THE CONTROL OF GENE EXPRESSION BY HIGH LIGHT SRESS IN CYANOBACTERIA THROUGH THE APPARENT TWO-COMPONENT NblS-RpaB SIGNAL TRANSDUCTION PAIR

by

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DEDICATION

Guimel Molina Kappell, my loving wife.

In the memory of David Anthony Kappell, my father.

The Kappell and Molina families.

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ABSTRACT

THE CONTROL OF GENE EXPRESSION BY HIGH LIGHT SRESS IN CYANOBACTERIA THROUGH THE APPARENT TWO-COMPONENT NblS-RpaB SIGNAL TRANSDUCTION PAIR

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In nature photosynthetic organisms including cyanobacteria are dependent upon their ability to acclimate to changes in environmental conditions including light and nutrient levels. The NblS sensor kinase of the cyanobacterium *Synechococcus elongatus* PCC 7942 regulates gene expression in response to a number of stress conditions including high-intensity light and nutrient limitation. NblS is known as DspA or Hik33 in the cyanobacterium *Synechocystis* PCC 6803. We determined that the high light-inducible *hliA* gene from S. *elongatus* is under negative control through NblS. We have identified the High Light Regulatory 1 (HLR1) sequence (two direct repeats of (G/T)TTACA(T/A)(T/A) separated by two nucleotides) upstream of a number of genes in *S. elongatus* and *Synechocystis* known to be regulated through NblS and DspA, including the high light-inducible *hli* genes, and found the HLR1 sequence is conserved upstream of *hli* genes in many other cyanobacteria, the *Cyanophora* cyanelle, and cyanophage. We have identified the response regulator RpaB as the factor that binds the HLR1 sequence upstream of high light-

regulated genes in *S. elongatus* and *Synechocystis* specifically the *hliB* and *hliC* genes from *Synechocystis* and *hliA* and *psbAI* genes from *S. elongatus*. In response to nutrient limiting conditions, the *S. elongatus nblA* gene is known to be regulated through NblS. We have found this control to be negative, with NblS repressing *nblA* expression under nutrient replete conditions, a repression which is relieved during nutrient limitation. We have shown that RpaB binds an HLR1 sequence found overlapping the promoters predicted from the two main transcriptional start sites of *nblA*, as well as binding the HLR1 site overlapping the predicted promoter of *hliA,* consistent with RpaB also acting as a negative regulator of these genes, as NblS is. A separate response regulator, NblR, is known to positively regulate *nblA.* We have found that the NblR response regulator is under redox control and binds upstream of *nblA* at a site containing two indirect repeats of (T/C)CT(C/G)AGAAAGG separated by six nucleotides (termed the reduced NblR binding (RNB1) element). Since *nblA* appears to be under positive control through the response regulator NblR binding to the upstream region, but under negative control through NblS, and apparently RpaB, and *hliA* is regulated by NblS and RpaB, but not NblR, NblS and NblR are not likely to form a direct cognate response regulatory pair. We hypothesize that NblS and RpaB do form a cognate two-component regulatory pair. In this work we present a model for gene control by NblS-RpaB and discuss the possible overlapping regulation of *nblA* by RpaB and NblR.

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CHAPTER 1

INTRODUCTION

1.1 Cyanobacteria and Environmental Stress

Cyanobacteria carry out oxygenic photosynthesis similar to that of eukaryotic algae and plants, and are considered to be the progenitors of plastids in these organisms. The photosynthetic activity of cyanobacteria is strongly controlled by environmental parameters such as light quality, light intensity, temperature, water availability, and nutrient status by influencing the capacity of the photosynthetic apparatus and the levels of pigments and the proteins associated with the complexes (Demmig-Adams and Adams 1992; Grossman *et al.* 1995; van Waasbergen *et al.* 2002). Disruption of homeostasis by changes in the environmental parameters causes aberrations in photosynthetic activity or stress which can cause damage to photosynthetic cells. To alleviate this stress, the cyanobacterial response involves a general stress response and various specific stress responses, which are often overlapping. The cyanobacterial general stress response involves the increase in catabolism, decrease in anabolism, protection of cellular components, degradation of phycobilisomes, changes in photosynthetic apparatus, and, ultimately, the degradation of the thylakoid membranes. (Reviewed in Grossman *et al.* 2001 and Schwarz and Forchhammer 2005). The specific response is dependent on the specific condition that is altered or nutrient that is limiting. The work in this dissertation focused on responses to high light intensity and general nutrient deprivation.

1.1.1 High light stress and hli *regulation*

Cyanobacteria and all photosynthetic organisms exist because of their ability to balance their absorbance and utilization of available light. Absorption of excess light beyond that which the photosynthetic apparatus can utilize causes overexcitation of the photosystems,

excessive reduction of electron carriers, accumulation of excited pigment molecules, and formation of damaging oxygen radicals that leads to photoinhibition and general cell oxidative damage (Aro *et al.* 1993; Nishiyama *et al.* 2004; Noguchi 2002). Therefore, photosynthetic organisms have evolved mechanisms to avoid, dissipate, or repair damage caused by excess light energy (Chow 1994; Demmig-Adams 1990; Demmig-Adams and Adams 1992; Horton *et al.* 1996; Niyogi 1999). The immediate response to excess light energy by the photosynthetic apparatus is to redistribute the energy utilization between photosystems and quench the excess energy within the antenna complexes (Bjorkman and Demmig-Adams 1994; Chow 1994; Demmig-Adams and Adams 1992; Grossman *et al.* 1995; Niyogi 1999; van Waasbergen *et al.* 2002). Long-term acclimation involves alterations in the composition of the photosynthetic apparatus or changes in processes that modulate the assembly or disassembly of specific protein complexes that function in photosynthesis (Bjorkman and Demmig-Adams 1994; Chow 1994; Demmig-Adams and Adams 1992; Grossman *et al.* 1995; Niyogi 1999; van Waasbergen *et al.* 2002). The physiological and structural changes of the photosynthetic apparatus reflect altered patterns of gene expression that may be triggered by the activity of specific photoreceptors that can detect light quality, the activity of redox-sensitive regulators that can detect changes in light intensity, or a mechanism that perceives the change in the redox state of a specific photosynthetic electron carrier (Anderson 1986; Argüello-Astorga and Herrera-Estrella 1998; Bauer *et al.* 1999; Chen *et al.* 2004; Danon and Mayfield 1994; Escoubas *et al.* 1995; Fankhauser and Chory 1997; Golden 1995; Kehoe and Grossman 1996; Li and Sherman 2000; Maxwell *et al.* 1995; Mullineaux 2001; Pfannschmidt 2003; Quail 2002; Thompson and White 1991).

One acclimation response that occurs when cyanobacteria are exposed to high-intensity light is the production of small chlorophyll a/b binding-like proteins also known as high light-inducible polypeptides (or HLIPs, encoded by *hli* genes). The first *hli* gene identified was *hliA* from *Synechococcus elongatus* PCC 7942 (hereafter, *S. elongatus*) (Dolganov *et al.* 1995).

Since then multiple *hli* genes have been identified in all sequenced cyanobacteria genomes, as well as in related genomes, those of cyanophages, red algae, and the *Cyanophora* cyanelle (Bhaya *et al.* 2002). The number of *hli* genes present vary among cyanobacteria with the highest number found in the genomes of marine cyanobacteria adapted to high light (Bhaya *et al.* 2002). The function of the HLIPs appears to be to stabilize PSI (Wang *et al.* 2008) and PSII (Promnares *et al.* 2006) and work is continuing to determine if their function is solely structural. The *hli* genes are found to be expressed in response to excess excitation energy stress such as chilling, nitrogen or sulfur deprivation, high light, the presence of the photosynthetic inhibitors, low intensity blue or UV-A light, salt stress, hydrogen peroxide, and hyperosmotic stress (Allakhverdiev *et al.* 2002; He *et al.* 2001; Hihara *et al.* 2001; Hihara *et al.* 2003; Hsiao *et al.* 2004; Kanesaki *et al.* 2002; Kanesaki *et al.* 2007; Mikami *et al.* 2002a; Salem and van Waasbergen 2004a; Salem and van Waasbergen 2004b; Suzuki *et al.* 2001; Tu *et al.* 2004). A number of these stresses have been shown to strongly limit photosynthetic carbon metabolism (Long *et al.* 1994). Left unchecked, this would cause decrease utilization of the reduced form of $NADP^+$, NADPH, in anabolism, depleting the pool of the terminal electron acceptor in photosynthetic electron transport, $NADP^+$ (Salem and van Waasbergen 2004b). This would then lead to overexcitation of the photosynthetic apparatus similar to that seen in high light (Aro *et al.* 1993; Nishiyama *et al.* 2004; Noguchi 2002).

1.1.2 Nutrient deprivation and nblA *regulation*

In nature photosynthetic organisms are also dependent upon their ability to acclimate to changes in nutrient conditions. Cyanobacteria have sets of specific and general responses allowing them to endure limiting concentrations of various specific nutrients. The specific response, depending on the limiting nutrient, includes the induction of efficient transport systems for acquisition of that limiting nutrient from various sources. Cyanobacteria also have more generalized responses to nutrient deprivation (*i.e.* not specific to any particular nutrient). One of these, for example, is that which *S. elongatus* under goes during starvation for nitrogen,

sulfur, or phosphorous: the nutrient deprivation causes, to varying extents, a decrease in anabolic metabolism and chlorosis or "bleaching" caused by degradation of the light-harvesting phycobilisome (Baier *et al.* 2004; Richaud *et al.* 2001; Sauer *et al.* 1999). Phycobilisome degradation provides some of the limiting nutrient and reduces the size of the light harvesting apparatus under periods of starvation when there is a decreased recycling of $NADP^+$ (the terminal electron acceptor in photosynthesis) due to the decreased utilization of NADPH in anabolism. If the phycobilisome antenna size weren't decreased under this condition there would be hyperreduction of (or redox stress on) the photosynthetic electron transport chain similar to that seen in high light.

The *nblA* gene was shown to be necessary for chlorosis in cyanobacteria under nitrogen and other nutrient deprivation conditions (Baier *et al.* 2004; Collier and Grossman 1994). Homologs of *nblA* are found in cyanobacteria and red algae (Baier *et al.* 2004; Baier *et al.* 2001; Sato *et al.* 2008). It is known that NblA binds the main light harvesting phycobilisome pigment, phycocyanin, yet it does not contain any detectable proteolytic activity. Therefore, it may be that NblA tags the phycobilisome for degradation, and a protein partner may then cause phosphorylation or proteolysis of phycobilisome protein to initiate degradation (Baier *et al.* 2004; Bienert *et al.* 2006; Ochoa de Alda *et al.* 2004). The precise function of NblA remains to be determined.

1.2 NblS and Putative Response Regulators

NblS (for non-bleaching sensor) appears to be a histidine sensor kinase, an apparent member of a two-component regulatory system, first identified in *S. elongatus* PCC 7942 (here after *S. elongatus*) (van Waasbergen *et al.* 2002). NblS appears to be important in the signal integration of a general stress response. In the prototypical two-component system, a histidine sensor kinase detects a specific stimulus through its signal input domain causing a conformational change that catalyzes the autophosphorylation of a histidine in its kinase domain. The phosphoryl group is then transferred to an aspartate residue on the receiver

domain of a cognate response regulator protein during protein-protein interactions between the kinase domain of the sensor kinase and the receiver domain of the response regulator. Phosphorylation causes a conformational change activating its response domain that can affect changes in cellular physiology, often by the binding of DNA by the response regulator's output domain, regulating gene expression appropriate to the original signal input. NblS controls the expression of photosynthesis-related genes during high light exposure and nutrient deprivation including the *hliA* gene and the *nblA* gene (van Waasbergen *et al.* 2002). The DspA sensor kinase, the probable NblS homolog, in *Synechocystis sp.* PCC 6803 (hereafter *Synechocystis*) was found to regulate a number of genes during high light, chilling, osmotic, hydrogen peroxide, and salt stress (Hsiao *et al.* 2004; Kanesaki *et al.* 2007; Suzuki *et al.* 2001; Tu *et al.* 2004). The structure of NblS and homologs contain an N-terminal putative signal input region composed of several domains. There is a region located between two transmembrane domains, which is localized within either the thylakoid lumen or the periplasmic space (depending on the localization of NblS within either the thylakoid membrane and/or the cell membrane (van Waasbergen *et al.* 2002). Following the second transmembrane domain is a HAMP domain (HAMP domains are involved in transmitting signals from outside the cell to the inside) followed by a PAS domain (possibly binding a redox active cofactor) (van Waasbergen *et al.* 2002). A point mutant of *nblS* in *S. elongatus* (*nblS-1*) showed decreased expression of *hliA* under high light conditions and decreased expression of *nblA* under nutrient deprivation, therefore exhibiting a lack of chlorosis or "bleaching" normally associated with this condition (van Waasbergen *et al.* 2002). The stresses that regulate *hliA* and *nblA* seem to be controlled through overreduction of the photosynthetic electron transport chain, and NblS may detect photosynthetic redox stress under a variety of stress conditions (van Waasbergen *et al.* 2002) possibly through the ligand binding of a photosynthetic electron carrier to the PAS domain (Salem and van Waasbergen 2004b).

Inactivation of a gene known as *nblR* in *S. elongatus*, also showed a non-bleaching phenotype under nutrient stress (Schwarz and Grossman 1998). *nblR* encodes a response regulator protein. Because of the similarity in phenotypes of the *nblS-1* and the *nblR*-inactivated mutants, it was originally believed that NblR was the cognate response regulator to NblS. However, NblR homologs seem to be limited to the nitrogen fixing bacteria, *Thermosynechococcus elongatus* BP-1, and *S. elongatus*, while NblS homologs are present in all sequenced cyanobacteria and red algae (Ashby and Houmard 2006). This is one indication that NblS-NblR are not actually a signal transduction pair. Furthermore, microarray work with gene knockouts and partial gene knockouts of histidine kinases and response regulators from *Synechocystis* suggested that RpaA (Rre31) (Shoumskaya *et al.* 2005) (and more recently, that both RpaA and RpaB (Rre26) (Reviewed in Novikova *et al.* 2007)) may be possible cognate response regulators of the NblS homolog, DspA. Also, by examining evolutionary conservation of histidine kinases and response regulators through plastid evolution, Ashby *et al.* (2002) proposed that RpaB (Rre26) may be the cognate response regulator to DspA. Interestingly, RpaA and RpaB were identified to antagonistically regulate the efficiency of energy transfer from the light harvesting phycobilisomes to photosystem II relative to photosystem I (Ashby and Mullineaux 1999). Herein, we examine the control of *hliA* and *nblA* expression and provide evidence that RpaB is the cognate response regulator to NblS.

1.3 Dissertation goals

In order to better understand the control of gene expression by light stress in cyanobacteria the goals of this dissertation work were discovery based and included:

1) identification of DNA elements upstream of *hli* genes that control their expression with a focus on *S. elongatus* and *Synechocystis*; and
2) identification of protein elements that bind to the DNA elements identified upstream of *hli* genes and in doing so
3) identification of the probable cognate response regulator of NblS and homologs,

4) identification of the binding site of NblR upstream of the *nblA* gene and possible mechanism for its control of gene expression, and

5) development of a model of regulation of *hli* genes and *nblA* by NblS.

