SCREEN OF NUCLEAR LOCALIZED EFFECTOR PROTEINS IN *Coxiella burnetii*

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Screen of Nuclear Localized Effector Proteins of *Coxiella burnetii.* (May 2013)

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In some pathogens such as *Ehrlichia chaffeensis*, *Anaplasma phagocytophilum*, *Chlamydia*, and *Shigella*, secreted effector proteins have been found to localize to the nucleus and alter the host's immune response by modulating chromatin or binding to regulatory transcription molecules. By doing so, the pathogen is able to successfully grow and replicate within the host cell causing infection. Several of *Coxiella burnetii*'s secreted effector proteins have similarly been found to localize to the nucleus but have no confirmed role. The objective of this study is to confirm nuclear localization of previously identified nuclear effector proteins and pinpoint any interactions between host DNA and protein. Each GFP tagged effector protein of interest- CBU0129, CBU0794, CBU0393, CBU1314, and CBU1524, was ectopically expressed in HeLa cells to perform sub-cellular fractionation. These sub-cellular fractions were run on a SDS-Page gel and probed with anti-GFP to identify any association with specific fractions within the host. Nuclear localization signal (NLS) truncations were then constructed using splicing by overlap-extension PCR (SOEing PCR) to remove predicted nuclear localization signals from each

effector. The ΔNLS constructs were also ectopically expressed in HeLa cells to visualize loss of localization. Chromatin Immunoprecipitation (ChIP) was performed to identify specific binding regions of each protein and further studies such as en Electrophoretic Mobility Shift Assay (EMSA) were attempted to confirm the binding of chromatin by the effector proteins. Sub-cellular fractionation confirmed the association of all five effector proteins to host chromatin, and the loss of nuclear localization by two effectors upon deletion of predicted NLS indicates that these effectors are not only specifically imported to the nucleus via NLS, but have some specialized role in the nucleus. Confirmation of these predicted protein-DNA interactions will allow *Coxiella* researchers to better understand how this organism survives and replicates. With more knowledge of these processes, scientists will be better equipped to prevent and treat future infections.

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CHAPTER I

INTRODUCTION

Coxiella burnetii is an obligate intracellular pathogen that uniquely uses its Dot/Icm type 4 secretion system (T4SS) for causing Q fever in mammals [1]. A list of proteins secreted through its pathogenic T4SS has been identified using a bioinformatics and bacterial-two hybrid approach; however the function of most of these bacterial substrates is unknown [2, 3]. The T4SS has been confirmed to play a key role in the pathogen's intracellular growth and replication, suggesting that specific effectors may play a role in intracellular replication and formation of an acidified parasitophous vacuole [1, 4]. In other pathogens such as *Ehrlichia chaffeensis*, *Anaplasma phagocytophilum*, *Chlamydia*, *Listeria* and *Shigella*, secreted proteins have been found to be localized to the nucleus in order to bind and alter the host cell's chromatin modulating its immune-response [5, 6]. By doing so, the pathogen is able to grow and replicate within the host cell successfully. In *Coxiella*, five of the secreted effector proteins have been shown to localize to the nucleus of the host cell by being ectopically expressed in HeLa cells and imaged using immunofluorescence [3]. Furthermore, a recent study demonstrated that *C. burnetii* infection leads to a change in the host transcriptional response and this transcriptional modulation may be mediated by specific effector proteins [7]. The localization of effector proteins to the nucleus paired with its intracellular lifestyle, similar to the bacteria listed above may indicate that these effectors may be responsible for the transcriptional modification s seen post-infection by binding to host chromatin. CBU1314, CBU0794, CBU0393, CBU0129, and CBU1524, have been observed to localize in the nucleus. Of these 5 nuclear localizing proteins,

CBU1314 was also predicted to contain a recognized nuclear localization signal in its domain using bioinformatics analysis and is currently under further studies.

