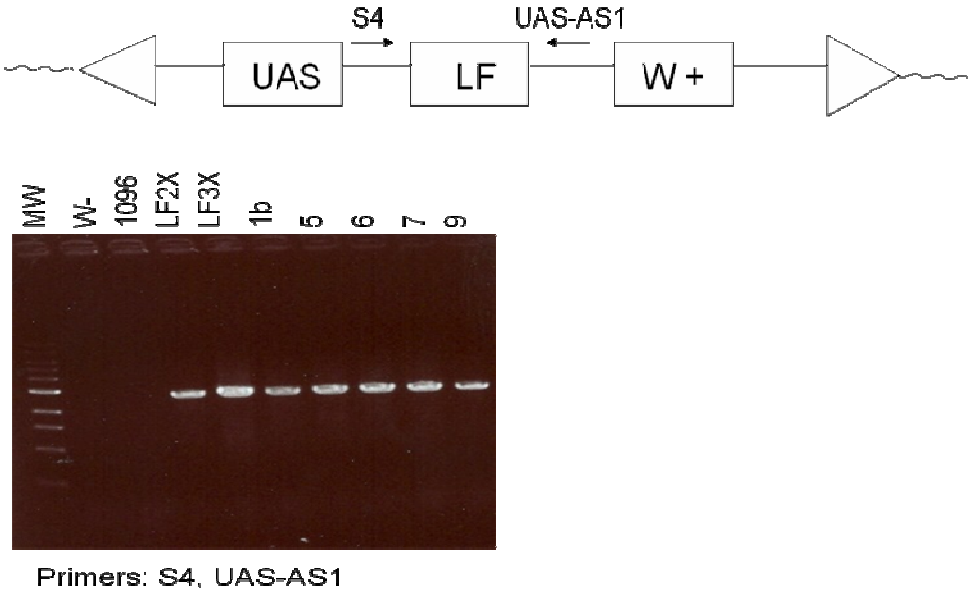


Figure 6. Regular PCR of control and mutant stocks and schematic depiction of primer locations on UAS-LF constructs.

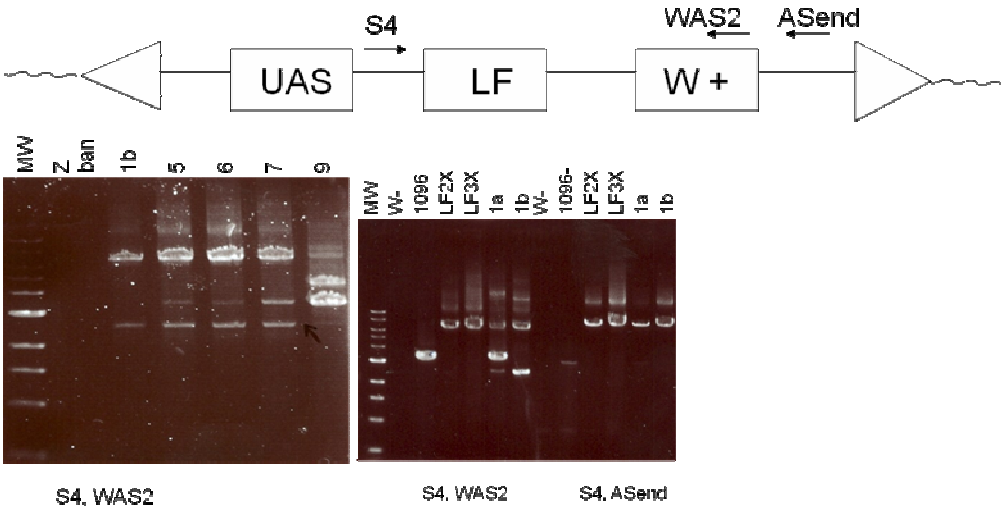


Figure 7. Regular PCR of control and mutant stocks and schematic depiction of primer locations. We did not restrict our analysis to the LF sequence. We amplified the biggest product possible.

We tried to find the best primer conditions to analyze the structure of the dominant negative lesion using PCR. We decided on LFS7 and LFS5UPAS1 because it yielded the largest band. LF3X genomic DNA was used as template for analysis.