CHAPTER 2

NEGATIVE CONTROL OF THE HIGH LIGHT-INDUCIBLE *hliA* GENE AND IMPLICATION FOR THE ACTIVITES OF THE NblS SENSOR KINASE IN THE CYANOBACTERIUM *Synechococcus elongatus* PCC 7942

2.1 Abstract

The *hliA* gene of the cyanobacterium *Synechococcus elongatus* PCC 7942 is known to be upregulated by high-intensity light through the activity of the NblS sensor kinase. In this work it was found that, within the *hliA* upstream region, changes to the sequence around -30 to -25 (relative to the transcriptional start site) resulted in elevated *hliA* expression, implicating this region in negative regulation of the gene. Electrophoretic mobility shift assays performed were consistent with a protein binding this region that acts to keep the gene off in lower light. A reduction in gene dosage of *nblS* in vivo resulted in enhanced *hliA* expression, suggesting that negative control of *hliA* is mediated through NblS. An extended version of the High Light Regulatory 1 (HLR1) motif (previously described in *Synechocystis* PCC 6803) was identified within the sequence surrounding -30 to -25 of *hliA*. The extended HLR1 sequence was found upstream of other NblS-controlled genes from *S. elongatus* and *Synechocystis* PCC 6803 and upstream of *hli* genes from a variety of cyanobacterial and related genomes. These results point to the evolutionary conservation of the HLR1 element and its importance in NblS-mediated signaling and yield new insight into NblS-mediated control of gene expression.

2.2 Introduction

Cyanobacteria are considered to be closely related to the progenitors of plant plastids, and they carry out oxygenic photosynthesis in a manner very similar to that in plastids. The ability to properly utilize light energy is critical for all photosynthetic organisms including cyanobacteria. If they receive too much light, the photosynthetic apparatus can become overexcited, and damage may occur (Aro *et al.* 1993; Nishiyama *et al.* 2004; Noguchi 2002).

Therefore, photosynthetic cells have evolved various acclimation mechanisms to deal with excess light exposure, many of which necessitate alterations in gene expression (Chow 1994; Demmig-Adams 1990; Demmig-Adams and Adams 1992; Horton *et al.* 1996; Niyogi 1999). The mechanisms by which high-intensity light (high light, HL) triggers changes in gene expression in photosynthetic cells are still being elucidated, including the ways in which HL stress is initially perceived. This may be through perception of light quality through the use of specific photoreceptors and/or the perception of the changes in cellular redox (such as changes in the redox state of specific photosynthetic electron carriers) (as representative recent reviews on these subjects in cyanobacteria see (Mullineaux 2001) in plants see (Argüello-Astorga and Herrera-Estrella 1998; Chen *et al.* 2004; Pfannschmidt 2003), and in photosynthetic bacteria see (Bauer *et al.* 2003)).

When cyanobacteria are exposed to HL, one acclimation response that occurs is the synthesis of photoprotective high light-inducible (Hli) polypeptides (HLIPs) (Dolganov *et al.* 1995; Havaux *et al.* 2003; He *et al.* 2001; Salem and van Waasbergen 2004a). The *hliA* gene of *Synechococcus elongatus* PCC 7942 (Dolganov *et al.* 1995) was the first of the *hli* genes to be identified. Since that time, multiple *hli* genes have been identified in the various cyanobacterial genomes (Bhaya *et al.* 2002; Funk and Vermaas 1999) and have also been identified in red algae(Bhaya *et al.* 2002), in higher plants (Jansson *et al.* 2000), and more recently in the genomes of certain cyanophages (Lindell *et al.* 2004). The *S. elongatus hliA* gene is upregulated by exposure to low-intensity blue/UV-A light as well as to HL (Dolganov *et al.* 1995; Salem and van Waasbergen 2004a), suggesting that a blue-light photoperceptive event may be involved in HL-mediated control of gene expression. The four *hli* genes of *Synechocystis* sp. strain PCC 6803 are upregulated in HL, and one or more of them are known to be regulated in response to a number of other stress conditions including chilling, osmotic stress, salt stress, UV-B treatment, and hydrogen peroxide treatment (He *et al.* 2001; Huang *et al.* 2002; Kanesaki *et al.* 2002; Li *et al.* 2004; Mikami *et al.* 2002a; Mikami *et al.* 2002b; Suzuki *et al.* 2001). A common feature of these latter stresses is that they would decrease anabolism, and thus the

use of photosynthate by the cell. This would cause hyperreduction of the photosynthetic electron transport chain and accumulation of reactive oxygen species, similar to that which is observed in HL, creating a similar “redox stress” or “light stress” on the cell. The induction of *S. elongatus hliA* by HL and UV-A light can be differentially affected by photosynthetic inhibitors in a manner that suggests that *hliA* upregulation is through a system monitoring the reduced state of cytochrome b_6f (or a carrier downstream) in photosynthesis (Salem and van Waasbergen 2004b).

Upregulation of *S. elongatus hliA* by HL and blue/UV-A light is controlled by the membrane-bound NblS histidine sensor kinase (van Waasbergen *et al.* 2002). During HL or UV-A light exposure, NblS also controls the expression of other genes important for acclimation of the photosynthetic apparatus to HL (van Waasbergen *et al.* 2002). This includes the three *psbA* genes, which encode forms of the D1 subunit of photosystem II, and the *cpcBA* genes, which encode for subunits of phycocyanin, the major component of the light harvesting phycobilisomes. The putative NblS homolog in *Synechocystis* PCC 6803, DspA (also known as Hik33), was also found to upregulate the *hli* genes and a number of other, overlapping sets of genes during exposure to HL, chilling, osmotic, and salt stresses (Hsiao *et al.* 2004; Marin *et al.* 2003; Mikami *et al.* 2002a; Suzuki *et al.* 2001; Tu *et al.* 2004). NblS/DspA may act as a sensor of photosynthetic redox stress under a variety of stress conditions (van Waasbergen *et al.* 2002) and may also act to help signal changes in membrane fluidity under certain stress conditions such as chilling, osmotic, and salt stresses (Suzuki *et al.* 2000).

In *S. elongatus*, NblS additionally controls expression of the *nblA* gene during nutrient deprivation (van Waasbergen *et al.* 2002), another condition that would reduce anabolism and place a redox stress on the photosynthetic apparatus similar to that seen in HL. The *S. elongatus nblA* gene is upregulated primarily in response to starvation for nitrogen or sulfur, and the NblA polypeptide triggers degradation of the phycobilisomes, resulting in a “bleached” phenotype (i.e., cultures look yellow-green instead of the normal blue-green color) (Collier and Grossman 1994). Expression of the *S. elongatus nblA* gene during nutrient deprivation is also

controlled by the NblR response regulator (Schwarz and Grossman 1998), and NblR has been found to bind upstream of *nblA* (Luque *et al.* 2001). However, NblR does not appear to be directly involved in *hliA* regulation (van Waasbergen *et al.* 2002), and there does not appear to be an NblR homolog in *Synechocystis* PCC 6803 (Morrison *et al.* 2005).

In this study, a region upstream of *hliA* was implicated as negatively regulating the gene under lower light conditions, and evidence suggests this control is through binding of a repressor protein whose activity may be modulated by NblS. Within that region we identified a modified version of a high light regulatory 1(HLR1) sequence that had been previously identified in *Synechocystis* PCC 6803 (Eriksson *et al.* 2000). Our analyses extended the original HLR1 motif to include additional conserved residues, and we explored the presence of the extended HLR1 motif upstream of other genes controlled by NblS/DspA in *S. elongatus* PCC 7942 and *Synechocystis* PCC 6803 and upstream of *hli* genes in a variety of species.

2.3 Materials and Methods

2.3.1 Strains, plasmids, and culture conditions

Synechococcus elongatus strain PCC 7942 was grown (Laudenbach and Grossman 1991) at 30°C in BG-11 medium under incandescent light (50 µmol of photons $m^{-2}s^{-1}$), and the cultures (50 mL in glass culture tubes (25 mm diameter)) were bubbled with 3% CO_2 in air during growth and light treatments. Cultures larger than 50 mL were grown and low light (LL) adapted in glass flasks placed on an orbital shaker and bubbled with 3% CO_2 in air. HL (800 µmol of photons $m^{-2}s^{-1}$) was supplied from incandescent white-light bulbs. UV-A light (350-400 nm with a peak at 366 nm) was supplied from black-light blue bulbs at 27 µmol of photons $m^{-2}s^{-1}$. Prior to the various light treatments, cultures were grown to an A_{750} (Spectronic Genesys 5 spectrophotometer) of approximately 1.0, diluted to an A_{750} of 0.2 with fresh BG-11 medium (to avoid self-shading of cells during exposure to light), and adapted to LL (10 µmol of photons $m^{-2}s^{-1}$) for 18 h before HL or UV-A light treatment (as 50 mL cultures in glass culture tubes). The plasmid pHLIP-GUS (Dolganov *et al.* 1995), which replicates in both *S. elongatus* and *E. coli*,

was used for the creation of altered promoter region constructs. This plasmid carries 508 bp upstream of, and slightly into the coding region, of *hliA* (-467 to +41 relative to the transcriptional start site) fused translationally to a β-glucuronidase (GUS) reporter gene. *Escherichia coli* bearing pHLIP-GUS constructs were grown in LB medium supplemented with 50 µg/mL ampicillin, and *S. elongatus* bearing these plasmids were grown in BG-11 medium supplemented with 2 µg/mL ampicillin or carbenicillin.

2.3.2 Construction and assay of hliA *promoter region-GUS reporter fusion strains*

Unless stated otherwise, all molecular genetic techniques were performed according to standard protocols (Ausubel *et al.* 2002; Sambrook *et al.* 1989) or as previously reported (van Waasbergen *et al.* 2002). Nested deletions were performed on the plasmid pHLIP-GUS (following digestion with *Sal*I and *Sph*I) with exonuclease III digestion to varying extents in the Erase-A-Base System (Promega) as directed by the manufacturer to generate pHG-0.5D, pHG-1.5B, pHG-2.0C, and pHG-3.0P. Plasmids pHG-del and pHG-pho were originally generated for another project. Plasmid pHG-del is pHLIP-GUS with a deletion of the *hliA* upstream region to -25 (generated by changing the AA to GC at -27 and -26 in the *hliA* upstream region pHLIP-GUS, which created a *Stu*I site, digesting the product with *Sal*I and *Stu*I, filling in of the *Sal*I site to generate a blunt end, and ligation of the two blunt ends). Plasmid pHG-pho is pHLIP-GUS with an 18-bp Pho box (5'-CTATTCTCAATCTCCTGT) inserted between -30 and -28 (with removal of the adenine at -29). Plasmids constructs were propagated in *E. coli* and then transformed (Laudenbach and Grossman 1991) into *S. elongatus*. Levels of *hliA* activity from the strains were quantified by assaying GUS activity following various light treatments as previously reported (Dolganov *et al.* 1995).

2.3.3 Formation of an NblS-disrupted strain

The *nblS* gene cloned in the plasmid pUC119 was interrupted by insertion of a spectinomycin (Ω) resistance gene (Elhai and Wolk 1988) at the *Eco*NI site located 418 nucleotides inward from the initial ATG of the gene. The plasmid bearing the interrupted gene was introduced into wild-type *S. elongatus* for *in vivo* gene disruption of the gene by

homologous recombination (selecting with spectinomycin at 25 μg/mL). Despite repeated transfer of strains in low light on media containing high levels (250 μg/mL) of spectinomycin, PCR analysis of genomic DNA isolated from transformants always indicated the presence of both wild-type and interrupted copies of *nblS*.

2.3.4. RNA isolation, RNA blot hybridization, primer extension analyses

Following light treatments, cultures were swirled briefly in flasks on liquid nitrogen, transported on ice in centrifuge tubes, and immediately centrifuged for 10 min at 4°C. Cell pellets were stored at -80°C. RNA was isolated from cell pellets as previously described (Bhaya *et al.* 1999). For RNA blot hybridizations, equal amounts of RNA (determined spectroscopically) were resolved by electrophoresis in formaldehyde gels. The plasmid pTHL (Salem and van Waasbergen 2004a) bears a fragment of *hliA* (extending from 26 bp upstream of the ATG start codon of the *hliA* gene to 3 bp downstream of the translation termination codon) cloned into the pGEM-T Easy vector (Promega). Transcription of *Nco*I-digested pTHL with SP6 RNA polymerase and the Strip-EZ RNA probe synthesis kit (Ambion) with [α^{-32}P] UTP generated the riboprobe used to detect *hliA*-encoding transcripts. Plasmid pTRP (Salem and van Waasbergen 2004a) bears a 303-bp internal fragment of the *rnpB* gene, which encodes the constitutively expressed RNA component of RNase P, of *S. elongatus*. As a control to confirm equal loading of RNA samples, Northern blots were stripped of the *hliA* probe and hybridized with an *rnpB* DNA probe prepared by using the *rnpB*-bearing *Not*I fragment of pTRP and labeled by using the Strip-EZ DNA probe synthesis kit (Ambion) with [α^{-32}P] dATP. Gel electrophoresis of RNA was performed by using standard protocols (Sambrook *et al.* 1989). Northern hybridizations were done by using ULTRAhyb Hybridization Buffer or ULTRAhyb Oligo Hybridization Buffer (Ambion) per the manufacturer's protocol with hybridizations and washes at 60°C for RNA probes and at 42°C for DNA probes.

The transcriptional start site for several of the *Synechocystis* PCC 6803 genes included in this study were determined using the Primer Extension System-AMV Reverse Transcriptase Kit (Promega) and RNA from HL-exposed *Synechocystis* PCC 6803 cells. Reaction products

were run on a sequencing gel next to sequencing ladders generated from the same primers using, as sequencing templates, plasmids bearing each respective gene and its upstream region, and the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (USB). Primers used for the transcriptional start site mapping were: for *hliA*, 5'-CTGGTTCGATGGCAA AGTTGTTGAGACGGT; for *hliB*, 5'-GGGTTCAATGGCGAAGTTGTTGAGACGGTT; for *hliC*, 5'-TTCGGCGAAAGCAGTGAATCCAAAT; for *hliD*, 5'-AAATTTGGGATCTTCCTGCACGGGGG TTTGGT.

2.3.5 Electrophoresis mobility shift assays

For use as probes and competitor DNA in electrophoresis mobility shift assays, complementary single stranded oligonucleotides (synthesized by Integrated DNA Technologies) were annealed according to the manufacturer's recommendation. Equal volumes of complementary oligonucleotides (500 μM each in annealing buffer (100 mM potassium acetate, 30 mM HEPES, pH 7.5) were mixed, heated to 94°C for 2 min, and allowed to cool gradually to generate double stranded DNA fragments. The following oligonucleotides and their reverse complementary sequences were used: SchA (*S. elongatus hliA* from positions -50 to +1 relative to the transcriptional start site), 5'-AAAGATTAAGAAAAACGTCACAGAACTTTACGTTGTGTTA CACTTCAAACA; Del2 (the sequence from pHG-del analogous to that in SchA on pHLIP-GUS), 5'-GAGCTTGCATGCCTGCAGGTCGACCTTTACGTTGTGTTACACTTCAAACA; Cod2 (*Synechocystis* PCC 6803 *hliB* from within the coding region +17 to +67 relative to the initial ATG), 5'-TTCGCCTCGACCAAGACAACCGTCTCAACAACTTCGCCATTGAACCCCCTG; PsbAI (*S. elongatus psbAI* from positions -115 to -54 relative to the transcriptional start site), 5'-GATCGCTCTAAACATTACATAAATTCACAAAGTTTTCGTTACATAAAAATAGTGTCTA CTTA; Sya2P is the same sequence as A2P in (Eriksson *et al.* 2000) (*Synechocystis* PCC 6803 *psbA2* from positions -52 to -13 relative to the transcriptional start site), 5'-CTTCCTGTTACAAA GCTTTACAAAACTCTCATTAATCCTT.

