

DETERMINING THE EFFECT OF LEF-12  
ON LATE VIRAL GENE EXPRESSION

A Senior Honors Thesis

By

APRIL LYNN PETERSON

Submitted to the Office of Honors Programs  
& Academic Scholarships  
Texas A&M University  
In partial fulfillment of the requirements of the

UNIVERSITY UNDERGRADUATE  
RESEARCH FELLOWS

April 2000

Group: Molecular Genetics

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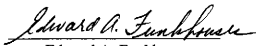
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RESEARCH FELLOW

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April 2000

Group: Molecular Genetics

## ABSTRACT

Determining the Effect of LEF-12  
on Late Viral Gene Expression. (April 2000)

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There are many advantages of using the baculovirus expression system, including: high expression levels, post-translational modifications, and the fact that the resulting proteins are correctly folded and biologically active. Late viral expression factors (LEFs) are required for transcription from late viral promoters, such as the polyhedrin promoter, under which the desired protein is overexpressed. For this reason, a better understanding of the LEFs is advantageous to better understand and improve upon the baculovirus expression system. The most recently identified LEF, LEF-12, was found to be necessary for transient late gene expression but its function has yet to be determined (5). We over-expressed and purified the LEF-12 protein and subjected it to DNA binding and transcription assays. Through these assays we determined that LEF-12 does not bind directly to the polyhedrin promoter and increases late viral transcription to a point. We also discovered significant sequence homology between LEF-12 and a subunit of eukaryotic RNA polymerase II. Although our next objective was to determine whether mutations in the conserved motifs abolished LEF-12 function, unforeseen difficulties have prevented this analysis up to this point. A recombinant virus in which the *lef-12* gene has been interrupted by the  $\beta$ -galactosidase gene has also been constructed. We are performing other experiments to better understand the function of LEF-12 in baculovirus late viral transcription which have not been completed at this time.

I would like to dedicate this thesis to my parents, Rodney and Rebecca Peterson who have always believed in me and have never thought twice about sacrificing of themselves so that I could achieve everything I've ever wanted.

To my sisters, Robin and Melissa Peterson  
who were great playmates  
and are great friends.

To my grandparents, Marlene and Pete Peterson who have made my college education possible with their generous contribution of financial and emotional support.

To my friends Cari Cowling and John Proctor  
for all the laughs to get me through the hard times  
and all the times you were there when I needed someone.

And finally, to Aaron Tabor Risinger  
for all his help and patience while I have been writing this  
and for the love he has shown me and the faith he has in me, and us.  
I love you.

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## INTRODUCTION

The baculovirus expression system is widely used for the production of medically important proteins for use as vaccines, diagnostic tools, and therapeutic agents. Hundreds of recombinant proteins have been produced and studied using the baculovirus expression system, which is one of the most documented expression systems. At this time, there are limitations to the baculovirus system due to the fact that expression of recombinant proteins is reduced when cellular processing pathways are compromised. Because of these prospective uses and results that indicate that the baculovirus late transcription system appears to be unique among eukaryotic viruses, there is an advantage to understanding the mechanisms involved in baculovirus gene expression. The genome of *Autographa californica* Nuclear Polyhedrosis Virus (AcNPV), the prototype baculovirus, is a double-stranded, super-coiled DNA molecule of 134 kbp, and potentially encodes 154 proteins. Eighteen proteins known as LEFs (late expression factors) that are required for baculovirus late gene expression were previously identified (6) and recently, a nineteenth protein, LEF-12, was discovered (5). LEFs are classified as such by providing at least a ten-fold stimulation to late reporter gene expression. Due to the recent discovery of LEF-12 and a lack of information on its role in baculovirus late viral transcription, the object of my thesis was to better elucidate the function of LEF-12 in late viral transcription.

The *lef-12* open reading frame was subcloned into an expression vector and the LEF-12 protein subsequently expressed and purified. This purified protein was then subjected to DNA binding and transcription assays to better understand LEF-12's role.

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This thesis follows the style and format of *Science*.