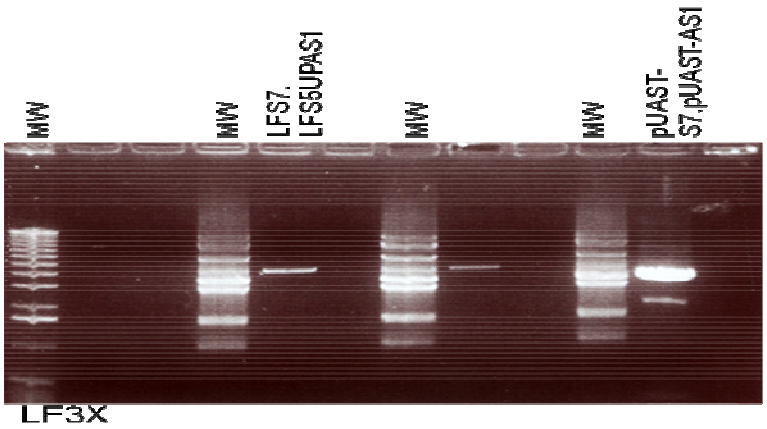


Figure 8. LF3X subjected to PCR with various combinations of N-Terminal primers.

In order to characterize the LF molecular lesion, we digested fly genomic DNA with various restriction enzymes and subjected them to inverse PCR as templates

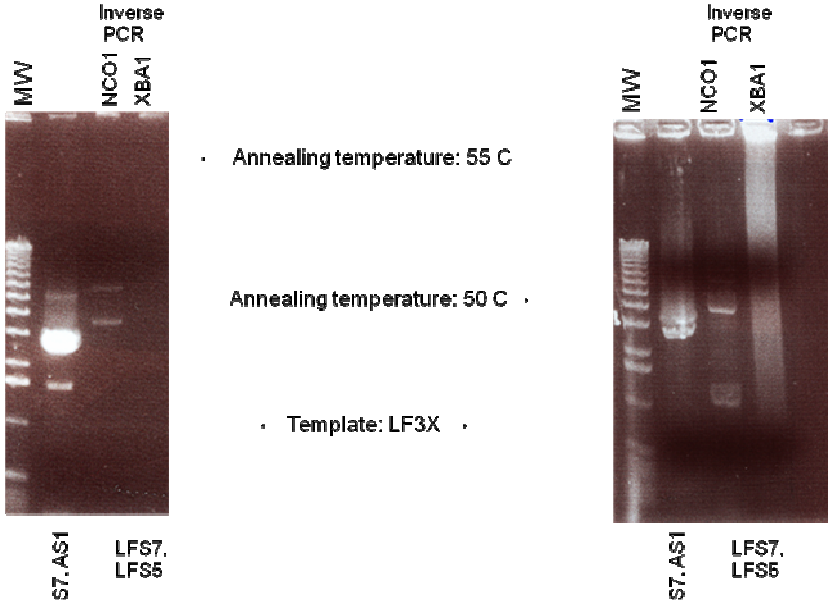


Figure 9. Inverse PCR of LF3X with N-terminal primers

After determining the best set of primers and restriction enzymes, we digested the genomic DNA of the mutant flies and subjected the product to inverse PCR with our N-Terminal primers. We were suspicious because the bands are all the same size.

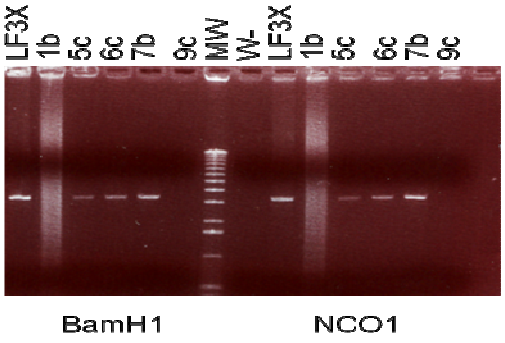


Figure 10. Inverse PCR of control and mutant stocks with N-terminal primers.

Due to the suspicious bands obtained in the previous analysis, we subjected the mutant DNA digested with BamH1 and Nco1 to inverse PCR using our N-terminal primers and another PCR kit.

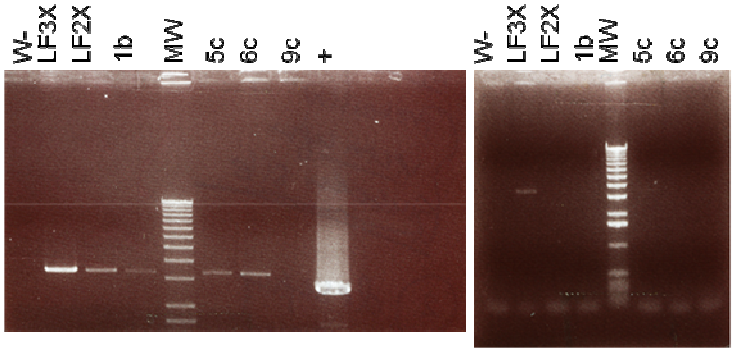


Figure 11. Inverse PCR of control and mutant stocks with N-terminal primers and another PCR kit.