Proteins were extracted from 1.5 L each of low light and high light (30 min)-treated cultures. Protein extractions and DNA-binding reactions were performed similar to that described by Onizuka *et al.* (2002). Low light- and high light-treated cyanobacterial cultures were harvested by centrifugation and resuspended in 10 mL of extraction buffer (10 mM Tris (pH 7.5), 1 mM EDTA, 250 mM KCl, 0.5 mM DTT and 10% glycerol). The cell suspension were frozen at -80°C and then divided into 1 mL portions in tubes to which approximately 500 µL of glass beads (0.1 mm average diameter) were added. The tubes were vigorously shaken (5,000 rpm) using a Mini Beadbeater (Biospec Products, Bartlesville, Okla.) for 5 to 8 min at 1 min intervals with cooling on ice between each cycle. Cell disruption (typically requiring 5-7 cycles of shaking) was confirmed microscopically. Lysates of a common sample type were combined and cellular debris were pelleted (15,000 X *g*, 20 min). Proteins were precipitated from the supernatants at 80% ammonium sulfate saturation. Protein precipitates were pelleted by centrifugation (15,000 X *g*, 20 min) and resuspended in 0.5 mL of extraction buffer without KCl. The protein extracts were dialyzed twice for 12 h against 2 L of extraction buffer without KCl using a Slide-A-Lyzer Dialysis Cassette (3500 MWCO, Pierce Biotechnology). All manipulations were performed at 4°C.

Double-stranded DNA fragments were 5' end-labeled using T4 polynucleotide kinase (USB Corporation) with [γ-^{32P}] ATP per the manufacturers instructions, and labeled products were purified using a QIAquick Nucleotide Removal Kit (Qiagen). Partially purified protein extract (20 µg) was incubated at room temperature for 20 min with 50 fmol of labeled DNA fragment in binding buffer (1 mM $MgCl_2$, 0.5 mM EDTA, 4% glycerol, 0.5 mM DTT, 50 mM NaCl, 0.1 mg/mL poly(dI-dC), and 10 mM Tris-HCl, pH 7.5) in a final volume of 20 µL. For competition assays, a partially purified protein extract in binding buffer was pre-incubated with an unlabeled competitor DNA fragment (at a concentration in fold excess of the amount of labeled DNA fragment used (50 fmol), as indicated) for 20 min at room temperature prior to incubation with the labeled DNA fragment. Samples were loaded onto a 4% non-denaturing

polyacrylamide gel (34.5:1 acrylamide-bisacrylamide, 0.5 X TBE, 2.5% glycerol). Electrophoresis was performed at 11°C in 0.5 X TBE running buffer at 350 V until the bromophenol blue dye had migrated two-thirds the length of the gel. Gels were dried and exposed to a BAS-MS imaging plate (Fujifilm) for radioisotope detection (Fujifilm FLA-3000 Image Analyzer).

2.3.6 Bioinformatic analyses

The motif-finding program ScanACE (Hughes *et al.* 2000; Roth *et al.* 1998) was used to scan genome sequences for sites within intergenic regions that were close matches to a given DNA motif (in the form of a weight matrix generated from a given set of input sites). A site thus discovered was then determined to be a good match if its score was within three standard deviations from the mean of the scores of the set of input sites used to construct the matrix (Roth *et al.* 1998).

2.4 Results

In order to identify light-responsive elements in the region upstream of *hliA*, we monitored *hliA* expression through GUS reporter activity following the various deletions and other alterations made to the upstream region of *hliA* carried on pHLIP-GUS, which bears approximately 500 bp upstream of the coding region on a plasmid that replicates autonomously in *S. elongatus* (Figure 2.2). Strains harboring constructs with deletions from the 5' extreme of this upstream region to a point -134 relative to the transcriptional start site showed no significant difference in GUS activity in low light (LL), HL, or UV-A light from that seen in the strain harboring the undeleted construct. A deletion to +2 eliminates the presumed promoter and the transcriptional start site and has background GUS activity. However, a deletion to -25 (on the plasmid pHG-del) generated an approximately eleven-fold increase in GUS activity in LL and an approximate four-fold increase in expression in HL and UV-A light. Similarly, an insertion of 18-bp between -30 and -28 (on the plasmid pHG-pho) resulted in around a nine-fold higher expression of the gene in LL and an approximately 2.5-fold higher expression in HL and UV-A light. These results indicated that the sequence around -30 to -25 is involved in negatively

regulating the gene, most actively in lower light. A possible scenario is that the changes to this region have disrupted the binding of a repressor protein that is most active in repression at lower light levels. This possibility is consistent with the results of gel shift assays, below. In strains bearing pHG-del and pHG-pho, the increase in expression observed upon shifting the strains from LL to HL and UV-A light (Figure 2.2) may thus be due to the ability of the putative repressor to still partially bind to that region and thereby exhibit weak light-responsive regulation (a possibility also consistent with gel mobility shift assays, below). The increase could also be due to some positive light-responsive control by factors downstream of position -25, but we have no evidence to support this. Since the changes in pHG-del and pHG-pho disrupt the putative -35 element of the promoter, presumably the sigma factor of RNA polymerase that recognizes the *hliA* promoter and helps promote expression of the gene under LL, HL, and UV-A light is able to recognize only the -10 element of the promoter. The putative *hliA* -10 hexamer (TACACT) is preceded by the sequence TGn, a feature of an extended -10 promoter, where contact between the sigma subunit and a -35 element is not required for transcription initiation (Kumar *et al.* 1993).

We had originally identified the NblS sensor kinase as controlling *hliA* expression in a chemically-generated mutant (*nblS-1*) in which *hliA* expression was significantly decreased in HL and UV-A light (van Waasbergen *et al.* 2002). Since the GUS reporter analyses suggested that the *hliA* gene is under negative control, we wished to explore further the nature of that control relative to control by NblS. We generated a merodiploid strain in which the *nblS* gene was inactivated in a portion of the chromosomes by insertion of a streptomycin resistance cassette. (We have been unable to completely segregate out chromosomes bearing the wild-type copy from the mutant, presumably because some NblS activity is essential for cell viability under normal growth conditions (van Waasbergen *et al.* 2002).) This mutant showed increased *hliA* expression in LL, HL, and UV-A light relative to the wild-type (Figure 2.1). A similar result was obtained for expression of the *hli* genes in *Synechocystis* PCC 6803 when the *dspA* gene (i.e. the putative *nblS* homolog) was inactivated (Hsiao *et al.* 2004). Thus, *hliA* appears to be

under negative control through NblS, and the *nblS-1* mutant originally isolated (van Waasbergen *et al.* 2002) is likely a gain-of-function mutant in *hliA* regulation by light, exhibiting negative control despite normally inducing light conditions.

The GUS reporter data suggested that the region around -30 to -25 upstream of *hliA* is involved in negative regulation of the gene. We noted within this region a stretch of sequence that is similar to an 18-bp sequence (GTTACATTTATTTACATA) we had observed in the upstream region of two of the four *hli* genes in the genome of *Synechocystis* PCC 6803 (*hliB* and *hliC*), which is itself identical within the first 16 bp to a sequence in the region upstream of the two *nblA* genes (*nblA1* and *nblA2* in tandem) in that organism. By visual examination, sequences with similarity to this 18-bp region were identified within the upstream regions of other genes in *S. elongatus* known to be regulated by NblS (van Waasbergen *et al.* 2002) (or assumed to be in the case of the *hli*2 and *hli*3 genes) and homologous genes in *Synechocystis* PCC 6803 (Figure 2.3). These include: the *nblA*, *psbAI*, and *cpcB1* genes of *S. elongatus*; two others of the six total *hli* genes apparent in the genome sequence of *S. elongatus* (which we termed *hli*2 and *hli*3); and two other *hli* genes (*hliA* and *hliD*), the *psbA2* gene, and the *psbA3* gene of *Synechocystis* PCC 6803. The 18-bp consensus sequence generated by the alignment of these genes (Figure 2.3) is composed of a pair of imperfect direct repeats of (G/T)TTACA(T/A)(T/A) separated by two nucleotides, and within certain of the genes, another direct repeat appears an even number of bases upstream or downstream of this sequence. Eriksson *et al.* (2000) had observed the presence of a 16-bp motif, TTACAA-N_4-TTACAA, which they termed HLR1 (High Light Regulatory 1), upstream of various light-regulated genes in *Synechocystis* PCC 6803 including *psbA2, psbA3, hliB, and nblA1*. The 18-bp sequence we observed encompasses the HLR1 for these *Synechocystis* genes, thus it is apparent that we had independently identified the HLR1 motif (in extended form) upstream of certain genes from *Synechocystis* PCC 6803 and had observed its conservation in those genes from *S. elongatus* PCC 7942.

Using the alignment shown in Figure 2.3 as input into the motif-finding ScanACE program, we identified this extended HLR1 motif upstream of numerous *hli* genes from the genomes of a variety of species (cyanobacteria, the *Cyanophora* cyanelle, and cyanophage) (Table 2.1). We also searched for the extended HLR1 motif upstream of all genes in the genome of *Synechocystis* PCC 6803. Of the 22 new matching sites discovered (i.e. sites other than those shown in Figure 3 used to generate the input alignment), 12 (54%) are upstream of genes recognized through microarray analyses (Mikami *et al.* 2002a; Paithoonrangsarid *et al.* 2004; Tu *et al.* 2004) as being directly controlled through the activity of the putative NblS homolog in that strain, DspA (Table 2.2), as are all those shown in Figure 3 except *Synechocystis* PCC 6803 *nblA*. This further indicates the importance of the HLR1 in conveying NblS/DspA-mediated signals.

In order to explore the activity of DNA-binding proteins to the HLR1 of *S. elongatus hliA*, we performed gel electrophoresis mobility shift assays using a radiolabeled 51-bp DNA fragment from the upstream region surrounding the HLR1 (from -50 to +1; SchA) and partially purified protein extracts from LL-adapted cells and LL-adapted cells that had been exposed to HL (30 min). The results (Figure 2.4) show a shifted complex that is specific for the SchA probe as evidenced by the ability of unlabeled SchA DNA fragment to compete with labeled SchA for binding and the inability of a non-specific DNA fragment (Cod2, a 51-bp DNA fragment from within the coding region of *hliB* of *Synechocystis* PCC 6803) to effectively compete for binding (Figure 2.4A). An unlabeled DNA fragment that has the sequence in SchA replaced by the comparable sequence present in pHG-del (the Del2 oligonucleotide) also competes, but more weakly than the SchA itself, for binding (Figure 2.4B). This result is consistent with our supposition based on the GUS reporter assays that the change in pHG-del (as represented by Del2), and thereby, possibly pHG-pho, although causing abnormal derepression of the gene in lower light, still allows some, still light-responsive, putative repressor binding. The region bearing the HLR1 from *psbAI* is also able to compete for binding (Figure 2.4B). This suggests that the same protein binding the *hliA* HLR1 is able to bind this sequence and, since this region

is well upstream of the apparent *psbAI* promoter (Golden *et al.* 1986), the binding we are seeing is likely not to be due to binding of RNA polymerase. The specifically shifted complex is more abundant in cell extracts from LL than from HL (Figure 2.4), indicating that the specific DNA-binding protein has higher affinity for the sequence in LL than in HL. This result is consistent with our GUS activity results indicating that a repressor protein binds to the upstream region and thereby negatively affects gene expression in LL. Similar results (a decrease in complex formation with an increase in light intensity) was seen in electrophoresis mobility shift assays by Eriksson, *et al.* (2000) using a DNA fragment surrounding the HLR1 from *psbA2* in *Synechocystis* PCC 6803. In fact, the same 40-bp *Synechocystis* PCC 6803 *psbA2* DNA fragment (-52 to -13 relative to the transcriptional start site within the *psbA2* upstream region), Sya2P, competes successfully for binding to the SchA sequence (Figure 2.4B), underscoring the evolutionarily conserved nature of this element.

2.5 Discussion

The results presented herein provide additional insight into the control of *hliA* and, directly and indirectly, into the activities of NblS. In this study, alterations in the *hliA* upstream region affect equivalent changes in *hliA* expression in HL and UV-A light as monitored in the GUS fusion strains (i.e. pHG-pho and pHG-del; Figure 2.2), and these two light conditions affect common changes in *hliA* expression as monitored by RNA hybridization in *nblS-1* (van Waasbergen *et al.* 2002) and interrupted *nblS* strains (Figure 2.1). Moreover, a previous study showed that photosynthetic inhibitors alter, in a common manner, HL- and UV-A-mediated *hliA* expression (Salem and van Waasbergen 2004b). Together, these results suggest that either there is a common sensory system that perceives these two stimuli or the signaling pathways for these stimuli converge at some point to act similarly through the HLR1 in a process requiring NblS input.

Since the HLR1 upstream of *S. elongatus hliA* and many of the other genes listed in Figure 3 overlaps with their putative promoters, a factor binding at this site would likely interfere with RNA polymerase binding and would be expected to act as a repressor. For *hliA*, this

scenario is consistent with our observations that the *S. elongatus* strains harboring pHG-del and pHG-pho, with mutations that interrupt the HLR1, exhibited elevated GUS activity. As the interrupted *nblS* mutant shows elevated levels of *hliA* transcripts (Figure 2.1) and the putative gain-of-function *nblS-1* mutant shows little or no increase in transcript levels of *hliA* under normally inducing conditions (van Waasbergen *et al.* 2002), the activity of this putative repressor may be controlled through the NblS sensor kinase.

The HLR1 also overlaps the putative promoter for the *S. elongatus nblA* gene (Figure 2.3). NblR binds upstream of the *nblA* gene somewhere between -206 and -1 (Luque *et al.* 2001) and likely acts to positively regulate the gene, as an *nblR* inactivated mutant shows an inability to upregulate *nblA* during deprivation for nitrogen or sulfur (Schwarz and Grossman 1998). Since the HLR1 overlaps the promoter and NblR appears to positively regulate the gene, it is unlikely that NblR binds to the HLR1 in upregulation of *nblA*. We had supposed that NblR was the cognate response regulator to the NblS sensor kinase under nutrient stress (van Waasbergen *et al.* 2002), however this may not be the case; NblR appears to positively regulate the gene and NblS likely negatively regulates the gene since the apparent gain-of-function *nblS-1* mutant acts to repress *nblA* mRNA levels (van Waasbergen *et al.* 2002). Since we have not seen significant constitutive bleaching of the strain in which *nblS* has been interrupted, as might be expected if *nblA* levels were significantly elevated due to loss of repressor activity, it may be that for significant *nblA* upregulation, in addition to having the absence of a negative regulator at the HLR1 (possibly mediated by NblS) during nutrient deprivation, it may also be necessary to have positive regulation by other regulators (i.e. NtcA during nitrogen stress (Luque *et al.* 2001; Sauer *et al.* 1999), and NblR during nitrogen or sulfur stress).

The HLR1 in the *S. elongatus psbAI* upstream region is from -85 to -68, well upstream of the promoter, so a regulatory protein binding at this site could act as an activator to promote transcription. In fact, there appears to be a positively acting element located between -115 and -54 in the *S. elongatus psbAI* upstream region (Nair *et al.* 2001). This same *psbAI* upstream region competes with the HLR1-containing *hliA* upstream fragment for binding (Figure 2.4),

indicating the same factor binds both HLR1-containing regions. In *S. elongatus*, NblS was found to be involved in regulation of the *psbAI* gene (van Waasbergen *et al.* 2002). The *psbAI* HLR1 may act as a positive element through the activity of NblS since, in the putative gain-of-function *nblS-1* mutant, *psbAI* transcript levels remained at or above the LL level in HL- and UV-A light (van Waasbergen *et al.* 2002)- conditions which would normally downregulate *psbAI* expression. Downregulation of the *cpcBA* genes in HL is also controlled through NblS(van Waasbergen *et al.* 2002). An HLR1 motif was observed in the *cpcB1A1*operon (Figure 2.3), just upstream of one of the two promoters that would be predicted from the two known transcriptional start sites (Kalla *et al.* 1988). The HLR1 in that case may, as we propose for *psbAI*, act as a positive element through the activity of NblS, as in the putative gain-of-function *nblS-1* mutant there was much less of the drastic HL-mediated decrease in *cpcBA* transcript levels than was seen in wild-type (van Waasbergen *et al.* 2002).

