REGULATION OF ADV-1 PROTEIN RHYTHMS BY THE TRANSCRIPTION FACTOR CSP-1 IN NEUROSPORA CRASSA.

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Regulation of ADV-1 Protein Rhythms by the Transcription Factor CSP-1 in *Neurospora crassa*.

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The circadian clock regulates the rhythmic expression of roughly half of the eukaryotic genome at the level of mRNA abundance. However, little is known about how rhythmic phase is regulated (Delaunay & Laudet, 2002). Chromatin-immunoprecipitation (ChIP)-seq revealed a network of transcription factors (TFs) downstream of the core oscillator component, the White Collar Complex (WCC) (Smith, et al., 2010). We hypothesize this TF network is important in phase regulation of downstream clock-controlled genes (ccgs). To test this hypothesis, we investigated the role of a smaller TF network upstream of ADV-1, a direct TF target of the WCC, and assayed the effect of single TF deletions on ADV-1 rhythms. We found deletion of CSP-1, a TF within this network, resulted in an ~3 hr phase delay of ADV-1 protein rhythms, without affecting FRQ protein rhythmic accumulation. ChIP-seq also revealed two CSP-1 consensus binding motifs near the start of *adv-1* transcription. If CSP-1 regulation of ADV-1 is through the aforementioned TF network, there will be little to no change from wildtype rhythms when the CSP-1 binding sites are deleted. However, if CSP-1 directly regulates ADV-1, then deletion of the binding sites will produce a phase delay similar to the single deletion of CSP-1. The binding site furthest upstream from the *adv-1* start codon has been deleted and shown to not

be solely responsible for ADV-1 phase regulation. However, the effect of the CSP-1 binding site nearest the *adv-1* start remains to be deleted, and thus, the full nature of CSP-1 regulation of ADV-1 phase remains unknown.

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NOMENCLATURE

BS1	First CSP-1 Binding Site on *adv-1* promoter
BS2	Second CSP-1 Binding Site on *adv-1* promoter
ChIP	Chromatin Immunoprecipitation
DD	Constant dark
FRH	FRQ-interacting RNA Helicase
FRQ	Frequency (negative element of the core oscillator in *Neurospora crassa*)
KO	Knock-Out
LL	Constant light
LUC	Luciferase
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
TF	Transcription Factor
TTFL	Transcription-Translation Feedback Loop
WCC	White Collar Complex (positive element of the core oscillator)
WT	Wildtype

CHAPTER I

INTRODUCTION

Background

Almost all eukaryotic organisms have rhythmic fluctuations in gene expression and activity in anticipation to changes in their environment (Baker, Loros, & Dunlap, 2012). The circadian clock is a conserved timing mechanism for organisms to anticipate 24 hr environmental cycles, such as the day/night cycle and the resulting changes in temperature (Pittendrigh, 1954). In humans, the clock controls many aspects of our physiology, including the timing of our sleep/wake cycle. Many human diseases have been linked to desynchronization between an individual's environment and their clock (Maury, Ramsey, & Bass, 2010). For example, shift-workers are active at the wrong time of the day and are out of phase with their internal clock. This leads to an increased risk for type-2 diabetes, heart disease, and metabolic syndromes (Turek, et al., 2005). Potential treatments for shift-workers and other such individuals require knowledge on how the phase of clock output is regulated.

The circadian clock mechanism is well conserved (Panda, Hogenesch, & Kay, 2002); therefore, we can use the simple model organism *Neurospora crassa* to understand how circadian phase is regulated. *N. crassa* is a bread mold that has played a fundamental role in the study of eukaryotic genetics due to its quick replication cycle and ease of genomic editing (Aramayo & Selker, 2013). *N. crassa* growth is characterized by the formation of interconnected hyphae called mycelia, which is similar in appearance to nervous tissue, giving the genus its name. *N. crassa* has two reproduction cycles: sexual and asexual. In the asexual cycle, *N. crassa* form aerial hyphae that then make spores called macroconidia. In nature, macroconidia spread to

new areas and start new colonies (Davis, 2000). Macroconidia are often multinucleated and the nuclei may have different genomes, however *N. crassa* also produces uninucleated microconidia (Horowitz & Macleod, 1960). Historically, *N. crassa* is used in circadian clock research because of its overt rhythms in conidiation (Sargent, Briggs, & Woodward, 1966).

The circadian clock is an endogenous oscillator that operates as a negative transcription-translation feedback loop (TTFL) (Dunlap & Loros, 2004). The oscillator has many rhythmic outputs, including gene expression and metabolism (Hurley, et al., 2014). Furthermore, environmental cues, such as changes in light and temperature, act as inputs to the clock and can entrain it to the local time (Pittendrigh, 1954). The oscillator consists of two main elements: a positive element and a negative element. In *N. crassa,* the TTFL is composed of the positive element White Collar Complex (WCC) and the negative element FREQUENCY (FRQ). In the presence of light, the blue-light photoreceptor WHITE COLLAR-1 (WC-1) will dimerize with WHITE COLLAR-2 (WC-2) to form the WCC. In the morning, the WCC binds to the *frq* promoter and activates *frq* transcription (Froehlich, 2002). As FRQ protein accumulates during the day, it homodimerizes and forms a complex with FRQ-interacting RNA helicase (FRH), and inhibits the activity of the WCC (Cheng et al., 2005). Throughout the circadian cycle, FRQ is progressively phosphorylated and degraded, allowing the WCC to activate transcription again the following morning (Liu & He, 2005). The output rhythms of the clock can be characterized according to their period and phase. The period is the length of the cycle, and phase is the time between some point in the cycle (such as an activation peak or trough) and a reference point in the cycle (such as daybreak or nightfall). Mutations in the genome of an organism may result is changes in period, phase, or both.