Previous studies have shown that virulence proteins localized to the nucleus have DNA binding motifs and contribute to the pathogen's survival by modulating the host's response to infection. Several of *Coxiella*'s secreted effector proteins have been found to localize to the nucleus but have no confirmed role. Our objective is to determine whether any of the five nuclear localized effectors bind to host DNA and identify the region where they bind using ChIP seq. We hypothesize that all the effector proteins not only possess a nuclear localization signal, but are required in the nucleus to modulate host immune response.

CHAPTER II

MATERIALS AND METHODS

Sub-Cellular Fractionation

To confirm that each of the proteins of interest were associated with the host chromatin, each GFP tagged effector was ectopically expressed in HeLa cells. The cells were seeded in tissue culture plates and cultured in DMEM with 10% FBS at 37° C and 5% CO_2. The cells were transfected using lipofectamine according to the Invitrogen protocol, and inspected using fluorescent microscopy 24 hours post transfection to confirm proper efficiency. The cells were then fractionated using the sub-cellular fractionation kit (Pierce). Five microliters of each fraction was run on an SDS-Page gel, transferred to a nitrocellulous membrane, and probed with anti-GFP antibodies.

Nuclear Localization Signal Truncations

To determine if the nuclear substrates encode a nuclear localization signal, bioinformatics analysis was conducted using cNLS mapper and NUCpredict for each protein. For substrates possessing a predicted nuclear localization signal, deletion constructs were generating using SOEing PCR. The regions upstream and downstream of the projected NLS were amplified using the primers listed in Table 1, and the resulting constructs were subsequently stitched together using overlap extension with the restriction enzyme primers indicated in the same table with an asterisk. Proper amplification and stitching were screened by gel electrophoresis and the positive constructs were cloned into pEGFS-C1 as *BamHI/SalI* or *BamHI/KpnI* fragments in *E. coli*. These ΔNLS plasmids were then used to transfected HeLa cells on 24 well glass-bottom plates using lipofectamine (Invitrogen). 24 hours post-transfection, the nuclei were stained with 1X

Hoechst and imaged using a Nikon A1 confocal microscope. The constructs that showed a loss of localization to the host nucleus were sent for sequencing to eurofins to confirm correct sequence deletion.

Table 1: Primers used in this study.

Primer	**Sequence**
CBU1314 del NLS R	TTCCTGAGTTAAGCCTTTTTCCTGTTGGCGGTGGAAAGAGA
CBU1314 del NLS F	AGGAAAAAGGCTTAACTCAGGAATCTCTTTCCACCGCCAAC
*CBU1314 BamHI F	CC**GGATCC**ATGATAAGAAGGAAAGAGGAAAAATAC
*CBU1314 SalI R	CC**GTCGAC**TCACGATCGCTTGGCA
CBU0393 del NLS R	CGGTTCCGTGACTATTGTTTCTTGTGTACAAAAAAAATCTACTTTTTTT
CBU0393 del NLS F	GAAACAATAGTCACGGAACCGAAAAAAAGTAGATTTTTTTTGTACACAA
*CBU0393 BamHI F	CC**GGATCC**ATGAGTCGGCAAGAAACAATAGT
*CBU0393 SalI R	CC**GTCGAC**TTACCCAGAAGGCGGTTTT
CBU01524 del NLS R	ATGAACACAAGTCCTACATCAACTAAAAAAGTTGACGAAGAAGAGTGG
CBU01524 del NLS F	AGTTGATGTAGGACTTGTGTTCATCCACTCTTCTTCGTCAACTTTTTT
*CBU1524 BamHI F	CC**GGATCC**ATGAACACAAGTCCTACATCAACTG
*CBU1524 KpnI R	CC**GGTACC**CTATGTCCTTTTGGGAGCGT
*CBU0129 BamHI F	CC**GGATCC**TTGTGGTTGAGAGCAGAAGAG
CBU0129 del NLS R	CC**GTCGAC**TCGCTGAACTGGCTCTTTTT
*CBU0129 SalI R	CC**GTCGAC**TCACTTTGGCCGATGATGT
*CBU0794 BamHI F	CC**GGATCC**ATGAAAATTATTAAATTAGTGG
*CBU0794 SalI R	CC**GTCGAC**TTATCTAAATCTGGCTTTTTGCCT
CBU0794 del NLS R	TCGCGCTGTCTTGAGCTGGTGAATAAAGGCGGTTCATTA
CBU0794 del NLS F	AGCTCAAGACAGCGCGATAATGAACCGCCTTTATTCACC