For all the *Synechocystis* PCC 6803 genes listed in Figure 2.2 (the *hli, psbA*, and *nblA* genes), the extended HLR1 overlaps their predicted promoters. In *Synechocystis* PCC 6803 where the *dspA* (putative *nblS* homolog) gene was inactivated, the *hli* genes and the *psbA* genes are all constitutively upregulated (Hsiao *et al.* 2004; Tu *et al.* 2004), consistent with negative control through DspA. The two *nblA* genes in *Synechocystis* PCC 6803 are located in tandem and are co-expressed in response to nitrogen, but not sulfur, deprivation, with subsequent bleaching (Baier *et al.* 2001; Richaud *et al.* 2001). They have also been found to be upregulated during iron starvation (Singh and Sherman 2000) and may also be elevated in response to high light exposure (Eriksson *et al.* 2000). As for the *nblS* interrupted strain, we have not observed constitutive bleaching typical to the *dspA* inactivated strain (and the mutant appears to bleach normally during nitrogen starvation (van Waasbergen, unpublished; (Morrison *et al.* 2005)). As proposed for *S. elongatus nblA*, the *Synechocystis* PCC 6803 *nblA* genes may be under negative control via DspA through repressor binding at their HLR1 under nutrient replete conditions (and derepressed during nitrogen deprivation), but may also require positive input during nitrogen deprivation for significant upregulation. This might explain the observation

that a phosphotransacetylase/*dspA* double mutant showed decreased phycobilisome degradation during nitrogen deprivation (presumably decreased *nblA* expression) (Morrison *et al.* 2005); it may be that in the double mutant, the lack of DspA activity causes derepression of the *nblA* genes, and the lack of small phosphodonor molecule contribution that would normally positively influence the activity of one or more separate, positively-acting response regulator(s), inhibits activation of the gene.

Finally, the presence of the extended HLR1 upstream of genes controlled by NblS/DspA from both *S. elongatus* and *Synechocystis* PCC 6803 as well as upstream of *hli* genes from a variety of cyanobacterial and related genomes (Figure 2.3 and Tables 2.1 and 2.2), points to the evolutionary conservation of this element upstream of high light/photosynthetic-redox stress-responsive genes and its likely importance in NblS-mediated signaling.

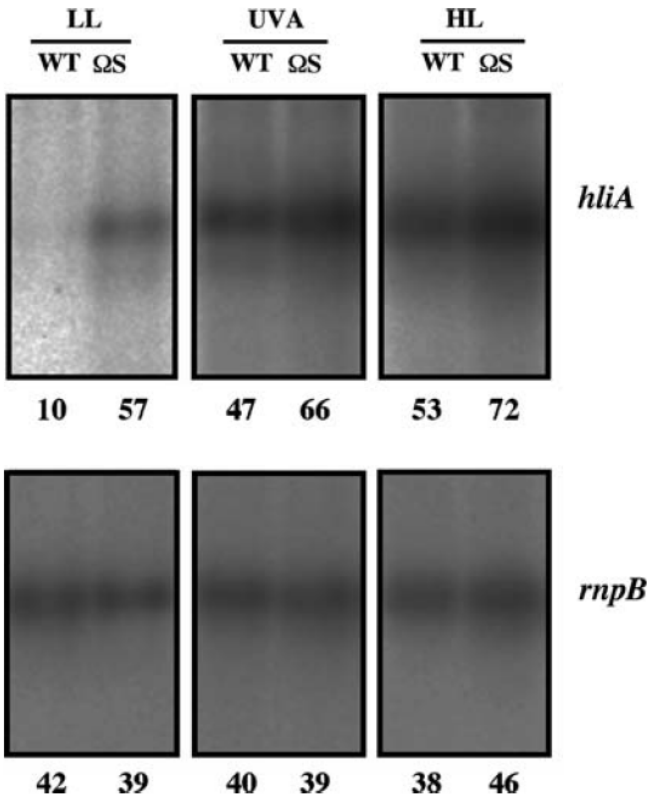

Figure 2.1 RNA hybridization analysis of *hliA* transcripts following various light treatments in a wild-type or *nblS*-interrupted (ΩS) background. Low light-adapted cells of each strain were exposed to high light or UV-A light for 30 min before harvesting for RNA. Hybridization of the RNA blot with an *rnpB*-specific probe serves as a loading control. Below each lane is presented the result of a densitometric analysis of the hybridizing signal (optical density in arbitrary units).

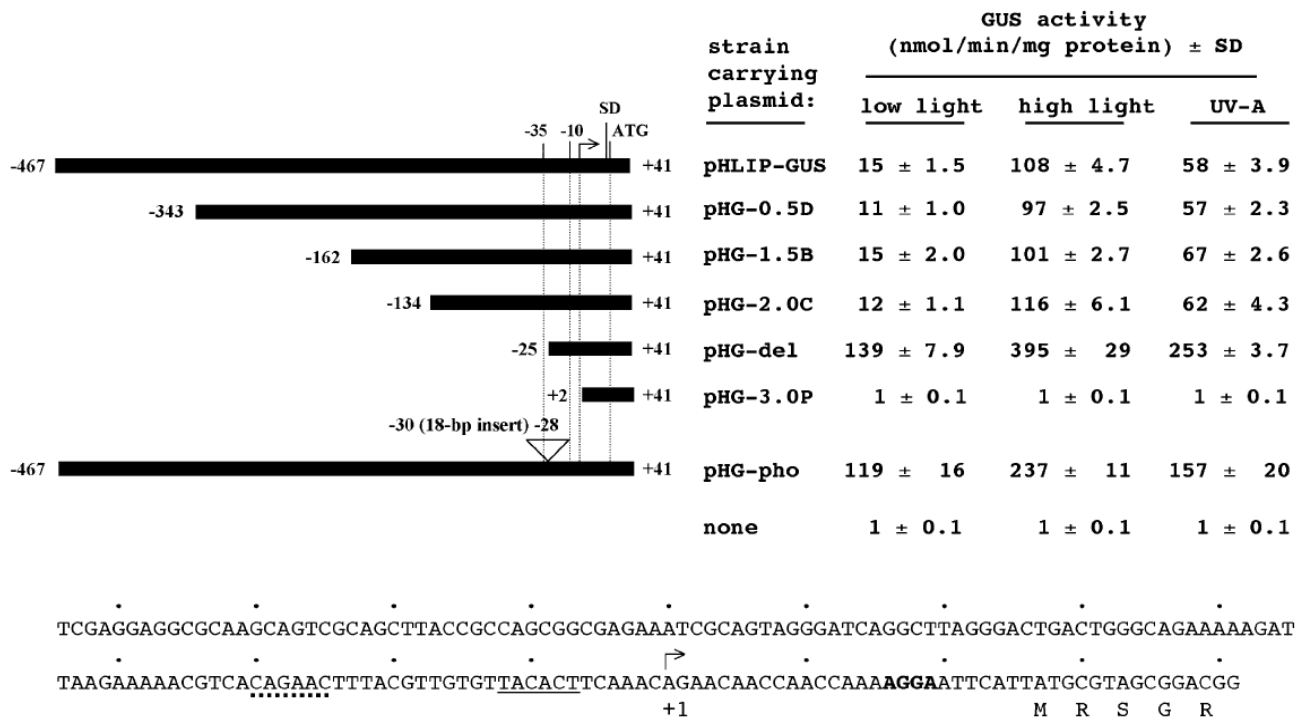

Figure 2.2 β-glucuronidase activities from *hliA*::GUS fusions under various light conditions. GUS assays were performed on *S. elongatus* carrying plasmids that bear various *hliA* promoter region fragments fused translationally to the GUS reporter gene. These promoter region fragments are diagramed with the indicated endpoints (relative to the transcriptional start site) and specific alterations noted. The diagrams also indicate the relative positions of the putative -35 and -10 promoter elements, the transcriptional start site (bent arrow), the possible ribosomal binding site (SD), and the initial ATG codon of *hliA*. The values shown are the averages and standard deviations of three independent replicates of the GUS activities determined from low light adapted cultures and from those shifted to high light or UV-A light for three hours. Shown at the bottom of the figure is the sequence of the largest promoter region sequence used (–134 to +41) with the transcriptional start site marked (+1, bent arrow) and the putative ribosomal binding site shown in boldface (Dolganov *et al.* 1995). On the sequence the putative -10 hexamer is underlined (solid line). Also underlined (dotted line) is the sequence from -30 to -25, alterations to which, as indicated in the top diagram in pHG-del and pHG-pho, caused elevated *hliA* promoter-driven GUS activity.

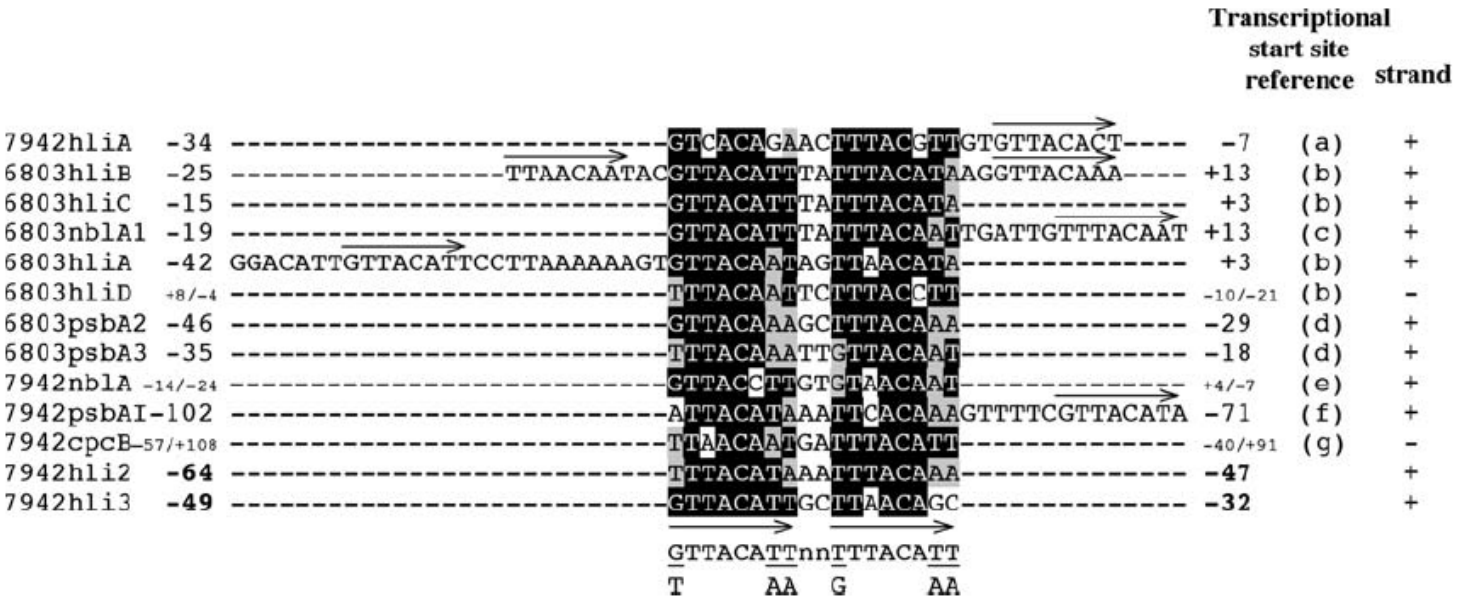

Figure 2.3 Alignment of putative light-responsive element (HLR1)-containing upstream regions for genes from *S. elongatus* and *Synechocystis* PCC 6803. Residues most highly conserved among the sequences in each repeat are shaded in black; residues next most conserved are shaded in gray. The direct repeats as they occur in the HLR1, the core two direct repeats, or elsewhere in the regions are indicated by arrows. Shown are regions from: the *hliA* gene and two others of the six total putative *hli* genes found in the genome sequence of *S. elongatus* (*S. elongatus* PCC 6301 Genbank accession numbers syc0429_d and syc1270_d, which we have termed hli2 and hli3, respectively); the *hliA, hliB, hliC,* and *hliD* genes from *Synechocystis* PCC 6803 (Cyanobase numbers ssl2542, ssr2595, ssl1633, ssr1789, respectively); the *nblA1A2* (nblA1), *psbA2*, and *psbA3* genes from *Synechocystis* PCC 6803; and the *nblA*, *psbAI,* and *cpcB1A1* (cpcB) genes of *S. elongatus*. Shown are the positions of the sequences relative to the transcriptional (plain-face type) or translational (bold-face type) start sites. References for the transcriptional start sites: a, (Dolganov *et al.* 1995); b, this work (start sites determined by primer extension, Figure 2.5); c, (Richaud *et al.* 2001); d,(Mohamed *et al.* 1993); e, (Luque *et al.* 2001); f,(Golden *et al.* 1986); g, (Kalla *et al.* 1988). *S. elongatus cpcB1A1* operon has two transcriptional start sites; the position of the sequences is shown relative to both. The *S. elongatus nblA* gene has multiple transcriptional start sites; the position of the sequences is shown relative to the major/minor start sites. We identified two possible transcriptional start sites for *Synechocystis* PCC 6803 *hliD*; the position of the sequences is shown relative to both.

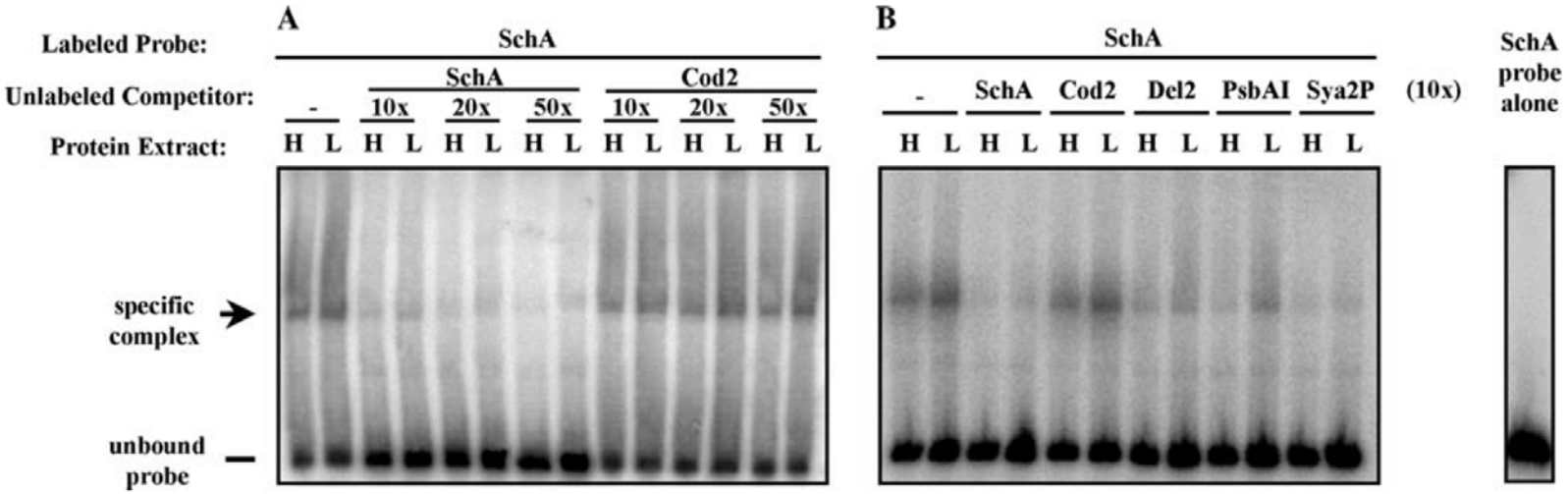

Figure 2.4. Competitive electrophoretic mobility shift assays show a specific protein species binding to the *hliA* upstream region (from positions –50 to +1 relative to the transcriptional start site, probe SchA). Electophoretic gel mobility-shift assays were performed with partially purified protein extracts prepared from low light-adapted cultures (L) or low-light adapted cultures that had been exposed (30 min) to high light (H). Protein extracts were incubated with radiolabeled SchA probe in the presence or absence of (A) either an unlabeled self (SchA) competitor fragment or an unlabeled non-specific competitor fragment from the coding region of the gene (Cod2), at the indicated fold excesses of the probe concentration; or (B) various unlabeled competitor fragments (at 10 fold excess of the probe concentration). A control sample with the SchA radiolabled probe alone with no protein extract added is shown in the final lane. The position of the unbound or "free" probe (migration of the probe when no protein extract is added) is indicated, as is the position of the complex that is specific for the SchA fragment versus a non-specific competitor (Cod2).