These assays indicated that LEF-12 does not bind directly to the polyhedrin late viral promoter and that increases in LEF-12 concentration increase transcription from the polyhedrin promoter, but only up to a point. The nineteen LEFs were all added to a reporter gene under control of the polyhedrin promoter, but to this date, we have not been able to reconstitute late viral gene activity in this manner. We have also discovered sequence homology between LEF-12 and a subunit of RNA polymerase II in yeast, arabidopsis, and humans (2,3). Four conserved motifs were identified and mutagenized to determine whether they are required for late viral transcription. If the mutant promotes transcriptional activity when added to the other eighteen LEFs, it would imply that the mutated motif is not required for LEF-12 functioning; however, if viral transcription is not supported, it would suggest the motif must be required for normal LEF-12 activity. The four conserved motifs were mutagenized and the mutants in two of these motifs have been positively screened. Ideally, the aforementioned reporter gene assay would be utilized to determine whether mutations in these conserved motifs eliminated LEF-12's effect on late viral transcription, however we have been unable to reconstitute late viral transcription from all nineteen wildtype mutants. The difficulty of transforming nineteen different plasmids into SF cells has prevented any experiments with the LEF-12 mutants. To avoid this obstacle, a recombinant baculovirus has been constructed in which *lef-12* is interrupted by the  $\beta$ -galactosidase gene. This mutant baculovirus is important because it will serve as an important tool in reconstituting late viral transcription with the LEF-12 mutants. By performing these experiments, we will come to a better understanding of the function of LEF-12 and whether it is truly analogous to the RNA polymerase II subunit with which it shares sequence homology.



## SUBCLONING, EXPRESSION, AND PURIFICATION

### Subcloning *lef-12* in an *E.coli* Expression Vector

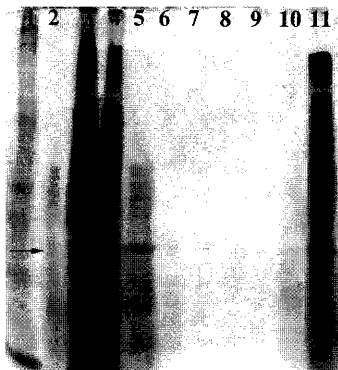
Site directed polymerase chain reaction (PCR) mutagenesis was performed on the Pst 1-F subclone of *Autographa Californica* Nuclear Polyhedrosis Virus (AcMNPV) to introduce a NdeI restriction enzyme site upstream of the *lef-12* open reading frame. Products of the PCR reactions were digested with DpnI restriction enzyme to fragment the parental DNA and then transformed into XL1-Blue competent cells and grown on LB ampicillin media. Ampicillin resistant colonies were amplified, their DNA extracted, and screened with NdeI to determine which cells carried the desired mutation. The *lef-12* open reading frame of the mutagenized construct was then digested with NdeI and EcoRV, gel purified, and directionally ligated under the T7 promoter of a PTYB12 expression vector cut with SmaI and NdeI. Digestion of PTYB12 with SmaI and NdeI removes an XhoI recognition site. Therefore, the restriction enzyme XhoI was added to the ligation reaction to ensure that no intact vector was present. Products of the digestion and ligation reaction were transformed into XL1-Blue competent cells and screened with NdeI for the desired directional ligation of *lef-12* into the vector. The insert was small in comparison to the vector so screening the colonies with NdeI (which only cuts once) was not sufficient to tell a difference between the parental and the subclone; therefore it was also screened with ApaI, which cuts in the insert but not in the vector, to confirm the proper ligation.

The desired DNA construct was used to transform BL21(DE3) GroESL competent cells. Colonies were grown in the presence of ampicillin and chloramphenicol to ensure only those GroESL cells containing the desired plasmid survived. Expression of LEF-12 under control of the PTYB-12 T7 promoter was induced with 0.1mM IPTG after a five hundred milliliter prep reached an OD600 of 0.65 and was then grown at 20°C overnight. The cells were harvested, sonicated to disrupt the cells, and centrifuged to pellet the cell debris.