Preliminary data

Twenty-four transcription factors (TFs) were revealed to be direct targets of the WCC using ChIP-seq (Smith, et al., 2010). These TFs form a complicated TF network, which we hypothesize functions to regulate circadian rhythms and phase (Figure 1). In the absence of one of the direct TF targets of WCC, ADV-1, developmental rhythms are abolished. These data suggested that ADV-1 is important for clock output (Dekhang, et al., 2017). In this study, ADV-1 is used as a proxy to understand how the network regulates phase. In preliminary experiments,

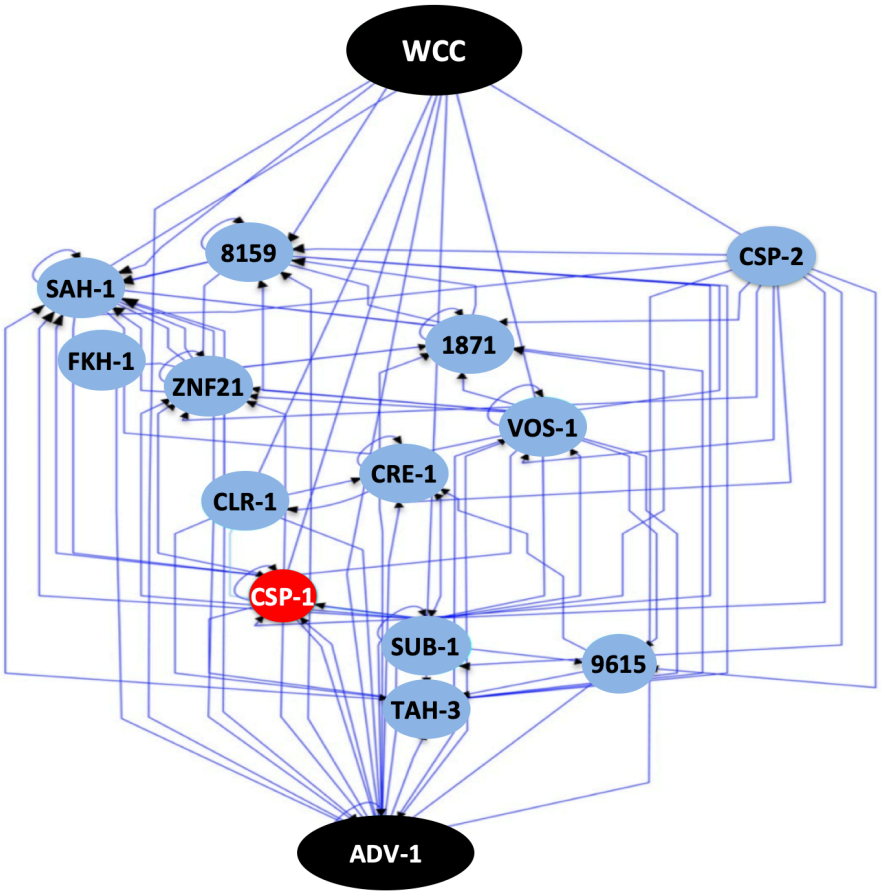

Figure 1. Proposed TF network that lies downstream of the FRQ/WCC clock and controls ADV-1 rhythmicity and phase. The circles represent individual TFs, and the lines represent binding of promoters of one TF by another as determined by ChIP-seq, and/or changes in expression of a TF when another TF is deleted as determined by RNA-seq in TF deletion strains.

we tested the effect of single TF knockouts (KOs) on ADV-1 protein rhythmicity using a luciferase reporter (ADV-1::LUC). We found that the KO of the clock-regulated TF CSP-1 delayed the phase of ADV-1 by ~3 hr, without affecting the period of ADV-1 rhythms. Furthermore, Δ*csp-1* did not alter the expression or rhythmicity of a FRQ-luciferase reporter (FRQ::LUC) (Figure 2). From this data, we hypothesized that CSP-1 affects ADV-1 rhythms either directly or through the TF network. If CSP-1 regulates ADV-1 directly, then deletion of the CSP-1 binding sites on the *adv-1* promoter will result in a phase delay of ADV-1::LUC similar to the delay observed in Δ*csp-1* cells. However, if CSP-1 regulation occurs through the TF network, then deleting the CSP-1 binding sites on the *adv-1* promoter will produce no noticeable change in ADV-1::LUC phase.