Table 2.1 Extended HLR1 sequences within the intergenic region upstream of *hli* genes from various species.

Organism	Gene	Extended HLR1[a]	Position[b] relative to the translational start site	Strand
Synechococcus sp. WH8102	SYNW1449 (*hli*1)	GTAACATTgtTTTACTTT	-11	+
	SYNW0817 (*hli*4)	TTTACACTccGTAGCAAT	-16	+
Thermosynech-ococcus elongatus BP-1	tsr0446 (CAB/ELIP/ HLIP superfamily)	GTAACAATtcTTTACCAA	-53	-
	tsl0446 (putative high light-inducible protein)	TTGAGAATccTTAACAAT	-79	-
	tsl0446 (putative high light-inducible protein)	GTTACCTGtgGTTACATC	-733	-
Prochlorococcus marinus MED4 (HL-Adapted Strain)	PMM1135 (*hli*14)	TTTACATTatTTAATACTtgtGTTACATTaaTTAACAAT	-53	+
	PMM1399 (*hli*6)	TTTACATTcaTTAATAGTtgtGTTATATTtgTTTACATA	-53	+
	PMM0818 (*hli*16)	TTTACATTcaTTAATAGTtgtGTTATATTtgTTTACATA	-53	+
	PMM0690 (*hli*21)	GTTACATTtaTTAATAAT	-14	+
	PMM1404 (*hli*5)	TTTACAGAaaGTAATATTaatGTTATATTtgTTTACGTA	-17	+
	PMM1404 (*hli*5)	TTTAGATAtcTTAACACA	-97	-
	PMM1384 (*hli*12)	GTTACATTaaTGTCCAAA	-119	-
	PMM1384 (*hli*12)	GTTATATTtaTTAATATA	-16	+
	PMM1118 (*hli*4)	TTTACAAAccTTCATATAtcctGTTACACTatTTAATATA	-18	+
	PMM1317 (*hli*13)	TTTGCATAtaTTTCCAAT	-229	+
Prochlorococcus marinus MIT9313 (LL-Adapted Strain)	PMT0051 (similar to high light inducible protein)	GTTACGTTccTTCATAAT	-74	-
Anabaena variabilis ATCC 29413	Ava_1411 (CAB/ELIP/HLI P superfamily)	GTTATATTaaTTTACATA	-58	+
	Ava_2916 (CAB/ELIP/HLI P superfamily)	TTTACAATagTTAATATAtctGTTACAGTtcTTAACAGA	-140	+
	Ava_0175 (CAB/ELIP/HLI P-related)	ATTACAATttGTTAAAAA	-178	+
	Ava_3554 (CAB/ELIP/HLI P superfamily)	TTCAACATctGTTACATTacGTTACAGA	-68	+

Table 2.1 Continued

Cyanophora paradoxa	CypaCp098 (ycf17)	GTTACAAAtgTTTACATA	-30	+
	CypaCp098 (ycf17)	GTTACTATagTTTACTTT	-369	+
P-SSM2 Cyanophage	PSSM2_272 (*hli*01)	GTTACACTtcTTTACAAA	-53	+
P-SSM4 Cyanophage	PSSM4_177 (*hli*01)	TGTACATAacTTTACATTagTTAACAAT	+2	+
	PSSM4_177 (*hli*01)	TTGACAAAacTTTACAAT	-39	+
	PSSM4_178 (*hli*02)	TTTACATTagTTAACAAT	-232	+

[a]The extended HLR1 sites listed were determined (using the ScanACE program (as described in the Methods section)) to be good matches to a motif generated using, as input, the set of all of the 18-bp direct repeats spaced two nucleotides apart (as shown in Figure 2.3) for *S. elongatus* PCC 7942 *hliA* and *nblA* and all the *Synechocystis* PCC 6803 genes shown in Figure 2.3; the non-conserved central two nucleotides were excluded as active columns. [b]With respect to the rightmost residue of the given sequence

Table 2.2. DspA-regulated genes from *Synechocystis* PCC 6803 newly identified as bearing an extended HLR1 motif within the intergenic region upstream.

Gene	**Extended HLR1[a]**	**Position[b] relative to the translational start site**	**Strand**
ssr2016 unknown	GTTACATTtaTTTACATA	-90	+
sll1797 ycf21	GTTACAAAacTTTACATT	-34	-
ssr2016 unknown	TTTACAAAaaTTTACAAA	-111	+
slr1634 unknown	TTTACATTtgTTAACAAT	-355	-
ssl3446 unknown	TTAACATAagTTTACAAA	-187	-
sll1783 unknown	TTTACAAAtaTTTACATG	-159	-
sll1541 unknown	GTTACATTtcTTTACTTT	-35	-
sll0819 *psaF*	GTTACGATtaTTTACAAT	-211	+
slr1634 unknown	TTTATAATtaTTTACATT	-142	-
slr0611 *sds*	TTTACGAAagTTTACATA	-43	-
sll1626 LexA repressor	GATACAAAatTTTACATT	-102	-
slr0897 probable endoglucanase	TTTACAATtaGTTGCATT	-204	-

[a]The extended HLR1 sites listed were determined (using the ScanACE program (as described in the Methods section)) to be good matches to a motif generated using, as input, the set of all of the 18-bp direct repeats spaced two nucleotides apart (as shown in Figure 2.3) for the *Synechocystis* PCC 6803 genes shown in Figure 3; the non-conserved central two nucleotides were excluded as active columns. [b]With respect to the rightmost residue of the given sequence.

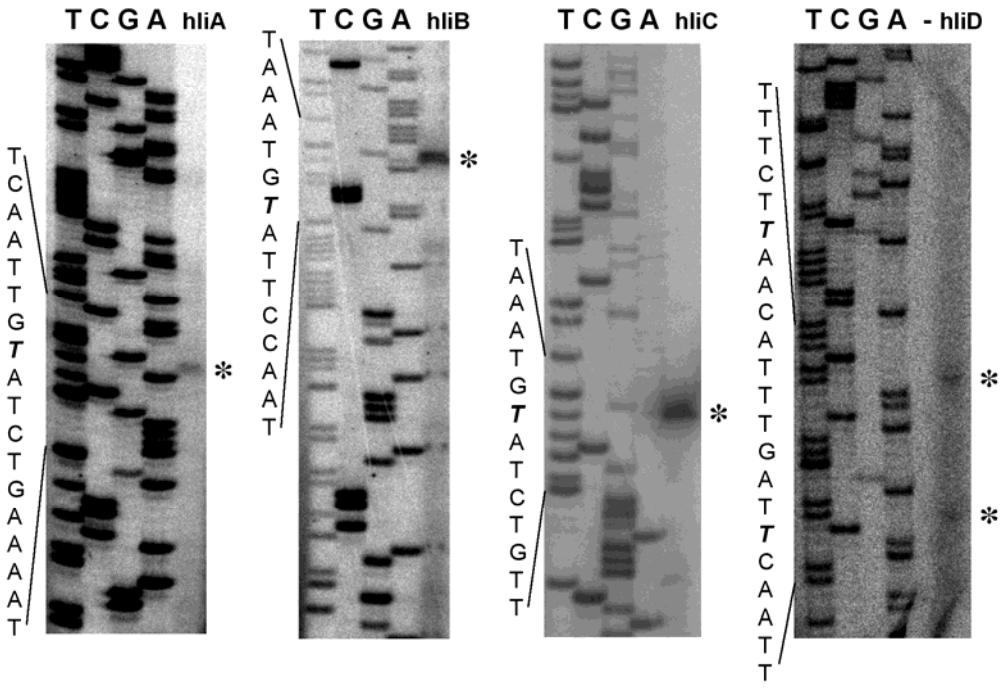

Figure 2.5 Transcriptional start site mapping for *Synechocystis* PCC 6803 *hliA*, *hliB*, *hliC*, and *hliD* genes. The sets of lanes TCGA are the sequencing ladders used to measure the primer extension reaction products for each of the genes. Asterisks indicate the locations of the major primer extension products denoting the transcriptional start sites. The sequences of the non-coding strands as read from the sequencing ladders are given with the transcriptional start sites shown in italics.

CHAPTER 3

THE RESPONSE REGULATOR RpaB BINDS THE HIGH LIGHT REGULATORY 1 (HLR1) SEQUENCE UPSTREAM OF HIGH LIGHT-INDUCIBLE *hliB* GENE FROM THE CYANOBACTERIUM *Synechocystis* PCC 6803

3.1 Abstract

Cyanobacteria, like other photosynthetic organisms, respond to the potentially damaging effects of high-intensity light by regulating the expression of a variety of stress-responsive genes through regulatory mechanisms that remain poorly understood. The High Light Regulatory 1 (HLR1) sequence can be found upstream of many genes regulated by high light stress in cyanobacteria. In this study we identify the factor that binds the HLR1 upstream of the high light-inducible *hliB* gene in the cyanobacterium *Synechocystis* PCC 6803 as the RpaB (Slr0947) response regulator.

3.2 Introduction

The existence of all photosynthetic organisms, including cyanobacteria, is dependent upon their ability to make proper use of the light energy they absorb. Absorption of too much light energy can lead to overexcitation of the photosynthetic apparatus and oxidative damage to the cell (Aro *et al.* 1993; Nishiyama *et al.* 2004; Noguchi 2002). Therefore, various acclimation mechanisms have evolved in these organisms to deal with excess light exposure, many of which require altering gene expression (Chow 1994; Demmig-Adams 1990; Demmig-Adams and Adams 1992; Horton *et al.* 1996; Niyogi 1999). However, the mechanisms by which high-intensity light is perceived and the signal transduced into the cell to trigger these changes in gene expression are still being defined.

The NblS sensor kinase in the cyanobacterium *Synechococcus elongatus* PCC 7942 is an important regulator controlling the expression of photosynthesis-related genes during high light exposure and nutrient deprivation (van Waasbergen *et al.* 2002). The probable NblS

homolog in the cyanobacterium *Synechocystis* PCC 6803, DspA (Sll0698, also known as Hik33), was similarly found to regulate a number of other, overlapping sets of genes during exposure to high light (HL), chilling, osmotic, and salt stresses (Hsiao *et al.* 2004; Marin *et al.* 2003; Mikami *et al.* 2002a; Suzuki *et al.* 2001; Tu *et al.* 2004). Like high light exposure, these other stresses, would reduce anabolism and thus decrease the recycling of the terminal electron acceptor in photosynthesis, NADP+. This would cause hyperreduction of the photosynthetic electron transport chain and accumulation of reactive oxygen species, creating a HL-like "redox stress" on the cell. Thus, NblS/DspA may act as a sensor of photosynthetic redox stress under a variety of stress conditions (van Waasbergen *et al.* 2002) and may, in addition, act to help signal changes in membrane fluidity under certain stress conditions such as chilling, osmotic, and salt stresses (Suzuki *et al.* 2000).

Upstream of a number of genes in *Synechocystis* PCC 6803 that are regulated by HL, (Eriksson *et al.* 2000) identified a sequence TTACAA-N_4-TTACAA, which they termed a High Light Regulatory 1 (HLR1) sequence. Recently, our group had found that the HLR1 motif could be extended, forming a pair of imperfect direct repeats of (G/T)TTACA(T/A)(T/A) separated by two nucleotides (Kappell *et al.* 2006). We found that many genes controlled by NblS/DspA in both *Synechocystis* PCC 6803 and *Synechococcus elongatus* PCC 7942 have one or more HLR1 motifs located upstream (Kappell *et al.* 2006). Included among those genes are the high light-inducible *hli* genes. Multiple *hli* genes have been found in the various cyanobacterial genomes, and in related genomes (Bhaya *et al.* 2002; Jansson *et al.* 2000; Lindell *et al.* 2004), and their products are likely to be involved in photoprotection (He *et al.* 2001). Bioinformatic analyses included in our previous study found HLR1 motifs upstream of *hli* genes from a variety of cyanobacterial, *Cyanophora* cyanelle, and cyanophage genomes (Kappell *et al.* 2006). Also in that study, as had been found for the *Synechocystis* PCC 6803 *psbA2* gene (Eriksson *et al.* 2000), DNA-binding activity was observed in electrophoresis mobility shift assays using the HLR1-containing region upstream of the *Synechococcus elongatus* PCC 7942 *hliA* gene and partially purified protein extracts from that organism (Kappell *et al.* 2006). However, it was not

known which protein actually bound the HLR1. Here we report the identification of the protein that binds the HLR1 of the *hliB* gene of *Synechocystis* PCC 6803 as the RpaB (Slr0947) response regulator.

3.3 Materials and methods

3.3.1 Cyanobacterial growth and protein extractions

Synechocystis species strain PCC 6803 was grown at 30°C in BG-11 medium at 50 µmol of photons m^{-2} s^{-1}, adapted to low light (10 µmol of photons m^{-2} s^{-1}), and exposed to HL (800 µmol of photons m^{-2} s^{-1}) as was described previously for *Synechococcus* PCC 7942 (Kappell *et al.* 2006). Protein extracts were prepared from *Synechocystis* PCC 6803 cultures as previously described for *Synechococcus* PCC 7942 (Kappell *et al.* 2006), lysing cells by bead-beating and partially purifying the proteins using an 80% ammonium sulfate precipitation step.

3.3.2 Overexpression of RpaA and RpaB in E. coli

We overexpressed RpaA and RpaB from *Synechocystis* PCC 6803 with a maltose protein binding (MBP) tag fused to their N-termini, essentially as outlined in the "Pilot Experiment" in the pMAL Protein Fusion and Purification System manual (version 5.1, New England BioLabs). The coding regions for the *rpaA* gene (slr0115) and the *rpaB* gene (slr0947) were PCR amplified (Sambrook *et al.* 1989) using *Synechocystis* PCC 6803 chromosomal DNA (extracted as in (Dolganov *et al.* 1995)) as a template and using, for *rpaA*, the forward primer 5'-C**ATG**CCTCGA(A/G)(T/A)A(C/T)T(G/C)ATCATTGACG and the reverse primer 5'-GAAATTAA(G/T)CTA(A/G)AAC**CTA**CGTTGGACTA and for *rpaB*, the forward primer 5'-ATTG**GTG**GTCGA(T/A)(G/T)(A/T)CGAGGCCAGC and the reverse primer 5'-CCTCGGTAAATC(C/T)CTAG(G/A)AA**TTA**CGGTT. (In the primers, the upper listed of the nucleotides in parentheses indicates the native sequence that was replaced by the lower of the nucleotides to introduce an *Eco*RI site in the forward primers and an *Xba*I site in the reverse primers and the boldfaced nucleotides indicate the predicted native start codon of the genes in the forward primers and the reverse complement of the predicted native stop codons of the genes in the reverse primers.) PCR products that had been cleaved with *Eco*RI and *Xba*I were

ligated with *Eco*RI-and *Xba*I-digested pMAL-c2X (New England BioLabs) and transformed into *E. coli* DH5α. Protein production was induced in the recombinant strains by the addition of isopropylthiogalactoside (IPTG) to log phase cultures (samples were periodically withdrawn to monitor protein induction), final lysates were prepared by beadbeating (Mini Beadbeater, Biospec Products, Bartlesville, Okla.), and the lysates were clarified by centrifugation (9,000 x g, 20 min, 4^{o}C). The MBP-tagged proteins were purified from the resulting clarified supernatants using amylose resin "in batch" essentially as in (Akerley and Lampe 2002), with column buffer, and column buffer with 10 mM maltose, substituted for TWB and TEB, respectively. Protein concentrations were determined by Bradford analysis (BCA Protein Assay, Pierce Biotechnologies).