## Purification of LEF-12

The soluble extract obtained from the expression of LEF-12 was loaded onto a chitin column. After extensive washing to remove unbound material, the column was incubated overnight in 30mM DTT. Then LEF-12 was eluted with five one-milliliter aliquots of low salt buffer. The chitin-binding column was used because the PTYB12 expression vector makes a fusion protein of the inserted gene and a chitin-binding domain linked to a self-cleaving intein. Incubation in the presence of KTT induces cleavage of the intein, releasing free LEF-12 that can be eluted with low salt buffer. Fractions from the pellet, the loaded supernatant, the flow-through, the elution and the stripping were run on an acrylamide gel to determine the location of LEF-12 (Figure 1). The LEF-12 protein was present in the supernatant, the flow-through, and the first eluted fraction. The first eluted fraction from the chitin column was then run on a MonoQ anion exchange column to further purify the protein. Since the pI of LEF-12 is 6.2 it carries a negative charge at neutral pH and should bind to the column and elute with a low salt buffer. Fractions from the MonoQ column were run on an acrylamide gel and it was discovered that the purified protein was too large to be LEF-12 (Figure 2). Since there were no other peaks on the chromatograph, the  $P_i$  of LEF-12 must not be low enough to exhibit sufficient binding to the MonoQ column at neutral pH.

More protein was acquired by repeating the expression protocol for LEF-12 in GroESL cells. This impure protein was again run on a chitin column, and then run on a gel filtration column which separates proteins by their size. By running several fractions on an acrylamide gel, we determined that LEF-12 was present in three fractions and was pure (Figure 3). These fractions were pooled and dialyzed against 50mM Tris (pH 8), 400mM KCl, 0.1mM EDTA, 50% glycerol, and 1mM DTT. After dialysis, the concentration of protein was measured, but was so low that it could not be accurately determined even using BSA standards.



**Fig. 1. Chitin Column Fractions.** The crude extract from the overexpression of LEF-12 in GroESL cells produced a LEF-12/chitin binding domain fusion protein. This protein bound to chitin beads when the extract or load was run over them while the majority of the other proteins flowed through the column. Samples of this load fraction (lane 3) and flowthrough fraction (lane 4) were run on an 8% acrylamide gel alongside a protein marker (lane 1), and stained with comassie blue. A sample from each of the five elutions (lanes 5-9) and the stripping fractions (lanes 10-11) were run and a sample of the pellet from the spinning down of the sonication was also run on the gel (lane 2). This gel illustrates that the 21kDa LEF-12 protein bound to the column and came off in the first elution fraction. There is sufficient protein in the first elution fraction to continue the purification process although there is a high concentration of LEF-12 in the stripping buffer. The arrow on the left points to the migration position of 21kDa proteins on the gel.



**Fig. 2.** MonoQ Column Fractions. The first elution fraction from the chitin-binding column was run on a MonoQ anion exchange column to further purify the LEF-12 protein. In this column, proteins that are negatively charged in neutral pH bind to the column and are then eluted with an increasing salt gradient. Samples of the loaded fraction (lane 2) and fractions corresponding to peaks in the chromatogram (lanes 3-7) were run on an 8% acrylamide gel alongside of a protein marker (lane 1). The protein which gave the peak in the chromatogram is seen in lane 7, but is too large to be LEF-12. The arrow on the left points to the migration position of 21kDa proteins on the gel.

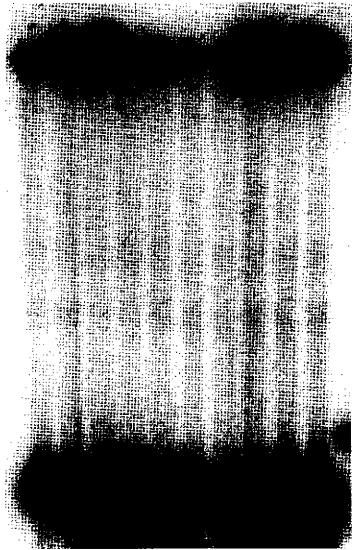


**Fig. 3.** Gel Filtration Column Fractions. The first elution fraction from the chitin-binding column was run on a gel filtration column to further purify the LEF-12 protein. In this column, proteins are separated by size; the larger proteins elute first and the smaller ones elute later. Since LEF-12 is a 21kDa protein, it was expected to elute fairly late. Fractions corresponding to peaks on the chromatogram were run on a 8% polyacrylamide gel (lanes 3-20) along side of a sample of the loaded fraction (lane 2) and a protein marker (lane 1). The chromatogram showed several peaks, so multiple fractions were run to determine the location of LEF-12. The arrow on the left points to the migration position of a 21KDa protein, therefore lanes 18-20 contain the purified LEF-12 protein.