ChIP-seq analysis of CSP-1 binding revealed two regions with considerable CSP-1 binding peaks on the *adv-1* promoter (Figure 3). One of these potential CSP-1 binding sites (BS1) had previously been deleted from the *adv-1* promoter, and deletion of this site did not alter ADV-1::LUC protein rhythms (Figure 4), suggesting that CSP-1 affects ADV-1 indirectly through the network. However, lack of CSP-1 binding has yet to be confirmed by qPCR in this binding site deletion, and the effect of the second CSP-1 binding site (BS2) on ADV-1 rhythms needs to be evaluated. Understanding how CSP-1 regulates ADV-1 will help characterize the WCC-associated TF network, including the role of the network on global phase regulation.

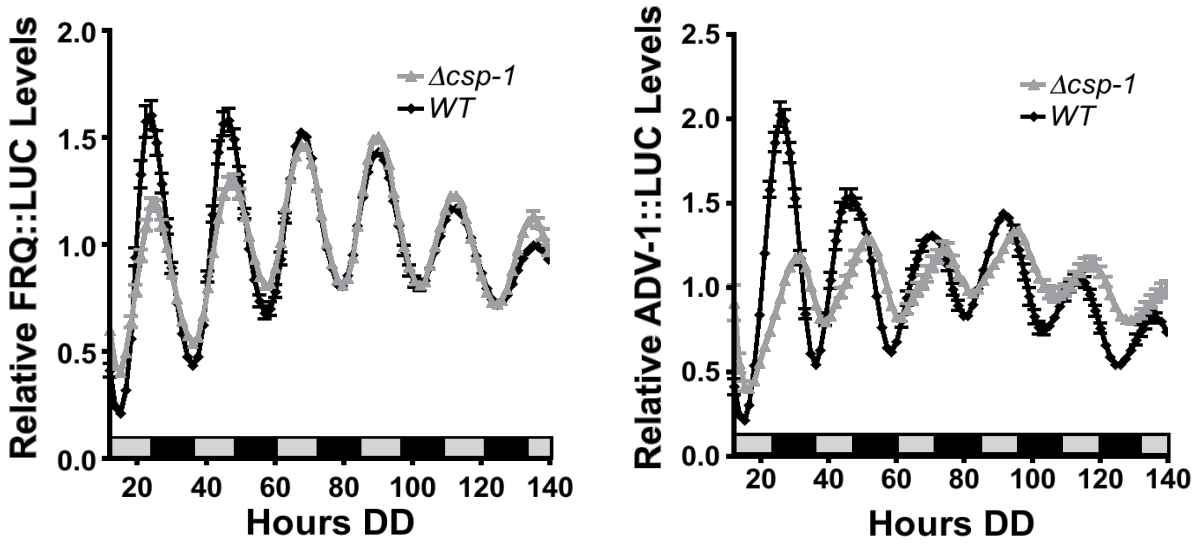

Figure 2. CSP-1 is required for proper phase regulation of ADV-1 rhythms. Plots of relative luciferase activity in FRQ::LUC (left) and ADV-1::LUC (right) reporters from cultures grown in constant dark (DD) and sampled every 90 min over 6 days. Deletion of CSP-1 (Δ*csp-1*) delayed the phase of ADV-1 by ~3 hr (left, grey), without affecting the period of ADV-1 rhythms (left, black). Furthermore, *Δcsp-1* did not alter the expression or rhythmicity of a FRQ-luciferase reporter (right, black). Gray and black bars represent subjective day and night. Error bars represent mean ± SEM. N≥24.

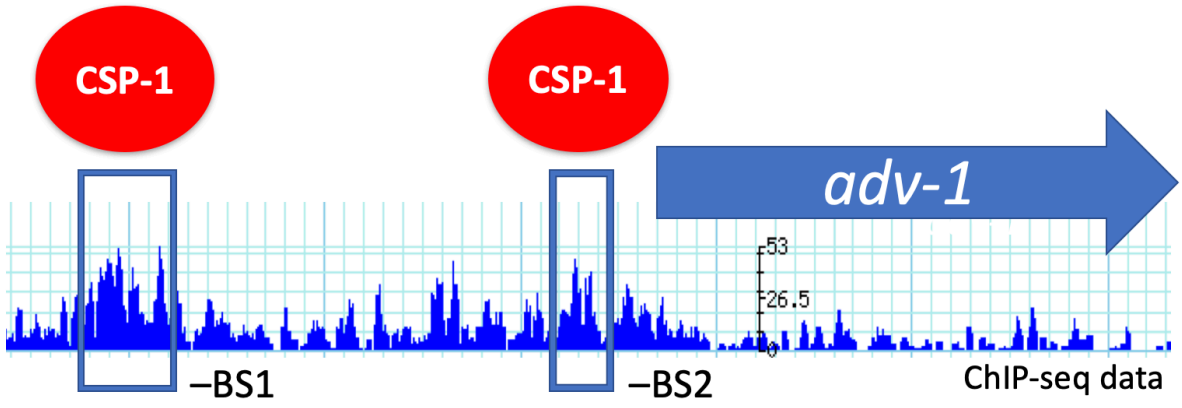

Figure 3. Areas of significant CSP-1 binding on the *adv-1* promoter from ChIP-Seq data. The boxed site closest to the *adv-1* transcription start site is CSP-1 binding site 2 (BS2); the boxed site further upstream from the *adv-1* transcription start site is CSP-1 binding site 1 (BS1). Sites are notated in order of deletion (discussed below), not strength or relation to the adv-1 promoter.