3.3.3 Electrophoretic gel mobility shift assays and DNase I footprinting

Electrophoretic gel mobility shift assays were performed as described previously (Kappell *et al.* 2006). The following oligonucleotides and their reverse complementary sequences were annealed and used as probes and competitor DNA in the assays: SyhB (*Synechocystis* PCC 6803 *hliB* from positions -31 to +21 relative to the transcriptional start site (Kappell *et al.* 2006)), 5'-AAAATCTTAACAATACGTTACATTTATTTACATAAGGTTACAAA ATAAAAA; Cod2 (*Synechocystis* PCC 6803 *hliB* from within the coding region +17 to +67 relative to the initial ATG), 5'-TTCGCCTCGACCAAGACAACCGTCTCAACAACTTCGCCA TTGAACCCCCTG.

DNase I footprinting was performed using a singly end-labeled, double-stranded DNA probe of the *hliB* upstream region (-388 to +114, relative to the transcriptional start point) that was generated in the following manner. The primer that had previously been used for primer extension of the *Synechocystis* PCC 6803 *hliB* gene (Kappell *et al.* 2006) was 5' end-labeled using T4 polynucleotide kinase with [γ^{-32}P]ATP, and labeled products were purified using a QIAquick Nucleotide Removal Kit (Qiagen). This labeled primer was used in a PCR reaction with an unlabeled upstream primer for the *hliB* gene: 5'-TTGAAGCCGCCGTC(G/C)(A/G)CCTGTAAAAA (in which the upper listed of the nucleotides

in parentheses indicates the native sequence that was replaced by the lower of the nucleotides, to introduce a *Sal*I site for use in another project). The purified PCR product (QIAquick PCR Clean-up Kit (Qiagen)) was used in binding reactions, performed as in the electrophoretic mobility shift assay, using varying amounts of purified MBP-RpaB. The binding reactions were each digested with 0.075 U RQ1 RNase-free DNase (Promega Corporation) in its reaction buffer at room temperature for one min. The reactions were stopped (with DNase STOP solution, Promega Corporation), extracted with phenol/chloroform, and run on a 6% polyacrylamide/urea sequencing gel alongside 5', [γ-^{32}P]ATP end-labeled ΦX174 DNA/HinfI markers (Promega Corporation).

3.4 Results and discussion

In the present study, we undertook a search for the HLR1 binding factor, acting under the assumption that it would be the cognate response regulator to NblS/DspA, since there was a link between genes being controlled by NblS/DspA and their having an HLR1 upstream (Kappell *et al.* 2006). As is true for many other cyanobacterial histidine kinases (Ashby and Houmard 2006), NblS/DspA-type kinases do not appear to have an obvious cognate response regulator encoded nearby in any of the cyanobacterial genomes. Two response regulators have been proposed to be the cognate response regulator to DspA (Sll0698) in *Synechocystis* PCC 6803. One is the RpaA (Slr0115) response regulator, because DspA (therein termed Hik33) and RpaA (therein termed Rre31) exhibited corresponding global gene expression profiles during salt and hyperosmotic stresses by microarray analyses (Shoumskaya *et al.* 2005). The other is RpaB (Slr0947), because RpaB is the only OmpR-type response regulator found in certain of the red algal chloroplast genomes in which the apparent DspA homolog (Ycf26) is the only histidine kinase (with DspA being a homolog of *E. coli* EnvZ and EnvZ and OmpR forming a two-component regulatory pair in *E. coli*) (Ashby *et al.* 2002). Interestingly, RpaA and RpaB have been found to oppositely control the flow of energy from the light harvesting phycobilisomes to the photosystems (Ashby and Mullineaux 1999). We therefore decided to test both RpaA and RpaB for their ability to bind an HLR1 in vitro.

A strong motif of two HLR1 direct repeats in tandem (from -25 to +13 relative to the transcriptional start site (Kappell *et al.* 2006)) is found upstream of the *hliB* gene of *Synechocystis* PCC 6803 (Figure 3.2A). The 18-bpthat makes up the central two of these four direct repeats is identical to the HLR1 sequence upstream of the *hliC* gene and identical within the first 16 bp to an HLR1 sequence found upstream of the *nblA* genes of *Synechocystis* PCC 6803 (Kappell *et al.* 2006). In a previous study we had identified in *Synechococcus elongatus* PCC 7942, as had been observed in *Synechocystis* PCC 6803 for the *psbA*2 gene (Eriksson *et al.* 2000), a factor in partially purified protein extracts that bound to the HLR1-containing sequence upstream of the *hliA* gene (Kappell *et al.* 2006). Thus, we checked first to see if we could detect binding activity to the *hliB* HLR1-containing region in partially purified protein extracts from *Synechocystis* PCC 6803. Electrophoretic mobility shift assays were performed using a radiolabeled 51-bp DNA fragment from the *hliB* upstream region surrounding the HLR1 (from -31 to +21; the SyhB probe, underlined sequence in Figure 3.2A) and partially purified protein extracts from LL-adapted cells and LL-adapted cells that had been exposed to HL (30 min). The results (Figure 3.2B) show two shifted complexes, one of which is specific for the SyhB probe as evidenced by the ability of unlabeled SyhB DNA fragment to compete with labeled SyhB for binding, and the inability of a non-specific DNA fragment (Cod2, a 51-bp DNA fragment from within the coding region of *hliB* of *Synechocystis* PCC 6803) to effectively compete for binding. As was seen with the Synechococcus PCC 7942 *hliA* gene (Kappell *et al.* 2006), the specifically shifted complex is more abundant in cell extracts from LL than from HL (Figure 3.2B), indicating that the specific DNA-binding protein has higher affinity for the sequence in LL than in HL and is consistent with a repressor protein binding to the *hliB* upstream region and thereby negatively affecting the gene's expression in LL.

We overexpressed in *E. coli* the *Synechocystis* PCC 6803 RpaA and RpaB proteins fused to the maltose binding protein and purified them using amylose resin. An SDS-PAGE gel run on samples taken during the extraction process showed, for each hybrid protein, a band of the expected molecular weight (estimated to be 69,757 Da and 68,746 Da for MBP-RpaA and

MBP-RpaB, respectively; with RpaA, 241 amino acids long, estimated to be 27,275 Da; RpaB, 234 amino acids long, estimated to be 26,264 Da; and the MBP at 42,428 Da), which were apparent at 1 h, 2 h, and 3 h post-induction and during the purification process (Figure 3.1).

We then repeated the gel mobility shift assays using the purified MBP-tagged proteins. The results show that MBP-RpaB and not MBP-RpaA binds to the *hliB* HLR1-containing, SyhB DNA fragment. A complex with the SyhB probe forms only in samples to which MBP-RpaB has been added (and not in those to which MBP-RpaA was added at the same concentrations), with a second complex forming at the higher MBP-RpaB concentrations (Figure 3.3A). The MBP-RpaB complex is specific for the SyhB probe as evidenced by the ability of unlabeled SyhB DNA fragment to compete with labeled SyhB for binding and the inability of the non-specific DNA fragment (Cod2) to effectively compete for binding (Figure 3.3B).

In order to confirm that RpaB bound the *hliB* HLR1 region and nowhere else in the *hliB* upstream region, we performed DNase I footprinting using a large (~500 bp) region upstream of, and slightly into, the *hliB* gene as a probe and increasing amounts of MBP-tagged RpaB. Only one protected region was identified (Figure 3.4), with the minimum protected region represented by probe fragments from 141 bp to 102 bp in size, representing bases from -28 to +12 relative to the transcriptional start site, which is the area of the HLR1 repeats (from -25 to +13).

In this report we have shown that the RpaB response regulator of *Synechocystis* PCC 6803 binds the HLR1 sequence repeats upstream of the *hliB* gene. It remains to be seen if RpaB binds the HLR1 sequences found upstream of other high light responsive genes (Eriksson *et al.* 2000; Kappell *et al.* 2006). However, we would suggest this is likely, especially given the high sequence conservation within the HLR1 sequences of *hliB* and those upstream of other genes, especially the *hliC* and *nblA* genes of *Synechocystis* PCC 6803 (Kappell *et al.* 2006). It also remains to be seen if RpaB actually is the direct cognate response regulator to NblS/DspA, and we are currently investigating this. This is important to determine because the NblS/DspA kinases are known to integrate a wide variety of important environmental stresses

and signals (Hsiao *et al.* 2004; Marin *et al.* 2003; Mikami *et al.* 2002a; Suzuki *et al.* 2001; Tu *et al.* 2004; van Waasbergen *et al.* 2002) and because NblS and RpaB and their probable homologs are among the most conserved of the two-component regulators in the cyanobacteria, found in all of the cyanobacterial genomes sequenced to date and in the chloroplasts of red algae (Ashby 2006). It should be mentioned that the RpaA response regulator was recently shown (Takai *et al.* 2006) to be the cognate response regulator to the SasA histidine kinase, which mediates signals from the central oscillator to control gene expression in response to the circadian cycle in cyanobacteria.

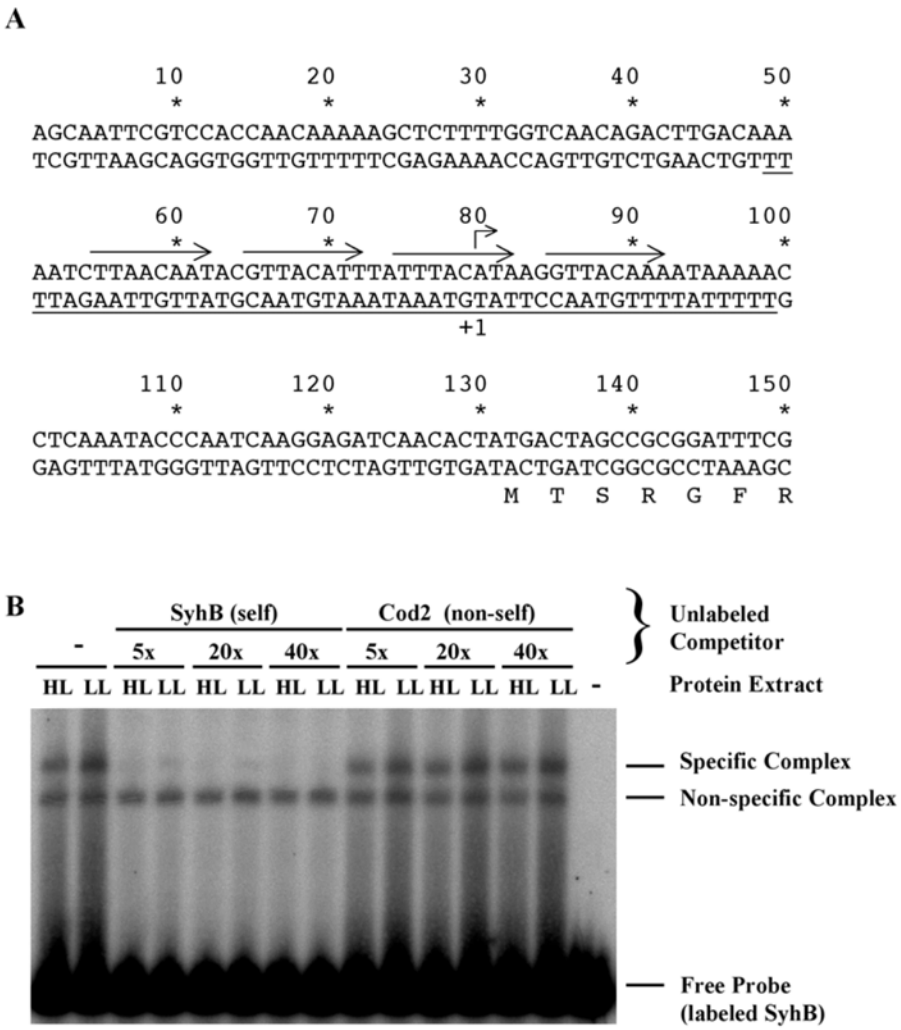

Figure 3.1 Competitive electrophoretic mobility shift assays show a specific protein species from partially purified protein extracts of *Synechocystis* PCC 6803 binding to the *hliB* HLR1-containing upstream region (probe SyhB). (a) The nucleotide sequence and the first several amino acids of the upstream region of *hliB* are shown. The four imperfect direct repeats that comprise the HLR1 pair (Kappell *et al.* 2006) are overlined with arrows. The transcriptional start point (Kappell *et al.* 2006) is indicated by a bent arrow at the (+1) site and the residues contained within the SyhB (double-stranded) probe are underlined. (b) Electrophoretic gel mobility-shift assays were performed with partially purified protein extracts (20 µg) prepared from low light-adapted cultures (LL) or low-light adapted cultures that had been exposed (30 min) to high light (HL). Protein extracts were incubated with radiolabeled SyhB probe in the presence or absence of either an unlabeled self (SyhB) competitor fragment or an unlabeled non-specific competitor fragment from the coding region of the gene (Cod2), at the indicated fold excesses of the probe concentration. The position of the unbound or "free" probe (migration of the probe when no protein extract is added) is indicated, as is the position of the complex that is specific for the SyhB fragment versus the non-specific competitor (Cod2).