## GEL SHIFTS AND TRANSCRIPTION ASSAYS

### Gel Shifts with LEF-12 and the Polyhedrin Promoter

Two fold dilutions of purified LEF-12 were added to radio-labeled DNA fragments containing the polyhedrin promoter and run on a 6% polyacrylamide gel to look for direct binding between LEF-12 and the polyhedrin late viral promoter (Figure 4). Each reaction contained 2M Tris (7.9), .5M EDTA, 1M DTT, BSA, .5M MgCl<sub>2</sub>, and xylene. The protein dilution buffer used contained 100mM KCl, 50mM Tris, 0.1mM EDTA, and 50% glycerol. The low salt concentration was attempted because it makes protein-protein and protein-DNA binding more favorable. Since no shift was seen, it appears that there is no direct interaction between LEF-12 and the polyhedrin promoter. Therefore, LEF-12 must impose its effect of increasing late viral transcription by some other means than direct interaction with the DNA.

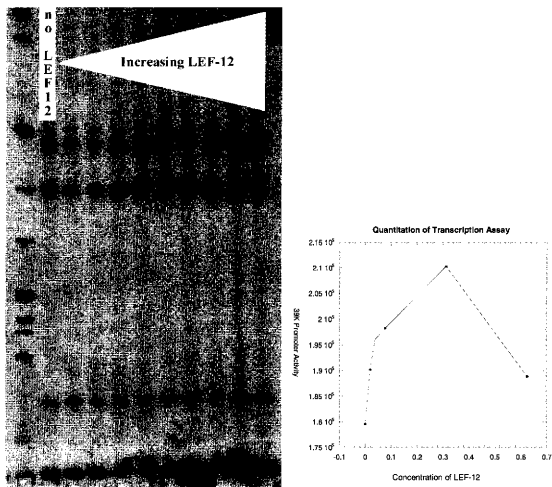


**Fig. 4.** Gel Shift with LEF-12 and Polyhedrin Promoter. Two fold dilutions of the LEF-12 protein were added to radiolabeled polyhedrin promoter to observe whether there was direct binding between the two. If there were direct binding, the radiolabeled probe seen at the end of the gel would have shifted up upon LEF-12 binding. Because no shift was observed with any protein concentration, there appears to be no direct binding between LEF-12 and the polyhedrin promoter; the increase in transcription due to LEF-12 probably occurs by some other mechanism.

### **Transcription Assays with LEF-12 on Polyhedrin and 39K templates**

Two fold dilutions LEF-12 were made in 50mM Tris (7.9), 400mM KCl, .1mM EDTA, and 50% glycerol. These successive dilutions were added to polyhedrin and 39K C-free cassettes, and purified AcMNPV RNA polymerase (1) in the presence of 2 mM  $Mg^{2+}$ , 5 mM DTT, 0.1% Chaps, RNasin, and PPase. 1 mM ATP and UTP nucleotides were added along with 20 mM  $^{32}P$  labeled GTP and the reactions were run on an acrylamide sequencing gel (Figure 5a). The C-free cassette allows transcription of the mRNA from the polyhedrin or 39K promoter in the absence of CTP and terminates at the end of the cassette when CTP needs to be incorporated. In this manner it is possible to get multiple transcripts of an exact size making quantitation much more exact. The presence of polyhedrin and 39K radiolabeled transcriptional product were detected upon exposure to a phosphoimager plate and the product quantitated on computer (Figure 5b). The quantitation showed a slight trend toward increasing product as more LEF-12 was added but only up to a certain point where it appeared to inhibit transcription. This may be the case, however, the baculovirus RNA polymerase used in the transcription assays was not entirely pure and may have had LEF-12 contaminants present. If this was the case then addition of LEF-12 may not make a marked difference in transcriptional levels because sufficient amounts of LEF-12 might already be present.





**Fig. 5.** Transcription Assay with LEF-12 on Polyhedrin and 39K C-Free Cassettes. LEF-12 has been shown to increase levels of transcription in CAT assays (5), therefore two-fold dilutions of LEF-12 were incubated with C-free cassettes under control of polyhedrin and 39K promoters in the presence of ATP, GTP, TTP, and  $^{32}$ P GTP. The reaction products were run on a sequencing gel (a) and the amount of product was quantitated. Panel B shows the amount of 39K product synthesized as a function of the concentration of LEF-12 present in each reaction (b). The graph indicates that an increase in LEF-12 leads to an increase in late-viral transcription up to a point where it becomes saturated or even begins to inhibit transcription.