Analysis with N-Terminal primers did not give any results, so we designed primers for the C-terminus of the LF coding sequence to analyze the DN activity.

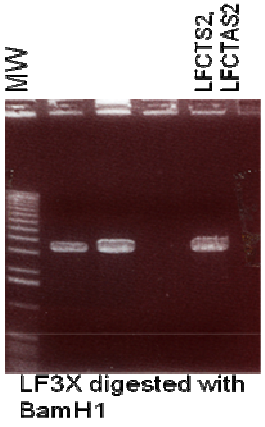


Figure 12. LF3X subjected to PCR with various combinations of C-Terminal primers.

After selecting the best set of c-terminal primers, we subjected the digested mutant DNA to inverse PCR analysis.

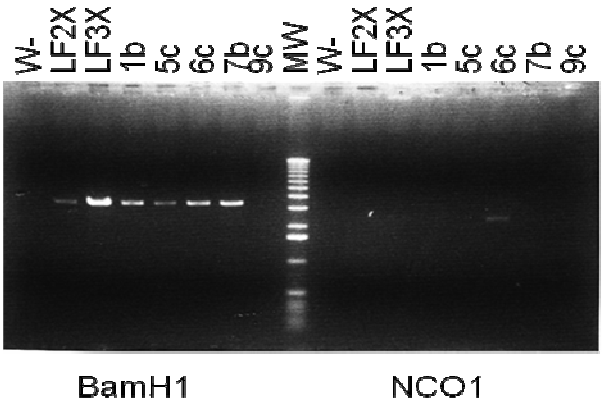


Figure 13. Inverse PCR of control and mutant stocks with C-terminal primers.

Since the enzymes BamH1 and NCO1 were not yielding any profound differences in the bands, we decided to digest DNA with different restriction enzymes and subject it to inverse PCR with our C-terminal primers

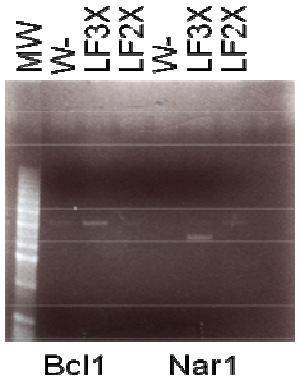


Figure 14. Inverse PCR analysis of control stocks with BclI and NarI and C-Terminal primers.

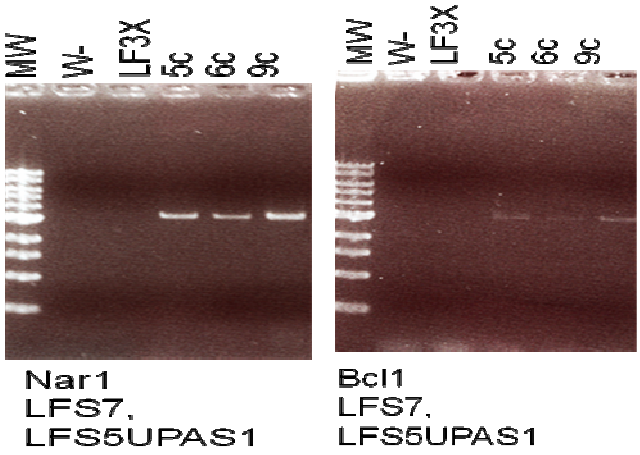


Figure 15. Inverse PCR analysis of control and mutant stocks with BclI and NarI and N-Terminal primers.

Further analysis with BclI and NarI enzymes was not successful.

In order to see if we could reveal more mutants, we performed the NOVA screen again.

Genomic DNA preparations are pictured below.

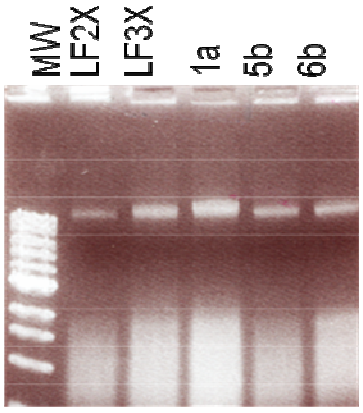


Figure 16. Genomic DNA extracted from mutant stocks.