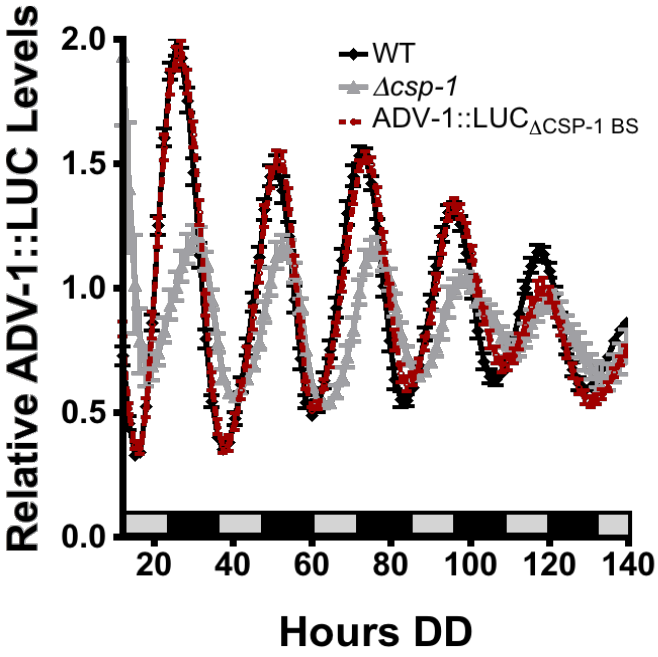

Figure 4. Deletion of CSP-1 BS1 region on the *adv*-1 promoter does not alter ADV-1 rhythmicity. Luciferase assay comparing CSP-1 knockout (dark grey), ADV-1::LUC with deleted BS1 (red), and wild type (black) grown in DD and sampled every 90 min over 6 days. Error bars represent mean ± SEM. N≥24.

CHAPTER II

METHODS

Strain upkeep and storage

Luciferase reporter strains were previously generated in the lab in a OR74A background (FGSC# 2489, Kansas State University, Manhattan, Kansas) and stored in desiccated silica gel stocks (Perkins, 1977). Stored strains were grown from silica stocks on minimal medium (1x Vogels, 2% glucose, and 1.5% agar) in constant light (LL) at 30 ºC for 5-7 days before storage at -20 ºC.

Crossing

N. crassa crosses were produced by co-inoculating two strains of different mating types on opposite sides of a petri plate containing basic synthetic crossing medium (Westergaard & Mitchell, 1942). After several weeks of growth at 25 ºC in LL, ascospores were collected individually on medium containing 1x Vogels, 2% glucose, and 1.5% agar. Spores were activated by heatshock at 65 ºC for 50 minutes.

PCR to generate BS2 deletion construct

The BS2 deletion construct was produced by assembly PCR to amplify fragments that contained homology to the promoter of ADV-1. The primers used to generate these fragments excluded 132 bp region containing the BS2 and a SexAI restriction site. PhusionTaq (Phusion® High-Fidelity DNA Polymerase) was used to generate individual and full fragments using the following PCR protocol: 98 °C for 2:00 min, (98 ºC for 15 s, 60 ºC for 45 s, 72 ºC for 2:30 min) x30, final elongation at 72 ºC for 10 min. PCR products were visualized on a 1% DNA agarose gel stained with ethidium bromide using a 1kb DNA ladder (ThermoFisher Scientific®) for size

reference.

Gel extraction and purification

DNA extractions were preformed using Genrta® Puregene® procedures for tissue (Quiagen®, Valencia, California), and PCR product purifications was carried out using QIAquick® Gel Extraction Kit protocol (Quiagen®, Valencia, California).

Ligation and cloning

The full BS2 construct was ligated into a pCR®-Blunt (Zero Blunt® PCR Cloning Kit) vector and transformed into competent DH5α *E. coli* cells via heatshock. Transformants were initially screened by kanamycin resistance and blue-white screening. Plasmids were extracted from kanamycin resistant white colonies by alkaline lysis (Plant Biotech Resource and Outreach Center, Michigan State University, East Lansing, Michigan) and screened by restriction digestion with BamHI-HF and PstI-HF (New England BioLabs®, Catalog numbers: R3136 and R0146).

Transformation procedures

N. crassa transformation was done as previously described by Navarro-Sampedro, Olmedo, & Corrochano (2019). Briefly, conidia were suspended and washed in ice-cold 1M Sorbitol, mixed with 1-2 µg of desired DNA construct, then electroporated using Bio-Rad Gene Pulser® Apparatus with the following parameters: 7.5 kV/cm (1.5 kV in 2 mm cuvette); resistance: 600 ohms; capacitance: 125 µF. Cells were recovered in 1xVogels solution and plated on 1xFGS media (2% L-sorbose, 0.05% fructose, 0.05% glucose) supplemented with 1xVogel's salts. Transformants were allowed to grow for 3-4 days. Transformants were screened by PCR and restriction digestion with SexAI (New England BioLabs®, Catalog number: R0605).