## SEQUENCE HOMOLOGY

### **Mast Search and Results**

The LEF-12 sequence was entered into the MAST database, which stands for Motif Alignment and Search Tool. This program compares the entered sequence to others in its database and finds proteins with significant sequence homology to the protein in question. The results of this search indicated that there was the expected sequence homology between six motifs in AcMNPV LEF-12 and its equivalent in two different baculovirus strains. However, there was also found to be sequence homology between four motifs in LEF-12 and a eukaryotic RNA polymerase II subunit. The level of sequence similarity was so great that there is calculated to be only a  $10^{25}$  chance that the match was due solely to coincidence. In yeast, arabidopsis, and humans, this protein has been found to be responsible for stress survival at high temperatures (2,3). It is not known what function LEF-12 has in late viral transcription, but if the conserved motifs are required for LEF-12 activity there is a strong likelihood that it has some of the same mechanistic properties as the eukaryotic RNA polymerase II subunit. The conserved motifs between LEF-12 and the RNA polymerase subunit were mutated using site-directed PCR mutagenesis on a plasmid containing the LEF-12 open reading frame. Charged residues, which are usually involved in protein-protein interactions were mutated to alanine which is a non-charged residue (Table 1). If a charged residue is conserved from the eukaryotic RNA polymerase to baculovirus because of its ability to interact with other proteins involved in transcription, then the mutation of that residue should decrease the level of late viral transcription. However, if late viral transcription is not altered when the conserved charged residues are mutated, then it decreases the likelihood that there is functional homology between LEF-12 and the RNA polymerase II subunit. Oligonucleotides for the site-directed mutagenesis were designed so that each mutant would contain the altered motif and a gain or loss of a restriction enzyme site making it possible to screen for the mutation by way of DNA digestions.

	Motif 1	Motif 2	Motif 3	Motif 4
AcNPV	MTAAWLC	PTTYMNYTLF	GYITQQDA	YFKFESLAQR
BMNPV	MTAAWLC	PTTYMNYRLF	GYITQQDA	HFKEESLAQR
OPNPV	MTAAWVC	PTTYMNYAFF	GAITRADA	HFMFESLAQR
Yeast		VYQCQVFRPFK	GTISGRHG	HLIPDMEFQA
Arabidopsis		VYQCQVFRPFK	GTISGRHG	HLIPDMEFQS
Human		VYRAVVFKPRK	GSITGKFG	HLMPDMITFNA
Consensus	MTAAWLC	PTTYMNYRPFK	GYITGQDG	HFMFDMLFQR

**Table 1.** Mutation of conserved motifs. The six motifs present in LEF-12 are conserved between three baculovirus proteins. Motifs 2, 3, 4, and 5 are also conserved between LEF-12 and the RNA polymerase II subunit found in yeast, arabidopsis, and humans. The marked charged residues have been mutated to alanine by site-directed mutagenesis along with the creation or deletion of a restriction enzyme site for screening purposes. In motif 2, two charged residues have been mutated in separate reaction to test for the transcriptional dependence of each residue independently. Motifs 5 and 6 are not shown because they have not been mutated by site directed mutagenesis.