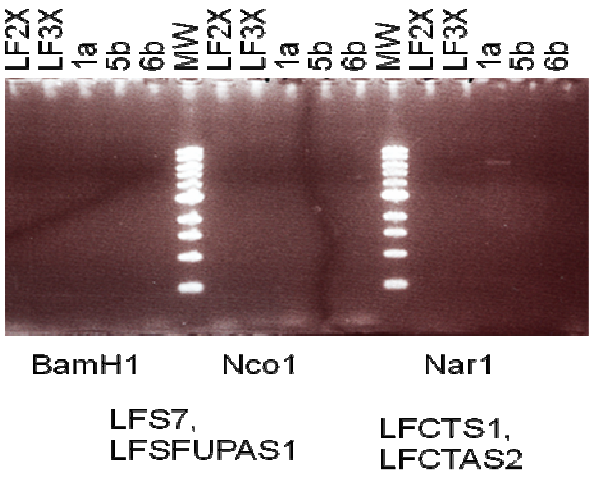


Figure 17. Inverse PCR analysis of new control and mutant stocks using N and C terminal primers.

In order to see if there were undetectable bands, another PCR reaction was done with the previous PCR as a template. Two mutant bands seemed promising, so I purified them.

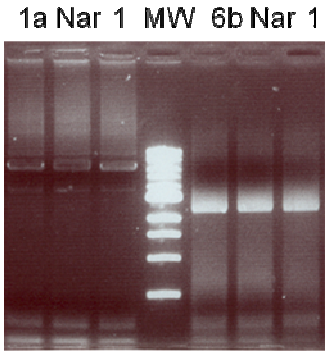


Figure 18. Re-PCR reaction with the previous PCR products as template.

I am currently in the process of cloning these PCR bands and sequencing them.

We were concerned that there was no separation that actually occurred in the second screen. Another PCR was done with primers UAS-S1 and pUAST with the original mutants and the new mutants. It looks as if no separation occurred.

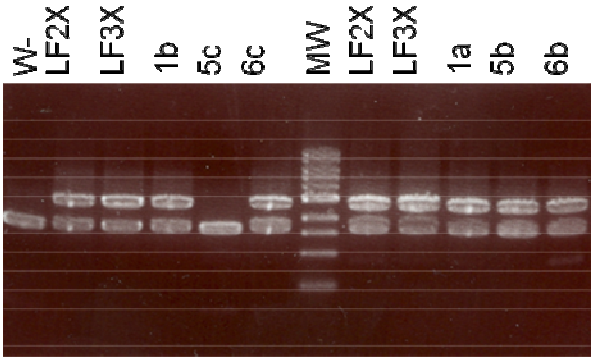


Figure 19. Regular PCR with control primers and both sets of mutant stocks.

Discussion

We performed a screen for new LF alleles exhibiting dominant negative activity and isolated six LF-DN mutants, on the basis of the reversion of a wing phenotype generated by the LF-wild-type toxin. We then analyzed the molecular lesions using PCR and inverse PCR. However, we did not manage to finalize this analysis. A part of the difficulty to determine the lesions causing the DN activities stems from the fact that at least 2 copies of UAS-LF used for the screen are inserted so close together that it is hard to separate out the wild-type copy of UAS-LF. In each UAS-LF-DN stock, a remaining copy of UAS-LF-wt was found, even after an attempt to recombine it out.

Although point substitutions are not known to be generated by the Δ2-3 transposase, we cannot completely rule out the possibility that some of the UAS-LFDN mutations are caused by such small mutations. Although the sequencing of the full-length LF PCR products in our LF-DN stocks did not reveal any mutation after careful examination, a potential point mutation may have been masked by the presence of the wild type LF sequence. On the other hand, inverse PCR using primers specific for LF did produce two promising bands found only in LF-DN6 and LF-DN1 mutant stocks, which are not fully sequenced yet. To obtain a satisfactory read of these products, we envision cloning them into a PCR vector first, although an initial attempt failed.