Western Blot

Protein was extracted from samples as previously described by Garceau, Liu, Loros, &

Dunlap (1997), and then separated by SDS-PAGE using an 8% gel and transferred to a nitrocellulose membrane. After blocking, the blot was probed with mouse anti-V5 and goat anti-mouse-HRP antibodies and detected with SuperSignal® (Thermo Scientific®, SuperSignal™ West Femto). The blot was exposed to film for 0.5-1 min. After exposure, the membrane was stained with amido black to visualize total protein.

ChIP-qPCR

Chromatin immunoprecipitation was performed as previously described in Dekhang, et al. (2017). Quantitative PCR (qPCR) was done using two sets of primers, each flanking one of the CSP-1 binding sites on the promoter of *adv-1*, iTaq Universal Green SYBR Mastermix (Bio-Rad®), and an Applied BioSystems QuantStudio6Flex qPCR machine. The PCR protocol was 95 ºC for 25 s; 95 ºC for 2 s, 60 ºC for 25 s, repeated 35 times; followed by a melting curve procedure from 65 ºC to 95 ºC with a 0.05 ºC change per second. Data was analyzed using the fold enrichment method (Haring, et al., 2007) comparing IP signal to mock-IP signal (background).

Luciferase assay

Strains were inoculated from a conidial suspension in water of $1x10^5$ cells/mL. 5 µl of this suspension were used to inoculate each well in 96 well Optiplates containing luciferase media (1x Vogel's salts, 0.05mg/mL biotin, 0.03% glucose, 0.05% arginine, 100mM quinic acid, 1.8% agar, and 10mM luciferase — added after autoclaving). Inoculated plates were incubated in LL at 30 ºC for 24 hrs, and then loaded into a Perkin-Elmer EnVision, located in a Percival set to 25 ºC DD. Luciferase activity in each well was measured every 1.5 hrs over the course of 99 iterations (~7 days).

CHAPTER III

RESULTS

Overview of project

The main goal of this project is to generate four *N. crassa* strains with ADV-1 fused to luciferase and a V5 epitope tag on CSP-1 (ADV-1::LUC, CSP-1::V5), with varying CSP-1 consensus binding sites deleted on the *adv-1* promoter. The V5 epitope on CSP-1 is required to confirm the absence of CSP-1 binding in the binding site deletion construct by ChIP-qPCR, as there is no commercially available antibody for CSP-1. The luciferase fusion (LUC) is used as a reporter to assay ADV-1 protein rhythms. One strain will be a control strain with both CSP-1 binding sites intact, and the remaining three strains will have either one or both of the CSP-1 binding sites on the promoter of *adv-1* deleted (Table 1). Once these strains are generated, the effect of each CSP-1 binding site on ADV-1 protein rhythms will be tested by luciferase assay.

Table 1. Summary of strains

Strain	**ADV-1 genotype**	**Description**
WTp	ADV-1::LUC	No CSP-1 binding sites deleted; used as control.
ΔBS1	ADV-$1_{\Delta BS1}$::LUC	BS1 deleted.
ΔBS2	ADV-$1_{\Delta BS2}$::LUC	BS2 deleted.
ΔBS1,2	ADV-$1_{\Delta BS1\Delta BS2}$::LUC	Both BS1 and BS2 deleted.

Generation of WTp and ΔBS1

The CSP-1 binding site deletion furthest upstream of the *adv-1* start site (BS1) was previously generated in a strain containing ADV-1::LUC, but it did not contain the CSP-1::V5 tag needed to confirm lack of CSP-1 binding by ChIP-qPCR. To generate the BS1 deletion in a

CSP-1::V5, ADV-1::LUC background (*ΔBS1*), the strain containing BS1 deletion and luciferase reporter was crossed to a strain of the opposite mating type containing CSP-1::V5. Progeny from this cross were screened by luciferase activity in duplicates to avoid false positives. Independent confirmation of the BS1 deletion was not necessary as it is linked to the ADV-1::LUC locus. CSP-1::V5 expression was verified by western blot (Figure 5). A control strain with a wildtype *adv-1* promoter was generated by a cross between parent strains containing ADV-1::LUC and CSP-1::V5, respectively, and screened in a similar fashion (*WTp*). Strains from this cross positive for luciferase activity and V5 expression were stored for future use (T1 and T4, Figure 5).