## CAT Assays

To determine whether late viral transcription occurs when the mutant LEF-12 is present instead of the wild-type LEF-12, a reporter construct was used. A late viral promoter, the polyhedrin promoter, was placed in front of the gene for chloramphenicol acetyl transferase. Since this assay was performed to first identify LEF-12 (5), it is an appropriate assay to identify active or inactive mutations in the protein. Originally, calcium phosphate precipitations were used to transfect the DNA into the SF9 cells. Because there were problems with consistency in the wildtype viral transfections using this method, we began using lipofectin for transfections. The Cellfectin recommended transfection protocol was followed using Grace's incomplete media instead of Sf-900 II SFM and the cells were harvested forty-eight hours post-transfection. This transfection method was more reliable and eliminated one of the variables involved in this experiment. After harvesting the transfection, the cells were lysed by three cycles of freeze/thaw and 25ml of the extract were added to 1M Tris (7.8), 5mM chloramphenicol, and tritium labeled acetyl CoA. Five milliliters of an organic scintillate were added and the counts per minute were measured in a scintillation counter every thirty minutes. The CAT gene was found to be active when transfected into SF9 cells along with viral DNA, but it was necessary to knock-out the function of wildtype LEF-12 to determine whether or not the LEF-12 mutants retained their activity when added in place of wildtype LEF-12. The first method used to knock-out LEF-12 activity was to digest wild-type viral DNA with EcoRI, which cuts in the open reading frame of LEF-12. Because EcoRI also cuts in the viral DNA polymerase open reading frame, the Bgl 1-F subclone containing the DnaPol open reading frame was also added to the transfection. However, when the digested viral DNA was transfected along with BglIF and wildtype LEF-12 there was no resulting CAT activity. This result is likely due to the low transfection rate of non-supercoiled DNA, especially when the uptake of twenty open reading frames are required.

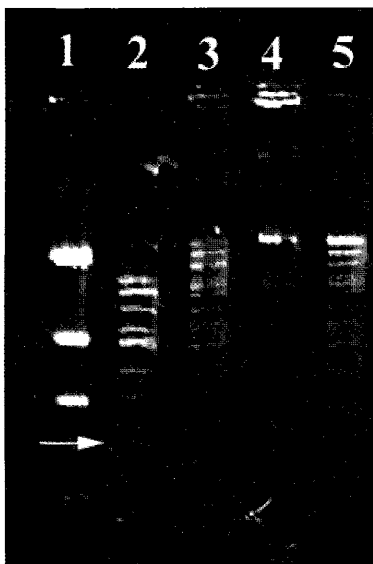
To bypass the inherent problems associated with the transfection of linear DNA, I attempted to subclone all the LEFs on smaller plasmids for use in the CAT assay with the initial objective of repeating the experiment used to discover LEF-12 (5). However, when I transfected the library including wild-type LEF-12 into SF9 insect cells along with the CAT gene under a late baculovirus gene promoter, there was no resulting CAT activity. Because of the difficult task associated with rescreening all twenty subclones, a complete LEF library containing all twenty plasmids shown to be required for late viral transcription was obtained from Dr. Joyce Wilson in the Department of Entomology at the University of Georgia. Each plasmid in this HSEpiHis LEF library contains a LEF open reading frame under control of the HSP70 promoter and was found to be active in the LEF assay. We digested each of these plasmids with SacII and BglII to cut out the open reading frame and determine if each plasmid was correct. When these plasmids were transfected into SF9 insect cells along with wildtype LEF-12 and the reporter plasmid, there was no CAT activity. The reason for this is unknown at this point, but it appears that one of the plasmids is incorrect due to the fact that the transfection of wildtype viral DNA resulted in high levels of CAT activity.

## LEF-12/ $\beta$ -GALACTOSIDASE RECOMBINANT VIRUS

### Construction of the Recombinant Virus

Due to the high levels of CAT activity resulting from the transfection of wild-type viral DNA, it would be ideal to construct a virus where the LEF-12 activity was knocked out, but was otherwise wildtype. This was found to be a possible endeavor because while LEF-12 is required for infection in SF9 cells, it is not essential for infection in *Trichoplusia ni* (T.ni) cells. LEF-7 also exhibits the same type of cell-line specificity (5). This fact allows us to knockout LEF-12 function and amplify the virus in T.ni cells and then perform the CAT assays in SF9 cells where functional LEF-12 is required for late promoter gene activity. The only difficulty in this method is that Tini cells can not be grown in spinners making it difficult to amplify large amounts of the mutant virus. To make a knockout of the *lef-12* gene, the  $\beta$ -galactosidase gene was first inserted in the *lef-12* open reading frame of the PstF fragment. The Kpn1 to Sma1 fragment of PstF was cut out and religated leaving only one Apa1 site in the plasmid interrupting the *lef-12* gene. This construct was digested with Apa1, ligated with BglII linkers and then transformed into JM83 competent cells and colonies screened for the desired construct. A plasmid containing the  $\beta$ -galactosidase gene was digested with BamHI and the resulting  $\beta$ -galactosidase fragment was gel purified and ligated into the BglII site of the mutated PstF plasmid. This construct was again transformed into JM83 competent cells and screened with x-gal. The correct construct was maxi-prepped and transfected into two million T.ni cells along with wild type AcMNPV DNA and left for four days. The resulting virus was spun down and the supernatant diluted to perform plaque assays. The plaque assays contained ten fold dilutions of the virus from  $1 \times 10^1$  to  $1 \times 10^7$  and X-gal to screen for those plaques that contained  $\beta$ -galactosidase, and were allowed to sit for one week. Blue plaques from the -1 and -2 plates were diluted ten and a hundred fold and replated to further purify the virus. The plaques were amplified in five milliliters of TNMFH for one week and the amplified virus was subjected to proteinase K, SDS,