Electrophoretic Mobility Shift Assay (EMSA)

The electrophoretic mobility shift assay (EMSA) was performed using fragmented DNA and ectopically expressed proteins from HEK293 cells. All cells were cultured in DMEM with 10% FBS at 37° and 5% CO_2. DNA was extracted, purified, and fragmented using the Chromatin Isolation kit (Pierce) and was nanodropped to record concentration. Biotin labeling was performed by following the Mirus Label-It Kit guidelines. The GFP-tagged nuclear effectors CBU0393, CBU0794, CBU0129, CBU1524, and CBU1314 as well as the positive control Ank-A from *Anaplasma phagocytophilum* were used to transfect HEK293 cells following the lipofectamine protocol (Invitrogen). Twenty-four hours post-transfection, cells were lysed using the Nuclear Extraction PER kit (Thermo) and treated with protease inhibitors according to the outlined protocol. Purification of these proteins was accomplished using magnetic protein G beads (Invitrogen) and an anti-GFP antibody (AbCam).

The purified proteins were mixed and incubated with the biotinylated DNA and allowed to bind as stated in the EMSA protocol. The reactions were then ran on a 6% EMSA DNA retardation gel, transferred to a nylon membrane (Thermo) in .5X TBE, probed with Streptavidin antibody and processed to be imaged according to the EMSA protocol using EMSA detection kit.

Chromatin Immunoprecipitation Assay (ChIP)

For the ChIP assays, HEK 293 cells were transfected using the Invitrogen Lipofectamine procedure to ectopically express each protein of interest (CBU0393, CBU0794, CBU0129, CBU1524, and CBU1314). Twenty-four hours post-transfection, DNA-protein interactions were stabilized by cross-linking with formaldehyde (Pierce). The chromatin fraction was then isolated and fragmented using micrococcal nuclease provided in the kit. Protein G beads and anti-GFP antibodies were used to separate the DNA and GFP-tagged effector proteins out of

solution. Once eluted, Proteinase K was added to degrade the bound effector proteins, resulting in the secluded DNA. The DNA was purified with reagents provided in the kit and concentration was measured using the Agilent 2100 Bioanalyzer.

CHAPTER III

RESULTS

Sub Cellular fractionation

All five nuclear localized effector proteins ectopically expressed in HeLa cells were observed to associate with the cell's chromatin when fractionated and separated into sub-cellular components (Figure 1). The vector, serving as the control, was shown to remain mostly in the cytoplasm, but traces were also noted in the soluble nuclear fraction. No traces were seen associated with the chromatin.

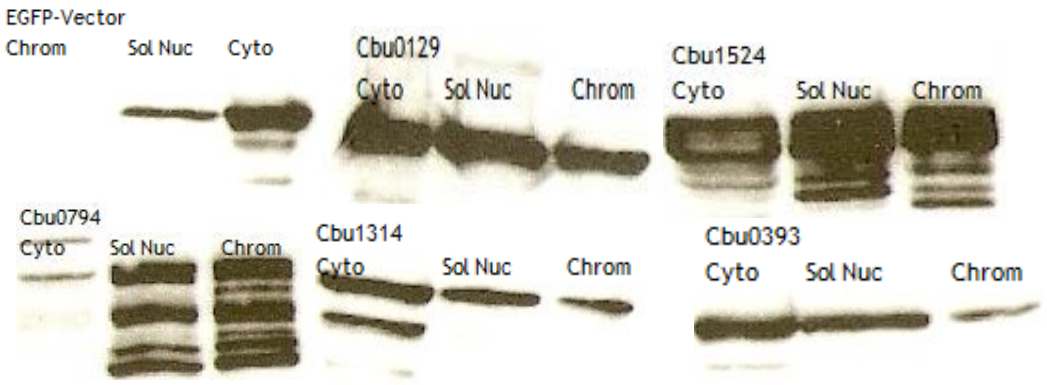

Figure 1: Subcellular fraction of transiently transfected HeLa cells. Cells were transfected using Lipofectamine, fractionated using Pierce kit and analyzed by western blotting. Each of the effectors localized to all 3 fractions, in contrast the vector control was excluded from the chromatin fraction and predominately assocaited with the cytoplasmic fraction.