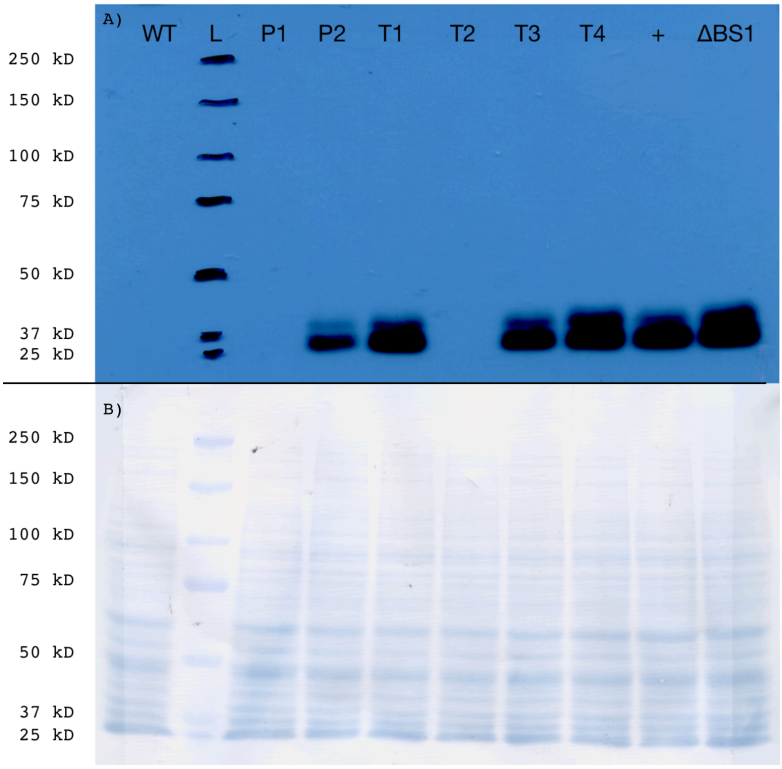

Figure 5. Western blot of CSP-1::V5 expression. A) P1 and P2 are control strains. P1 contains ADV-1::LUC and no CSP-1::V5 (negative control); P2 expresses CSP-1::V5 (positive control). T1-4 denote cross progeny to generate WTp. The plus sign (+) denote an unrelated strain known to express CSP-1::V5. ΔBS1 generated in the previous cross was included in this blot to confirm equivalent CSP-1::V5 expression to WTp (Table 1). T1 and T4 were stored as WTp. B) Amido stain blot to confirm protein loading is equivalent in each lane.

CSP-1 no longer binds to BS1 region of adv-1 promoter in ΔBS1

To confirm that CSP-1 no longer binds to the BS1 region on the *adv*-1 promoter in the ΔBS1 shown, we used primers to amplify ~200 bp of each binding site region and performed ChIP-qPCR. WTp strain had enrichment of the BS1 signal compared to the mock IP. However, the ΔBS1 strain did not, confirming the absence of CSP-1 binding to the BS1 region. Additionally, both strains had similar signal (p=0.2) within the BS2 region, confirming the continued binding of CSP-1 at the undeleted BS2 region (Figure 6).

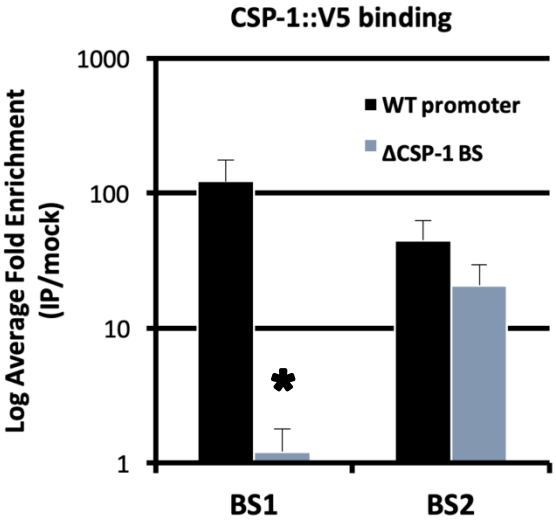

Figure 6. ChIP-qPCR data of deletion of BS1 led to loss of CSP-1 binding. CSP-1 binding was observed in WT (black) but not in ΔBS1 (light blue). Deletion of BS1 did not affect CSP-1 binding to BS2 (p=0.2). Asterisk indicates $p<0.05$, student's two tailed t-test.

Generation of ΔBS2 and ΔBS1,2

To produce a construct to delete BS2, two amplicons were generated via PCR which had partial homology to each other, and excluded 132 bp region containing BS2 (Figure 7). These two DNA fragments, fragment 1 (1448 bp) and 2 (1409 bp), were purified by PCR purification and fused together by assembly PCR using the furthest outside primers (Figure 7). The region on

the promoter deleted contains a SexAI site, which was used to screen *N. crassa* transformants.