phenol/chloroform extractions, and finally ethanol precipitated, digested with EcoR1, and run on an agarose gel (Figure 6). A virus containing the desired mutation was further amplified in thirty milliliters of TNMFH for one week and then pelleted, extracted with phenol/chloroform, and precipitated in ethanol.



**Fig. 6.** *Lef-12*/ $\beta$ -galactosidase recombinant virus. The four blue plaques obtained from the plaque assay were amplified and the DNA digested with EcoRI. The fragment that contains LEF-12 in the wildtype virus, EcoRI-K, is marked with an arrow in lane 2 and is absent in the viral mutants present in lanes 3 and 5. This fragment is not present in the mutant virus because the  $\beta$ -galactosidase gene, which also contains an EcoRI site, has been inserted into this fragment. The absence of this fragment in the mutant viruses is an indication that the desired interruption of *lef-12* by  $\beta$ -galactosidase has been successful.



### **Southern Blot of Wildtype and LEF-12/B-galactosidase Baculoviruses**

The amplified mutant virus appeared to contain the desired construct containing the  $\beta$ -galactosidase gene interrupting the *lef-12* gene but, to confirm this, wildtype and mutant viral DNA were subjected to southern blots using the  $\beta$ -galactosidase gene and PstI-F inserts as probes. Wildtype AcMNPV and LEF-12/B-galactosidase mutant virus were each digested with XhoI and PstI in separate reactions and run on a .8% agarose gel. The concentration of mutant virus was not high enough to see with ethidium staining, so wild-type viral DNA was also used to probe the mutant and wild-type digests making it possible to see all of the viral bands on the southern. The agarose gel was subjected to alkaline denaturation, neutralization, and the DNA transferred to nitrocellulose filter paper overnight. Probes were constructed by the addition of random primers, dNTPs, <sup>32</sup>P dCTP, and Klenow enzyme to LMP gel purified fragments of PstF and  $\beta$ -galactosidase and to viral DNA. The nitrocellulose filter paper was cut into three portions each containing wild-type and mutant viral DNA and separately probed with the three radio-labeled probes.

## SUMMARY AND CONCLUSION

### Summary

*Lef-12* was successfully subcloned into an expression vector and subsequently overexpressed in *E.coli*. The LEF-12 protein was then purified by running the sonicated cell extract over chitin and gel filtration columns. This purified LEF-12 was subjected to gel shifts to determine whether it directly bound to the polyhedrin promoter and transcription assays to determine LEF-12's effect on transcription when added to purified RNA polymerase. It was discovered that LEF-12 contains four motifs that are conserved in an RNA polymerase II subunit responsible for stress survival in high temperatures. Charged residues in these motifs were mutated by site-directed mutagenesis to uncharged alanines in order to determine if they are essential for LEF-12 transcriptional activity. CAT assays were performed to assay for transcription of a reporter gene under a late viral promoter. A recombinant virus was also constructed which contains a  $\beta$ -galactosidase gene interrupting the *lef-12* gene. This is similar to the approach used to analyze LEF-7, which also exhibits cell-line specificity (5). With the construction of this recombinant virus, it is possible to transfect the LEF-12 mutants along with it and the CAT reporter gene to measure late viral transcription when different LEF-12 motifs have been mutated.