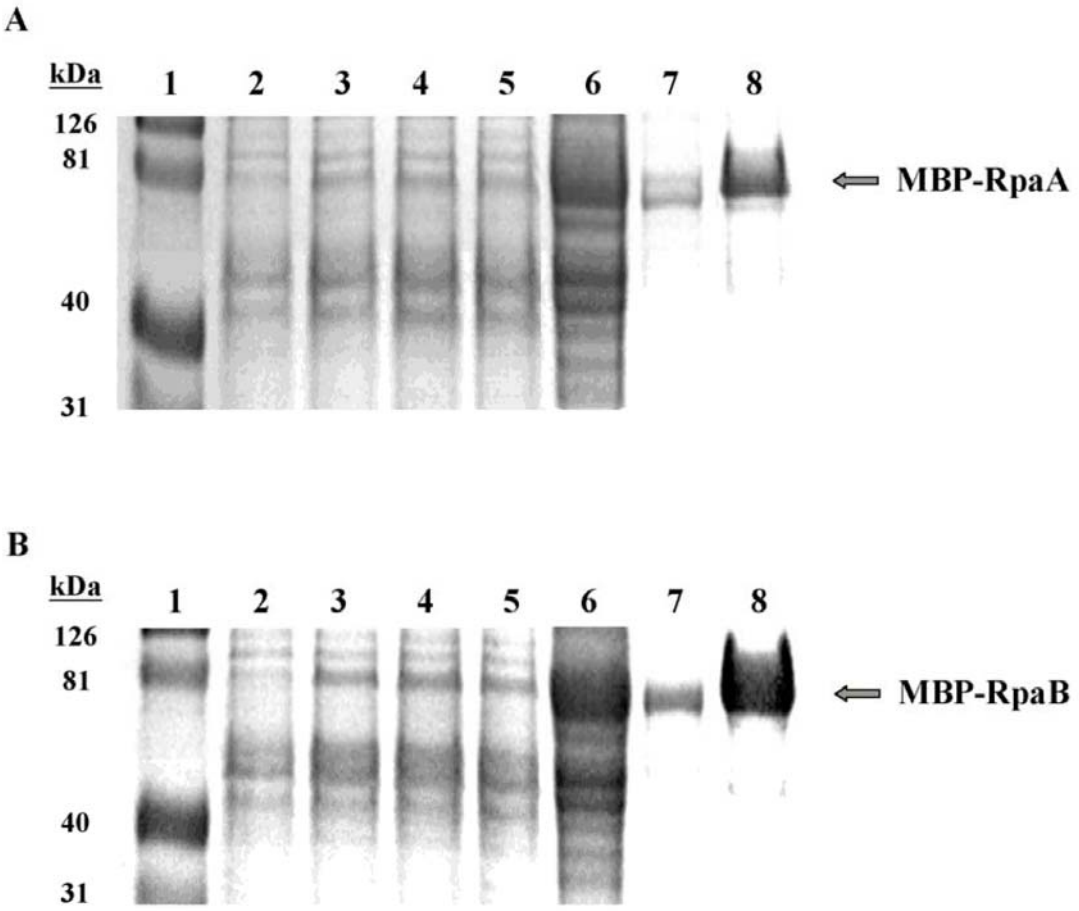

Figure 3.2 Purification of MBP-RpaA (a) and MBP-RpaB (b). Fractions from various steps of the overexpression and purification were analyzed by SDS-PAGE (16% polyacrylamide gel), and the gel was stained with Coomassie brilliant blue. Arrows mark the position of the purified MBP-bound proteins. Lane1, Kaleidoscope Prestained Protein Standards, Broad Range (Bio-Rad); lanes 2-5, crude lysates (2 µg protein each) from: uninduced cells (lane 2), cells 1 h after induction (lane 3), cells 2 h after induction (lane 4), cells 3 h after induction (lane 5); lane 6, clarified crude extract from 3 h induced cells (7 µg protein, MBP-RpaA; 14 µg protein, MBP-RpaB); lane 7, pellet fraction (500 ng protein, MBP-RpaA; 1 µg protein, MBP-RpaB); lane 8, elution collected from amylose resin containing the purified MPB-tagged proteins (4 µg protein, MBP-RpaA; 15 µg protein, MBP-RpaB).

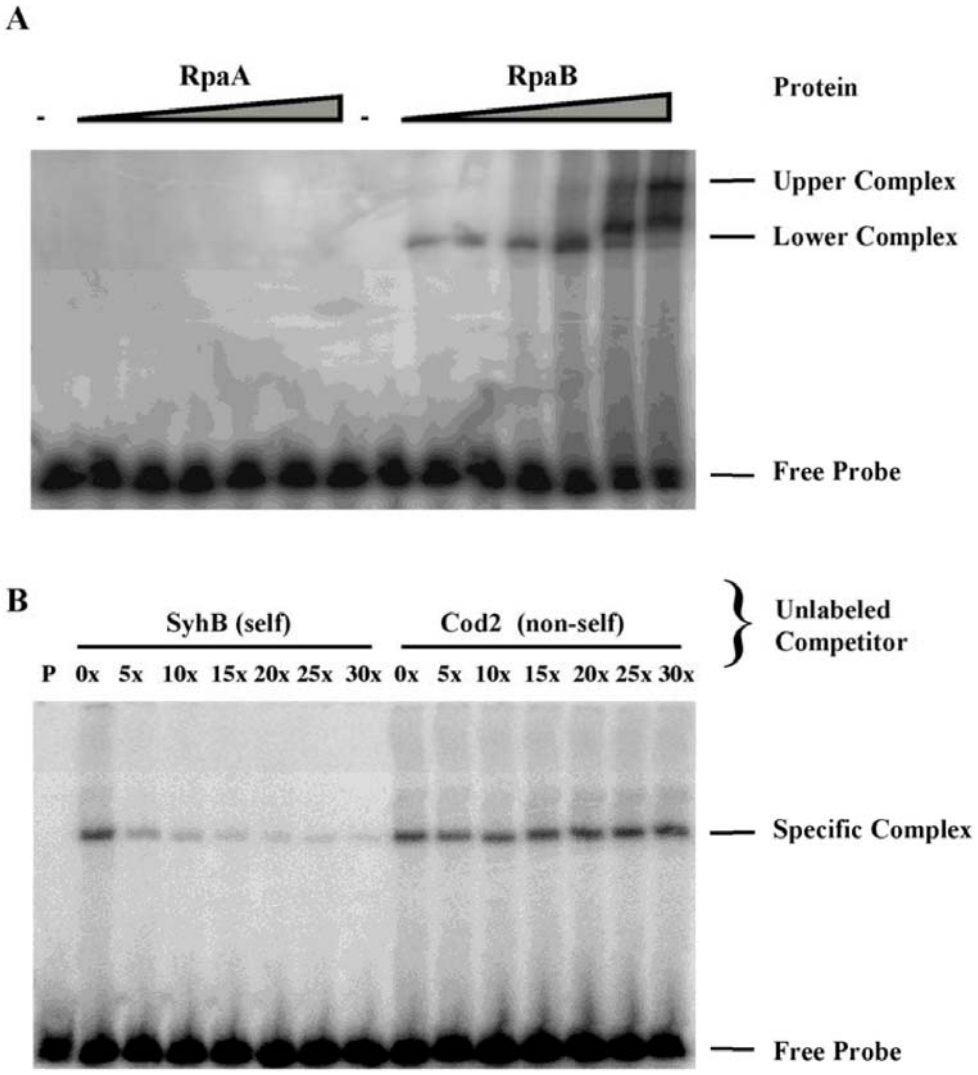

Figure 3.3 Electrophoretic mobility shift assays show that RpaB and not RpaA specifically binds to the *hliB* HLR1-containing region (probe SyhB). (a) Assays were performed with MBP-tagged, purified proteins. Increasing amounts of protein of each type (indicated by the wedge) were incubated with radiolabeled SyhB probe before electrophoresis. The amounts of protein used in the binding reactions were: no protein (lane 1 and lane 8); for MBP-RpaA (lanes 2-7) and for MBP-RpaB (lanes 9-14), respectively: 450 nM, 900 nM, 1.8 μM, 3.6 μM, 7.2 μM, and 14.4 μM. (b) Competitive assays were performed with MBP-tagged, purified RpaB (3.6 μM) in the presence or absence of either an unlabeled self (SyhB) competitor fragment or an unlabeled non-specific competitor fragment from the coding region of the gene (Cod2), at the indicated fold excesses of the probe concentration. The position of the unbound or "free" probe (migration of the probe when no protein extract is added) is indicated, as is the position of the complexes that are specific for the SyhB fragment versus a non-specific competitor (Cod2).

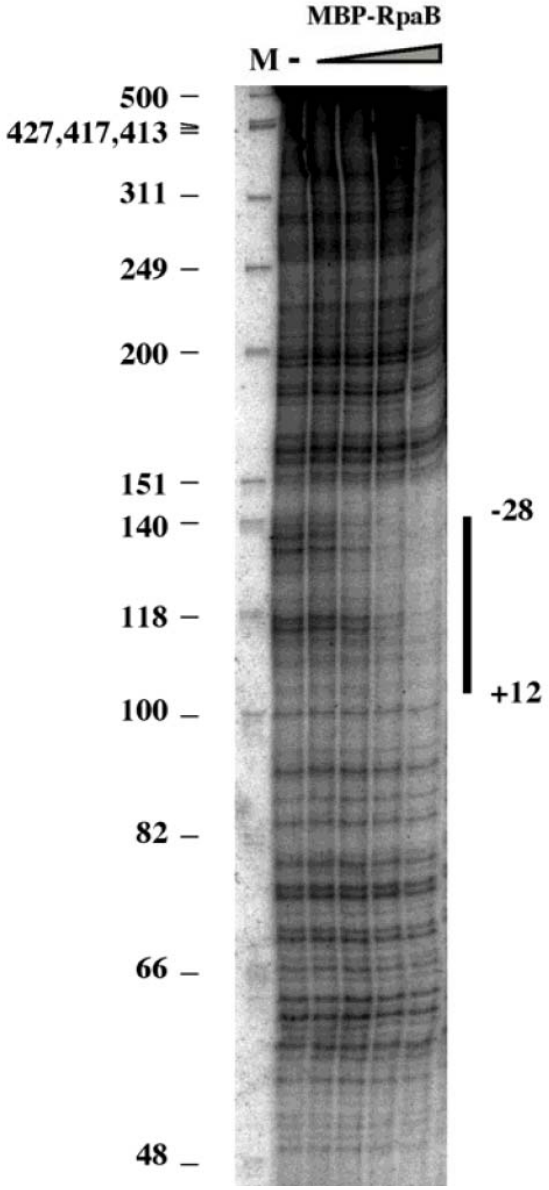

Figure 3.4 DNase I footprinting assays of the *hliB* upstream region bound by RpaB. MBP-tagged RpaB was added at increasing concentrations (indicated by the wedge) to DNase I footprinting reaction mixtures containing the radiolabeled *hliB* upstream fragment. The bold line indicates the location of the RpaB protected region with the positions (relative to the transcriptional start site) marked. Lane 1, radiolabeled marker (M) with band sizes indicated (in bp); lane 2, no protein; lanes 3-6, increasing amounts of MBP-RpaB protein included in the binding reaction with the *hliB* upstream region probe before DNase digestion: 1.8 μM, 3.6 μM, 7.2 μM, 14.4 μM.

CHAPTER 4

THE BINDING OF RpaB TO THE HLR1 ELEMENT AND NblR TO THE RNB1 ELEMENT AND THEIR REGULATION OF *hliA* AND *nblA* IN *Synechococcus elongatus* PCC 7942

4.1 Abstract

In *Synechococcus elongatus* PCC 7942 the NblS sensor kinase (called DspA or Hik33 in *Synechocystis* PCC 6803) regulates gene expression in response to a number of stress conditions including high-intensity light and nutrient limitation. In previous work, High Light Regulatory 1 (HLR1) sequences (two direct repeats of (G/T)TTACA(T/A)(T/A) separated by two nucleotides) were identified upstream of a number of genes in *S. elongatus* PCC 7942 and *Synechocystis* PCC 6803 known to be regulated by light and nutrient stress through NblS and DspA including the high light-inducible *hli* genes and the nutrient stress induced *nblA* gene. In subsequent work, we identified the response regulator RpaB as the factor that binds the HLR1 sequence upstream of the *hliB* gene from *Synechocystis* PCC 6803. Here we characterize binding to the HLR1 sequence upstream of genes *in S. elongatus*. Gel mobility shift and DNase I footprinting assays using *S. elongatus* RpaB overexpressed and purified from *E. coli* showed binding of RpaB to the HLR1-containing regions upstream of *hliA,* overlapping the promoter, *psbA*, upstream of the promoter, and overlapping the promoter of *nblA*. Work here suggests that *nblA* is negatively regulated through NblS (and by RpaB through binding of the HLR1), whereas the response regulator NblR positively regulates *nblA*, further supporting our supposition (Kappell and van Waasbergen 2007) that NblR is not the cognate response regulator to NblS, but that RpaB is. Using gel mobility shift assays and DNase I protection assays, we have found that the NblR response regulator binds upstream of *nblA* protecting an area from -183/-193 to -131/-141 relative to the transcriptional start site. Within this region a pair of indirect repeats were found consisting of (T/C)CT(C/G)AGAAAGG separated by six nucleotides, which we have

termed the reduced NblR binding 1 (RNB1) element because high levels of reducing agent was required for binding *in vitro*. This redox dependence is presumably through two conserved cysteine residues found in the receiver domain and the possible importance of this redox dependence *in vivo* is discussed.

4.2 Introduction

In nature photosynthetic organisms are dependent upon their ability to acclimate to changes in light and nutrient conditions. The means of their existence is the integration of the responses to both light intensity and nutrient availability through a general stress response. The cyanobacterial general stress response involves an increase in catabolism, a decrease in anabolism, protection of cellular components, degradation of phycobilisomes, changes in photosynthetic apparatus, and degradation of the thylakoid membranes. (Reviewed in Grossman *et al.* 2001 and Schwarz and Forchhammer 2005). The mechanisms by which the signals are perceived, transduced, and integrated into the general stress response are still being defined.

The NblS sensor kinase in cyanobacterium *Synechococcus elongatus* PCC 7942 (hereafter, *S. elongatus*) appears to be important in the signal integration of the general stress response. NblS controls the expression of photosynthesis-related genes during high light exposure and nutrient deprivation (van Waasbergen *et al.* 2002). The DspA sensor kinase, the probable NblS homolog, in *Synechocystis sp.* PCC 6803 (hereafter, *Synechocystis*) was found to regulate a number of genes during high light, chilling, osmotic, hydrogen peroxide, and salt stress (Hsiao *et al.* 2004; Kanesaki *et al.* 2007; Suzuki *et al.* 2001; Tu *et al.* 2004). A number of these stresses have been shown to strongly limit photosynthetic carbon metabolism (Long *et al.* 1994) and therefore cause overexcitation of the photosynthetic apparatus similar to that of high light (Aro *et al.* 1993; Noguchi 2002; Nishiyama *et al.* 2004). Thus NblS/DspA may detect photosynthetic redox stress under a variety of stress conditions and presumably be involved in transducing that signal to a cognate response regulator that binds DNA and affects gene expression forming a traditional two-component regulatory system (van Waasbergen *et al.*

2002). Originally it was assumed that the response regulator NblR (Schwarz and Grossman 1998) was the cognate response regulator to NblS (van Waasbergen *et al.* 2002). However, in subsequent work we discovered that RpaB binds the High Light Regulatory 1 (HLR1) sequence found upstream of NblS/DspA controlled genes in *Synechocystis* and it is likely that RpaB is the cognate response regulator to NblS (Kappell and van Waasbergen 2007). HLR1 sequences are also found upstream of NblS-, high light-, and redox stress-controlled genes in *S. elongatus* including *hliA* and *nblA* (Kappell *et al.* 2006).

The cyanobacterial general response to nutrient deprivation (such as sulfur-, nitrogen-, and phosphorous-limiting conditions) includes the decrease in anabolic metabolism and chlorosis or "bleaching" caused by the degradation of the light harvesting phycobilisome (Baier *et al.* 2004; Richaud *et al.* 2001; Sauer *et al.* 1999). Phycobilisome degradation provides some of the limiting nutrient and reduces the size of the light harvesting apparatus under periods of starvation when there is a decreased recycling of $NADP^+$ (the terminal electron acceptor in photosynthesis) because of the decreased utilization of NADPH in anabolism. If the phycobilisome antenna size weren't decreased under this condition there would be hyperreduction of (or redox stress on) the photosynthetic electron transport chain similar to that seen in high light.

A gene involved in phycobilisome degradation during nutrient limitation, *nblA*, was shown to be regulated through NblS in *S. elongatus* (van Waasbergen *et al.* 2002). The response regulator NblR in cyanobacterium *S. elongatus* is necessary to fully activate *nblA* expression under nutrient limiting conditions (Schwarz and Grossman 1998). Interestingly, it was demonstrated that phospho-transfer to the conserved aspartate at the phosphorylation site in NblR was not necessary for activation of *nblA* expression (Kato *et al.* 2008), and therefore a different mechanism may be present for the control of NblR-DNA interaction. While NblS and RpaB are evolutionarily conserved among cyanobacteria, red algae, and related plastids, NblR is present only in nitrogen fixing bacteria, *Thermosynechococcus elongatus* BP-1 and *S. elongatus* PCC 7942 (Ashby and Houmard 2006).