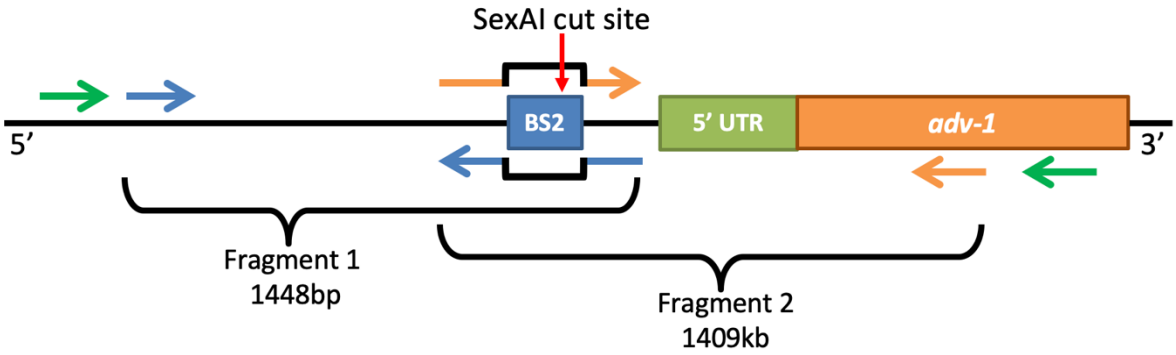

Figure 7. Diagram of *adv-1* promoter and primer locations. The second CSP-1 binding site on the *adv-1* promoter (132 bp) is excluded by the construct design. The primer pairs shown in blue amplified the region upstream of BS2, and the orange pair amplified the region downstream of BS2. This excludes the BS2 region and a SexAI restriction site. Primer pair in green amplifies the full region and is used to screen for *N. crassa* transformants that targeted to the endogenous locus. DNA isolated from transformants that did not integrate the construct at the correct site will generate two bands (2642 bp & 3742 bp) upon digestion with SexAI. Successful transformants will display an undigested band of 6252 bp. Diagram not to scale.

Ligation and transformation into E. coli (DH5a) cells

After generation of the full construct by PCR, the fragment was ligated into a commercially available vector, pCR®-Blunt, which contains a gene conferring kanamycin resistance and allows for blue-white screening through the LacZ gene. The LacZ gene encodes a β-galactosidase which, in the presence of X-gal (5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside), results in blue colonies. However, the proper insertion of our construct will disrupt the LacZ gene and successful transformants can be distinguished by white-colored colonies. To confirm the sequence of the cloned construct, plasmids were extracted from each transformant and digested with BamHI-HF and PstI-HF. The expected sizes after digestion were 3.5 kb, 1.4 kb, 1.0 kb, and 0.5 kb (Figure 8). Several positive bacterial strains were isolated and allowed to grow before being mixed with glycerol for longterm storage at -80 ºC. For *N. crassa*

transformation, the stored plasmid was digested with BamHI and XhoI to excise the liner DNA construct, which was isolated via gel purification.

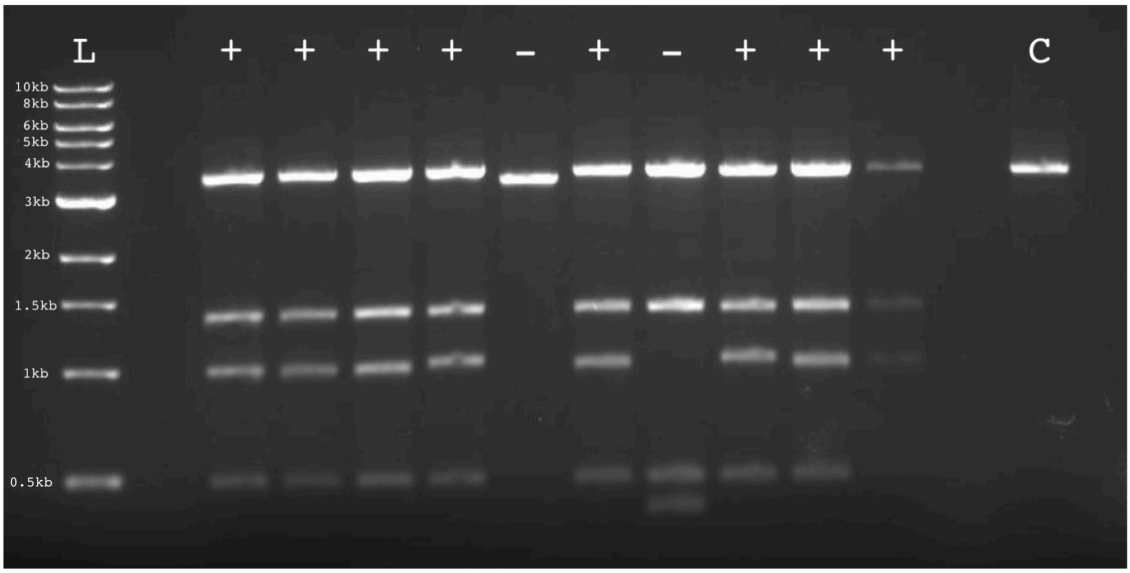

Figure 8. Composite gel photograph showing the separated fragments of the digested pCR®-Blunt plasmid from several colonies with BamHI-HF and PstI-HF. L denotes ladder. C denotes a control plasmid of pCR®-Blunt with no insert. A plus sign (+) denotes a successful ligation of the construct into pCR®-Blunt. A minus sign (-) denotes bacterial transformants that did not have proper insertion of the ΔBS2 construct.