ΔNLS Truncations

Proper nuclear localization signals were identified and successfully removed from two proteins of interest (CBU1524, and CBU0794). These ΔNLS constructs were ectopically expressed in HeLa cells and found to lose localization to the nucleus by fluorescent microscopy. The deleted amino acid sequences predicted for nuclear localization are listed in Table 2 below. Sequencing

result for, CBU1524, and CBU0794 have confirmed the deletion of said amino acid sequences. Figure 2 shows the loss of localization when the ΔNLS constructs of CBU1524 and CBU0794 were viewed under the confocal microscope.

Table 2. Amino acid sequences for NLS truncations.

CBU Effector	Amino Acid Sequence Deleted	Location
CBU1314 del NLS R	RNRARLRNRRR	starts at AA100 (del 100-120)
CBU1314 del NLS F	RNRARLRNRRR	starts at AA121 (del 100-120)
CBU0794 del NLS R	RIRKRKLLSISSIDEDKAFKKKIGE	starts at AA228 (del 228-253)
CBU0794 del NLS F	RIRKRKLLSISSIDEDKAFKKKIGE	starts at AA254 (del 228-253)
CBU0393 del NLS R	PPTKRPRGLAIKEKKL	starts at AA11 (del 11-26)
CBU0393 del NLS F	PPTKRPRGLAIKEKKL	starts at AA27 (del 11-26)
CBU0129 del NLS R	RPPKKHHRPK	starts at AA106 (del 106-115) (NLS at end of protein, no additional primer required)
CBU01524 del NLS R	PKRTRVD	starts at AA182 (del 182-188)
CBU01524 del NLS F	PKRTRVD	starts at AA189 (del 182-188)