Southern Blotting with various probes covering the whole LF sequence, parts of the LF sequence, and vector sequences, is another type of analysis which can be performed. This will involve digesting the genomic DNA of the UAS-LF-DN mutants and UAS-LF-wt stocks with various restriction enzymes and then probing the resulting blot with the different LF sequence probes. Although Southern blotting will not uncover precise breakpoints, it will unequivocally reveal the presence or absence of large and

medium scale rearrangements affecting the LF sequence. In addition, a Northern blot could be used to reveal whether the LF-DN mutants encode transcripts of novel lengths.

Some mutants generated by the Δ2-3 transposase can encode hairpin RNA with RNA interference activity (Guichard et al, 2002) because this mutagen can induce the formation of inverted duplications. To test whether some LF-DN activity can be attributed to RNA interference, we could make use of GST-His-LF transgenic flies. We could cross these flies to each of our LFDN stocks and a control wild type stock. Subsequent immunoblotting with anti-GST antibodies would determine whether some of our UAS-LF-DN mutants are able to reduce the levels of the GST-HisLF protein, as would be expected if RNAi activity has been generated. A complementary approach for this question is to use immunofluorescence in wing imaginal discs to assess the effect of LF-DN alleles on the protein levels of LF-wt. If some mutants prove to have RNAi activity, then these should not be further studied, as they represent no potential therapeutic value for the treatment of anthrax.

If we find any LF-DN mutants that do not have RNAi activity, we would expect that they encode a protein product able to exert the DN activity toward LF-wt. Although it is hard to predict what kind of mechanism would underlie the DN activity without molecular data, we can suspect that a titration of co-factors (MKK substrate or unknown co-factors) is a likely possibility. Another hypothesis is that the LF-DN proteins bind and inactivate the LF-wt protein itself, although there is no current evidence that LF-wt forms homodimers.

In future screens, other mutagens, such as EMS, can be chosen that commonly generate point mutations but don't specifically affect P-element transgenes. Although the mutation rate is expected to be significantly lower in such screens, the subsequent

molecular analysis should involve a simple sequencing of the mutated LF sequence, and therefore be much easier. It may be helpful also to use a tagged version of LF (N and C-terminal tags) to initiate a new screen, in order to readily identify mutants able to encode a protein product. In the future, close double insertions of UAS-LF should be avoided.

When we are able to successfully analyze the molecular lesions in our LF-DN mutants, we will then test whether they have dominant negative activity in mammalian systems. For example, we will induce human cells to express the dominant negative toxins, to see if they can counteract the effects of LF-wt or anthrax exposure. If such activity is observed, then animal infection systems could be envisioned.

DN strategies are not currently pursued by other groups in the search for anthrax adjunctive therapies, because of their possible higher cost, and perhaps lower stability of protein products in comparison to chemical compounds. Nonetheless, it seems that a DN LF that is able to enter specifically cells attacked by anthrax toxins (cells carrying ATR/PA multimers at their surface) would be able to block toxin activity within infected cells, and might have less side effects than chemical compounds. Thus, DN strategies should still be pursued as a way to aid patients in the later phase of an anthrax infection.

References

Infect Immun. 1991 Oct;59(10):3472-7. Contribution of individual toxin components to virulence of Bacillus anthracis. Pezard C, Berche P, Mock M.

Singh Y, Khanna H, Chopra AP, Mehra V. A dominant negative mutant of Bacillus anthracis protective antigen inhibits anthrax toxin action in vivo.J Biol Chem. 2001 Jun 22;276(25):22090-4.

Characterization of dominant-negative forms of anthrax protective antigen. Mol Med. 2003 Jan-Feb;9(1-2):46-51

Jonah G, Rainey A, Young J. Antitoxins: Novel Strategies to Target Agents of Bioterrorism. Nature Reviews, volume 2. 2007 September; 721-726

Tournier JN, Quesnel-Hellman A, Cleret A, Vidal D. Contribution of toxins to the pathogenesis of inhalational anthrax. Cellular Microbiology 2007 Jan 11; 9(3), 555-565

Guichard A, Park JM, Karin M, Cruz-Moreno B, Bier E. Anthrax lethal factor and edema factor act on conserved targets in Drosophila. Proc Natl Acad Sci U S A. 2006 February 28; 103(9): 3244–3249.

Bier E. Drosophila, the golden bug, emerges as a tool for human genetics. Nature Reviews Genetics 6, 9-23 (January 2005)

Guichard A, Srinivasan S, Zimm G, Bier E. A screen for dominant mutations applied to components in the Drosophila EGF-R pathway. Proc Natl Acad Sci U S A. 2002 March 19; 99(6): 3752–3757.