### Conclusions

While LEF-12 was purified to apparent homogeneity, the concentration of protein was not high enough to measure accurately making it difficult to perform gel shifts and transcription assays. The gel shift assays indicated that the activity of LEF-12 in late viral transcription is not due to direct binding of LEF-12 to the polyhedrin late viral promoter. The transcription assay gave inconclusive results due to the fact that the baculoviral RNA polymerase was not entirely pure. The quantitation of the assay, however, indicates that LEF-12 may interact directly with the baculoviral RNA polymerase to increase transcriptional levels up to a point where it appears to inhibit or at least ceases to increase them. It would be worth repeating this experiment with a

completely purified RNA polymerase to better understand the function of LEF-12 in baculoviral late transcription. The site-directed mutagenesis performed to construct the LEF-12 mutants has, to this point, only been successful in mutating two of the conserved motifs. Motif 3 has not been mutated because there has been difficulty in constructing an oligonucleotide that would mutate the conserved cysteine residue and introduce a restriction enzyme site for screening. The mutants in motif 1 and 4 have been elusive likely due to the larger degree of mutagenesis required to mutate two consecutive residues. The two mutations that have been successful are the mutation to alanine of two separate conserved charged residues in motif 2. The difficulty I have had in reconstituting late viral transcription from a complete set of LEF plasmids has, to this point, made it impossible to test whether the mutated LEF-12 is active. The lack of late viral activity from the addition of the LEF plasmids is probably the result of one or more of the plasmids being incorrect. The process to find which one(s) are incorrect will be difficult because they have already been screened with different restriction enzymes and all appear to coincide with their accompanying restriction map. When the mutated virus has been amplified and shown to be correct by southern hybridization, we may be able to bypass the need for the LEF library because the mutant virus will carry the entire genome with the exception of LEF-12 which can be supplemented on a plasmid in its wildtype or mutant forms. Although it would be ideal to reconstitute late viral transcription by both means and obtain data on the activity of LEF-12 mutants in each manner, the method of using the LEF library may take much more time do to the extensive screening of each of the plasmids involved.

In conclusion, we know that LEF-12 is required for late viral transcription of the baculovirus AcMNPV in SF9 insect cells. We also know that LEF-12 shares high sequence homology with a subunit found in eukaryotic RNA polymerase II (2,3). The objective of my research was to further understand the function of LEF-12 and to determine whether the conserved motifs were required for LEF-12 functioning, giving a

basis for functional homology between the proteins. The fact that I have purified the LEF-12 protein, performed some basic binding and transcriptional assays, and constructed a mutant virus that lacks the LEF-12 will allow further studies to be completed where this thesis left off. Due to unforeseen difficulties in carrying out the experiments outlined, however, I have been unable to fulfill my final objective of determining the function of LEF-12 in baculovirus late viral gene expression. I believe that these obstacles can be overcome in time and, with the help of the work I have done, it will eventually be possible to obtain the results I was looking for.

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## VITA

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Although I am completing my third year, I am currently classified as a senior at Texas A&M University. I will be graduating in December of 2000 with my bachelors of science degree in biochemistry. To this point in my college career, I have maintained a 4.0 cumulative grade point ratio. I plan to attend graduate school beginning in the fall of 2001 to obtain my Ph.D. in biochemistry. I have completed two years of undergraduate research in Dr. Linda Guarino's laboratory and plan to continue undergraduate research for the remainder of my time at Texas A&M University.

### HONORS AND AWARDS

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|-----------|--|
| 1999-2000 | <i>Undergraduate Research Fellow</i>   |
| 1999      | <i>Sigma Chemical Company Award for Outstanding Junior in Biochemistry</i>   |
| 1999      | <i>National Undergraduate Bioethics Conference</i><br>Selected as one of 250 undergraduates to attend the first ever national undergraduate bioethics conference held at Princeton University.                           |
| 1998      | <i>Outstanding Sophomore in the College of Agriculture and Life Sciences</i> Recognized by Gamma Sigma Delta (Honor Society of Agriculture and Life Sciences) as the outstanding sophomore in the college.               |
| 1997-2000 | <i>Dean's Honor Roll</i><br>Named to the Dean's Honor Roll all semesters for maintaining a 4.0 cumulative grade point ratio.   |
| 1997      | <i>College Board Distinguished Scholar</i><br>Received title of College Board Distinguished Scholar by the College Board for achieving a grade of three or higher on four or more College Board Advanced Placement exams |