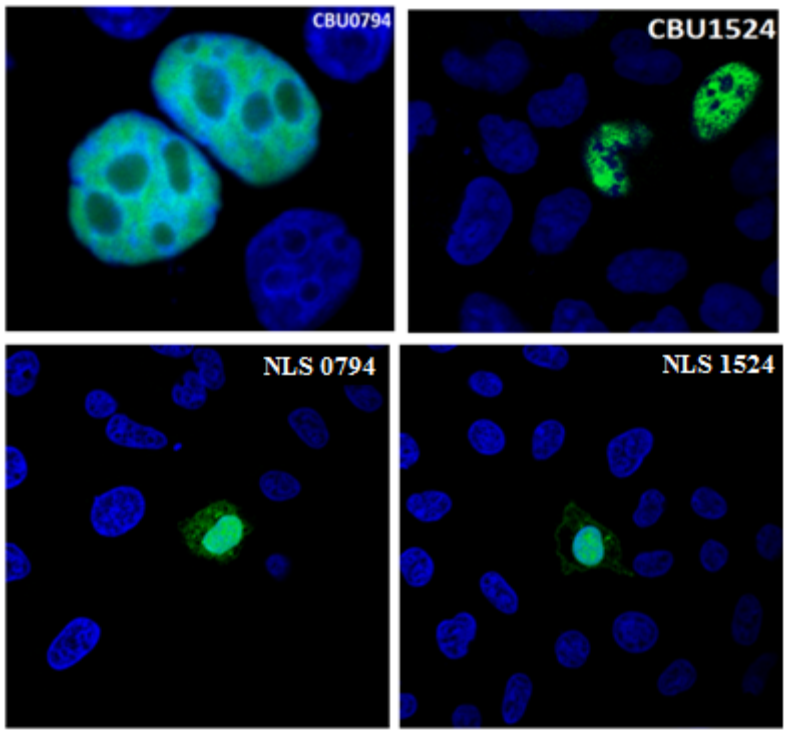

Figure 2. Loss of Nuclear Localization in ΔNLS effectors. Images A and B picture CBU0794 and CBU1524 ectopically expressed in HeLa cells stained with 1X Hoechst, magnification 100X. Images C and D are the ΔNLS constructs of CBU 0794 and CBU1524 expressed in HeLa cells and stained with 1X Hoechst, magnification 60X. Both images C and D indicate loss of nuclear localization.

Electrophoretic Mobility Shift Assay

Although several attempts were made to confirm binding between the nuclear effector proteins and fragmented host DNA using EMSAs, no shifts were noted. The control reactions provided in the kit resulted in clear bands as indicated in the protocol, but given that the positive control AnkA didn't give similar results suggest that further troubleshooting is needed.

Chromatin Immunoprecipitation

Small amounts of DNA from the following ChIP reactions were successfully detected and illustrated in figure 3; CBU1524, CBU1314, CBU0129, and CBU0393. Bioanalyzer results indicated that the concentration of each of these DNA samples was less than 10ng/ml, which is below the threshold needed for ChIP-seq. Currently larger scale ChIP is underway to get more DNA in an attempt to sequence the host sites bound by these effectors.