Transformation of WTp and ΔBS1

The two strains previously described (WTp and ΔBS1) were transformed with the ΔBS2 DNA construct by electroporation. Screening revealed one transformant that lacked the SexAI restriction site found in the WT promoter (Figure 9). It is highly likely this is due to successful transformation of the BS2 construct into the *adv-1* promoter. However, this strain is a heterokaryon, and a homokaryon is needed before any further experiments can be done. To purify for the transformed genome, a microfiltration, as described in Ebbole and Sachs (1990), will be performed to isolate for the uninucleate microconidia.

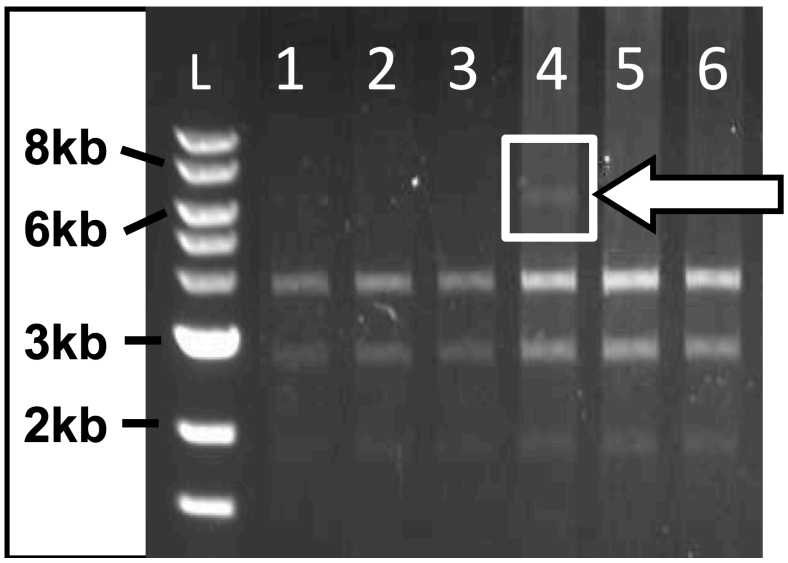

Figure 9. Gel photograph of ΔBS1,2 transformants. ΔBS1,2 heterokaryon identified by undigested band of the expected size (white arrow). Lanes 1-6 show SexAI digested genomic DNA of heterokaryon transformants 1-6. Only transformant 4 had a band of expected size (boxed with arrow, 6.384 kb). L denotes DNA ladder.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

Previous research revealed a TF network downstream of the WCC that was hypothesized to regulate phase of rhythmic mRNAs in *N. crassa*. Further research demonstrated that the deletion of one of these TFs, CSP-1, resulted in an ~3 hr phase delay of ADV-1 protein rhythms. Thus, CSP-1 is known to be necessary for the proper regulation of ADV-1 protein rhythms. Further still, ChIP-seq revealed two sites of considerable CSP-1 binding in the ADV-1 promoter. This offers two possible models of CSP-1 regulation of ADV-1: that CSP-1 regulation is primarily direct by use of the CSP-1 binding sites in the ADV-1 promoter; or that CSP-1 regulation of ADV-1 is primarily through the TF network.

This study attempted to elucidate the role of CSP-1 in ADV-1 phase regulation by deleting these binding sites and observing if any changes occurred in ADV-1 phase. An ADV-1 phase delay similar to Δ*csp-1* observed after deletion of one or both CSP-1 binding sites would give strong evidence of direct CSP-1 regulation. However, failure to alter ADV-1 protein rhythms would provide evidence that CSP-1 regulation is indirect and likely through the previously mentioned TF network.

Unfortunately, difficulties in spore germination during crossing and problems obtaining a reliable PCR primer pair needed to screen potential transformants caused delays. While at the present time of writing these issues have been resolved, they prevented the acquisition of data needed to fully test the hypothesis originally laid out. Thus, the effect of BS2 on ADV-1 phase has not been evaluated by luciferase assay. Further, confirmation that integration of the BS2 deletion construct results in loss of CSP-1 binding at BS2 has not been evaluated by ChIP-qPCR.

However, a heterokaryon containing nuclei that successfully integrated the BS2 deletion construct into a ΔBS1 genome has been generated. Steps have been taken to develop a homokaryon with this genome by isolating the microconidia from the heterokaryon strain. After isolation, this strain *(ΔBS1,2)* will be subjected to luciferase assays to determine if the deletion of both CSP-1 BSs on the ADV-1 genome have altered ADV-1 phase. ChIP-qPCR will also be performed to confirm the loss of CSP-1 binding at both sites. Transformation of WTp with the BS2 deletion construct is also currently being performed to generate ΔBS2. This strain will be subjected to the same procedures described above.

In summary, we are only able at present to discuss the role of the first CSP-1 binding site on ADV-1 phase. The BS1 region alone is not necessary for proper regulation of ADV-1 phase. This could be due to compensation through the TF network or binding of CSP-1 to BS2 in the ΔBS1 strain. The data from this project will allow us to determine which of the two hypothesized models of CSP-1 regulation of ADV-1 protein rhythms is more accurate and, once obtained, these data will be integrated into a computational model, not discussed in this thesis, to predict the effect of the TF network on phase. As such, this work will be important to our longterm goal of characterizing the mechanism of global phase regulation in *N. crassa*.

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