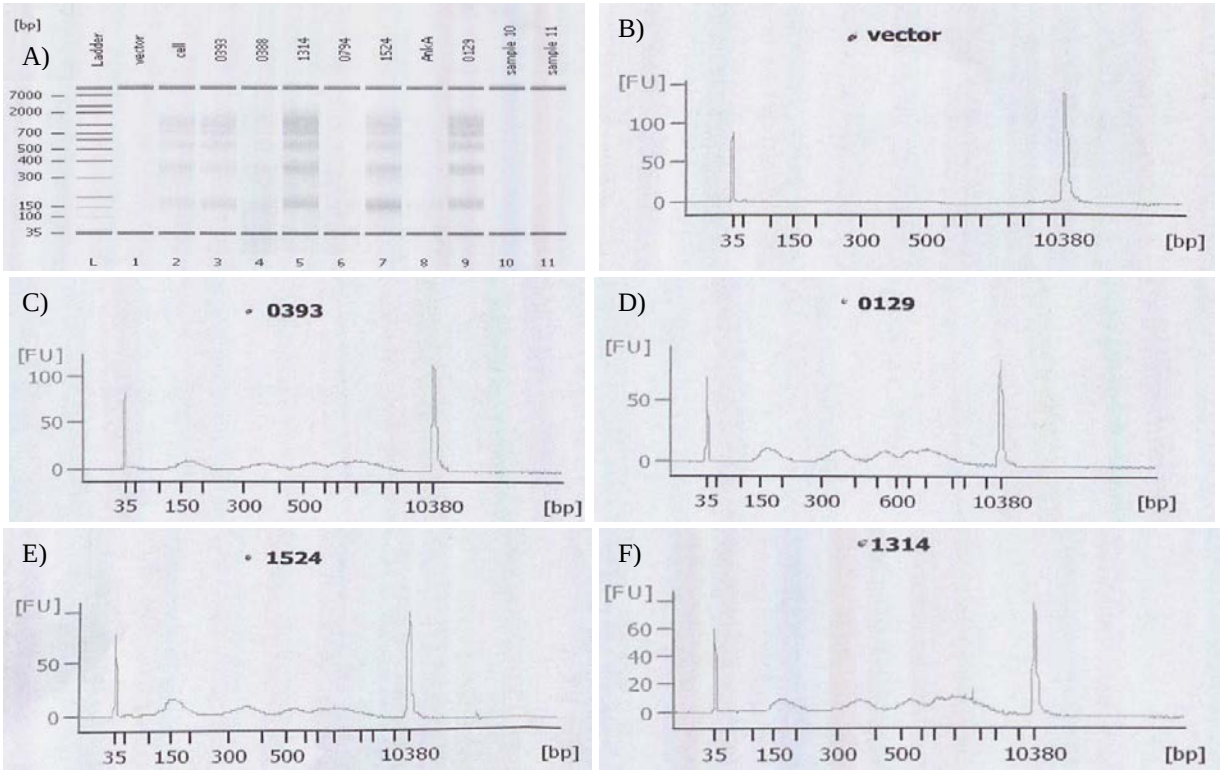

Figure 3. ChIP high sensitivity DNA Assay. ChIP protocol was performed according to manufacturer's guidelines for all nuclear effector proteins of interest plus the control-AnkA, empty vector, and untransfected cells. Figure A shows the electrophoresis on each of these samples, figures B-F correspond to the electropherograms of CBUvector, CBU0393, CBU0129, CBU1524, and CBU1314 respectively.

CHAPTER IV

DISCUSSION

Five of *Coxiella*'s effector proteins have previously been found to localize to the nucleus of host cells when ectopically expressed (images A and B in Figure 1). In this study, we provide evidence that these effectors not only localize to the nucleus, but are associated with the chromatin. Through sub-cellular fractionation of HeLa cells ectopically expressing these effectors, we identified that all five effectors are found in the cytoplasmic fraction, soluble nuclear fraction, as well as in the chromatin fraction. Since the vector, our control, was observed mainly in the cytosol and not in the chromatin fraction, we can infer that these proteins are indeed associating with host chromatin.

The loss of nuclear localization seen in ectopically expressed ΔNLS CBU0794 and ΔNLS CBU1524 indicates the successful identification of the NLS. The sequencing analysis further confirmed that the deleted region consisted of only the predicted NLS and nothing more (Table 2). The fact that these regions were also rich in Arginine and Lysine, a common characteristic of nuclear localization signals, contributes as further evidence of proper NLS deletion.

The inability to determine a shift from our positive control, AnkA on the EMSA indicates that additional troubleshooting is required before analyzing data. Since the control reactions provided in the kit revealed clear bands, and our purified samples were blurs, we came to the conclusion that the DNA fragmentation was the problem. DNA fragmentation does not guarantee equal sized fragments, and carries the risk of fragmenting within a possible binding

motif. Identifying the binding motif of each effector protein through ChIP sequencing can aid in future EMSA attempts. By using the binding motif sequence to make protein specific probes, protein-DNA interactions could be more easily detected since these probes would be equal in size. Results should show clearly defined bands, allowing easy identification of shifts. Although the DNA isolated from ChIP was too low to send for deep sequencing, the detection of small amounts of DNA from four effectors provides some evidence of chromatin binding. The lack of DNA from the vector, used as our control, additionally supports this information. The small amounts of DNA collected from this ChIP will be combined with that obtained from future or larger scale ChIPs in order to obtain enough DNA for sequencing.

CHAPTER V

CONCLUSIONS

By confirming the association of *Coxiella burnetii*'s nuclear localized effector proteins to host chromatin, we have provided supporting evidence to our hypothesis: *Coxiella* effector proteins bind host DNA in order to alter immune response during infection. Previous studies on other intracellular pathogens such as *Chlamydia* and *Shigella* have found nuclear effector proteins to bind host cell DNA, altering immune transcriptional response. Although we know that *Coxiella* also alters host immune response, we cannot infer from this evidence that the association of these proteins with the chromatin is the sole cause.

By additionally confirming nuclear localization signals in two of these effector proteins, we can infer the presence of NLSs in the remainder three effectors. Nuclear localization signals indicate that the effectors have specific targets within the nucleus, allowing our hypothesis to remain valid.

With this evidence, future studies can aim at identifying the specific transcriptional modifications caused by these effectors. Comparing a wild type infection with a ΔNLS construct can give insight on the roles of each effector within the nucleus. By identifying survival mechanisms, scientist can better work at finding a better way to target and prevent *Coxiella* infections.

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