In this study we show in *S. elongatus* (like we had previously shown in *Synechocystis* PCC 6803 (Kappell and van Waasbergen 2007)) that the RpaB homolog binds the HLR1 element upstream of genes controlled by NblS, and we also demonstrate that NblR binds to a separate region upstream of *nblA* and that binding is redox controlled. We also present evidence that NblS negatively regulates *nblA* expression (as we had found for *hliA* (Kappell *et al.* 2006)), providing further proof that NblS and NblR do not form a two-component signal transduction systems as had been originally proposed (van Waasbergen *et al.* 2002).

4.3 Materials and Methods

4.3.1 Strains, culture conditions, and quantitation of pigments

S. elongatus was grown at 30°C in BG-11 medium under incandescent light (50 µmol of photons $m^{-2}s^{-1}$) (Laudenbach and Grossman 1991), and the cultures (50 mL in glass culture tubes (25mm diameter)) were bubbled with 3% CO_2 in air during growth and nutrient deprivation treatments. Cultures were starved for sulfur and nitrogen as previously described (Schwarz and Grossman 1998). Briefly, cells were harvested by centrifugation (5,000 X g, 10 min, room temperature (25°C)) after reaching an A_{750} of 0.8 (Spectronic Genesys 5 spectrophotometer). Pellets were resuspended in an equal volume (50 mL) of resuspension medium (BG-11 without nitrogen and sulfur), repelleted by centrifugation, and resuspended in onefifth volume with resuspension medium. 3.2 mL of this resuspension was added to medium lacking sulfur or nitrogen and 1.6 mL was added to replete medium to a total volume of 50 mL. When appropriate, antibiotics were added to a final concentration of 25 µg/mL for kanamycin and 200 µg/mL for spectinomycin. Chlorophyll levels were approximated from whole-cell absorbance at A_{680} (Collier and Grossman 1992) of samples diluted to equal A_{750}. Phycobilisome (PBS) content was estimated by relying on approximation of phycocyanin (PC) by whole-cell absorbance at A_{620} (Collier and Grossman 1992) of samples diluted to equal A_{750} (as an approximation of cell density).

The *nblR* mutant analyzed was the *nblRΩ* strain, in which the *nblR* gene was disrupted by insertion of a kanamycin resistance gene (Schwarz and Grossman 1998). The *nblS* mutants analyzed were the *nblS-1* strain, in which the parental strain was chemically mutagenized (van Waasbergen *et al.* 2002), and the *nblSΩ* strain, in which the *nblS* gene was disrupted by insertion of a spectinomycin resistance gene (Kappell *et al.* 2006). The *nblSΩ* mutant is a merodiploid strain in which the *nblS* gene was inactivated in a portion of the chromosomes by insertion of a streptomycin resistance cassette. (We have been unable to completely segregate out chromosomes bearing the wild-type copy from the mutant, presumably because some NblS activity is essential for cell viability under normal growth conditions (van Waasbergen *et al.* 2002).)

4.3.2 RNA isolation and RNA blot hybridizations

Cultures were swirled briefly in flasks on liquid nitrogen following nutrient deprivation treatments, transported on ice and centrifuged for 30 min at 4°C. Cell pellets were stored at -80°C. RNA was isolated from cell pellets as previously described (Kappell *et al.* 2006). For RNA blot hybridizations, equal amounts of RNA (determined spectroscopically) were resolved by electrophoresis in formaldehyde gels. Ethidium bromide was added to the loading buffer to visualize rRNA bands to confirm equal loading. The gene-specific probe for *nblA* were prepared as previously described (Collier and Grossman 1994). Northern hybridizations were done using ULTRAhyb Hybridization Buffer (Ambion) per the manufacturer's protocol with hybridizations and washes at 60°C.

4.3.3 Overexpression of RpaB and NblR in E. coli

RpaB and NblR from *S. elongatus* were overexpressed in *E. coli* with a maltose protein (MBP) tag fused to the N-termini, essentially as previously described (Kappell *et al.* 2006; Kappell and van Waasbergen 2007). Briefly, the coding region of *rpaB* gene and the *nblR* gene were PCR amplified from chromosomal DNA extracted from *S. elongatus* and using, for *rpaB*, the forward primer 5'-GAAAATCGCAAG(G/T)(A/C)(A/T)A(A/G)AATCCTCGTTG And the reverse primer 5'-GATCGCTGC(C/T)(G/C)G(C/A)GCTGGCTGCT, and for *nblR*, the forward

primer 5'-AACTATGATC(G/T)C(G/T)(C/A)(C/G)AGCCTCGCCAC, and the reverse primer 5'-GAGAGGTCGA(T/C)CGCTAAAACTTAG. (In the primers, the upper listed of the nucleotides in parentheses indicates the native sequence that was replaced by the lower of nucleotide to introduce an *Xba*I site in the forward primers and an *Xho*I site for *rpaB* reverse primer and *Sal*I site for the *nblR* reverse primer.) PCR products were cleaved with *Xba*I and *Xho*I or *Sal*I and ligated into *Xba*I and *Sal*I digested pMAL-c2x (New England BioLabs) and transformed into *E. coli* DH5α. Protein production, purification, and quantification was performed as previously described (Kappell and van Waasbergen 2007).

4.3.4 Electrophoretic gel mobility shift assays and DNase I footprinting

Electrophoretic gel mobility shift assays were performed as described previously (Kappell *et al.* 2006). The following oligonucleotides and their reverse complementary sequences were annealed and used as probes or competitor DNA as previously described (Kappell *et al.* 2006): SchA (*S. elongatus hliA* from positions -50 to +1 relative to the transcriptional start site), 5'-AAAGATTAAGAAAAACGTCACAGAACTTTACGTTGTGTTAC ACTTCAAACA; Cod2 (*Synechocystis hliB* from within the coding region +17 to +67 relative to the initial ATG), 5'-TTCGCCTCGACCAAGACAACCGTCTCAACAACTTCGCCATT GAACCCCCTG; PsbAI (*S. elongatus psbAI* from positions -115 and -54 relative to the transcriptional start site), 5'-GATCGCTCTAAACATTACATAAATTCACAAAGTTTTCGTTA CATAAAAATAGTGTCTACTTA.

The creation of singly end-labeled, double stranded PCR products of *hliA* and *nblA* was performed as previously (Kappell and van Waasbergen 2007). Briefly, the reverse (downstream) primers for PCR were 5' end-labeled using [γ^{-32}P] ATP, the primers were purified, and a PCR was performed using for *hliA*, the forward primer 5'-GAAGCTGATAGAC(A/C)(T/C)CGGGAACATTCA and the reverse labeled primer 5'-ACCCAAAGCAACTTCAGTCAGGATCAAAGCCACA and for *nblA*, the forward primer 5'-ACGCACTGACCC(A/G)G(C/G)CTGAGCGATC and the reverse labeled primer 5'-CAGATCAAACTGCTGTTCAAGACTGAGGGAA (in the primers the upper nucleotides in

parenthesis were replaced by lower nucleotides for the introduction of an *Sma*I site for a different project), the PCR product was purified, and used as probes for electrophoretic gel mobility shift assays and DNase I footprinting with varying amounts of purified MBP-7942RpaB or MBP-NblR. The Electrophoretic gel mobility shift assays and DNase I footprinting were performed essentially as described previously (Kappell and van Waasbergen 2007). In binding of MBP-NblR to *nblA*, 1 M DTT was added to the binding buffer to create a final concentration of 100 mM DTT.

4.4 Results and Discussion

4.4.1 nblA *appears to be under negative control by NblS, but under positive control through NblR*

In previous work (Kappell *et al.* 2006) we showed that the *hliA* gene is under negative control through NblS. We did so, in part, by examining *hliA* expression in two *nblS* mutants: the apparent gain-of-function mutant *nblS-1* (where *hliA* expression was decreased (constantly repressed) under normally inducing conditions: blue/UV-A and high light) and the partial knock-out mutant *nblSΩ* (where *hliA* was always "on," even in low light). Here we examine *nblA* expression and associated bleaching during nutrient deprivation in these two mutants and compare it to that found in an *nblR* mutant, (Note that NblR does not appear to regulate *hliA* (data not shown).) As reported previously by van Waasbergen *et al.* (2002), the *nblS-1* mutant exhibited: very little loss of pigmentation or bleaching (Figure 4.1A), very little loss of phycobilisomes as tracked by changes in A_{620} peak (Figure 4.1C), and a decreased accumulation of the *nblA* transcript (Figure 4.1B) as compared to that of wild-type cells during nutrient deprivation. The *nblSΩ* mutant exhibited a moderate increase in bleaching (Figure 4.1A) and decreased phycobilisomes levels (Figure 4.1C), and an increase in *nblA* transcript accumulation (Figure 4.1B) during nutrient deprivation as compared to wild-type. These contrary results between the *nblS-1* point mutation and the partial deletion mutant *nblSΩ* can be explained by assuming that as for *hliA*, *nblA* is under negative control through NblS: the *nblS-1* mutant allele encoding a gain-of-function form of NblS that causes increased repression of *nblA* expression (decreased *nblA* expression and bleaching), while partial deletion of *nblS* in the

*nblS*Ω mutant causes a decrease in repression, (increased *nblA* expression). However, as reported by Swartz and Grossman (1998), the *nblR*Ω mutant showed very little bleaching and decrease in phycobilisome levels, and showed decreased *nblA* transcript accumulation compared to that of wild-type cells (Figure 4.1). These results suggest that *nblA* is under positive control through NblR and NblR is necessary for activation of *nblA* expression and the normal degradation of the phycobilisome during nutrient limitation. These results suggest that NblS acts as a negative regulator of *nblA* expression, while NblR is a positive regulator of *nblA* expression, necessary for activation of *nblA* expression, and that NblS and NblR are part of separate signal transduction systems. Since, in our previous study (Kappell and van Waasbergen 2007), the RpaB response regulator was found to bind the HLR1 found upstream of many genes regulated by NblS (DspA/Hik33 in *Synechocystis* PCC 6803), we assumed that NblS and RpaB may comprise a two-component system (with NblR not being found in *Synechocystis* PCC 6803, at least not in a conserved manner). We wished to explore, then, RpaB and NblR DNA-binding in *Synechococcus elongatus* PCC 7942.

4.4.2 Successful overexpression and purification of MBP-tagged RpaB and NblR from Synechococcus elongatus *PCC 7942*

We overexpressed in *E. coli,* RpaB (Synpcc7942_1453) and NblR (Synpcc7942_2305) from *Synechococcus elongatus* PCC 7942 fused to the Maltose Binding Protein (MBP) and purified them using amylose resin. An SDS-PAGE gel (Figure 4.2) run on samples taken after the extraction process showed bands of expected molecular weight (estimated to be 69.2 kDa and 68.3k Da for MBP-7942RpaB and MBP-NblR, respectively). We used the purified MBP-tagged proteins for gel mobility shift assays and DNase I footprinting.

4.4.3 RpaB binds the HLR1-containing regions upstream of the hliA*,* nblA*, and* psbAI *genes in* Synechococcus elongatus *PCC 7942*

In a previous study we had identified DNA-binding activity in *S. elongatus* partially purified protein extracts that was specific for an *hliA* upstream fragment (from -50 to +1 relative to the transcriptional start site, the SchA probe) bearing a motif of direct repeats consisting of three half sites of an HLR1 in tandem (Kappell *et al.* 2006). We previously identified in

Synechocystis PCC 6803 that the RpaB response regulator is the factor that binds the HLR1 (Kappell and van Waasbergen 2007). Thus, we wished to see if RpaB is the factor binding the HLR1 in *S. elongatus* as well. We checked first to see if we could detect RpaB binding to a large fragment of the HLR1-containing region of *hliA*. Electrophoretic mobility shift assays were performed using a radiolabeled PCR fragment consisting of the region from -510 to +179 of *hliA* relative to the translational start site, and increasing concentrations of purified MBP-7942RpaB. The results (Figure 4.3) shows a complex that is unique in samples containing MBP-7942RpaB that increase in intensity with increasing concentration of MBP-7942RpaB. Electrophoretic mobility shift assays were performed to verify that the binding of RpaB could be localized to the HLR1 motif upstream of *hliA* by using a radiolabeled 51-bp DNA fragment from the upstream region surrounding the HLR1 (from -50 to +1 relative to the transcriptional start site; the SchA probe) and purified MBP-7942RpaB. The results (Figure 4.4A) show four bound complexes, two of which are specific for the SchA probe as evidenced by the ability of unlabeled SchA DNA fragment to compete with the labeled SchA probe for binding, and the inability of non-specific DNA fragment (Cod2, a 51-bp DNA fragment from within the coding region of *hliB* of *Synechocystis* PCC 6803) to effectively compete for binding. The position of RpaB binding at a site overlapping the promoter (-10) suggests that RpaB binding could affect *hliA* expression by RNA polymerase exclusion.

We identified a putative HLR1 site upstream of *nblA* in a previous study (Kappell *et al.* 2006). We tested the binding of RpaB to the upstream region of *nblA* by electrophoretic mobility shift assays. The assays were performed using a radiolabeled PCR fragment consisting of the region from -440 to +53 of *nblA* relative to the translational start site, and increasing concentrations of purified MBP-7942RpaB. The results (Figure 4.3) shows a complex that is unique in samples containing MBP-7942RpaB that increase in intensity with increasing concentration of MBP-7942RpaB.

In the previous study we showed that an HLR1 site upstream of *psbAI* from *S. elongatus* could compete with the binding of crude protein extracts and the SchA probe

suggesting specific binding upstream (Kappell *et al.* 2006). We tested the specific binding of RpaB to the upstream region between -115 and -54 relative to the transcriptional start site of *psbAI* (the PsbAI probe), which contains three half sites of HLR1, by electrophoresis gel mobility shift with the PsbAI probe and purified MBP-7942RpaB. The results (Figure 4.4B) show a single complex with specific binding for the PsbAI probe as supported by the ability of unlabeled PsbAI DNA fragment to compete with the labeled PsbAI probe and the inability of the non-specific Cod2 DNA fragment to compete. The binding of RpaB upstream of the promoter region suggests an ability to interact with and stabilize the RNA polymerase, activating *psbAI* expression. This is supported by the accumulation of *psbAI* transcripts in the *nblS-1* (gain-of-function) mutant in high light compared to the parental strain (as previously reported by van Waasbergen *et al.* (2002)). However, this would need to be confirmed experimentally.

We performed DNase I footprinting using singly end-labeled PCR products, as used in the electrophoretic mobility shift assays, consisting of the upstream region and part of the transcribed regions of *hliA* and *nblA,* with increasing amounts of MBP-7942RpaB, to confirm binding of RpaB to the HLR1-containing sites, and identify other areas in the region that RpaB may bind. Only one region protected by MBP-7942RpaB binding was identified upstream of *hliA* with the protected region representing bases from -47 to +3 relative to the transcriptional start site (Figure 4.6). MBP-7942RpaB binding protected two regions of the *nblA* probe representing the bases from -119/-129 to -89/-99 and -29/-39 to +22/+12 relative to the two transcriptional start sites, with the latter showing enhanced protection compared to the former (Figure 4.7A). The result also shows hypersensitivity for DNase I digestion upstream of the -119/-129 bases also suggestive of binding immediately downstream.

4.4.4 NblR binds upstream of nblA *gene and binding depends upon thiol-reducing conditions*

Currently the only gene in *S. elongatus* known to be regulated by NblR is the *nblA* gene. NblR was shown previously to be necessary to fully activate *nblA* expression during nutrient stress and for moderate activation during nutrient replete conditions (Schwarz and Grossman 1998). Electrophoretic mobility shift assays were performed to demonstrate NblR

binding to the upstream region of *nblA*. The assays were performed using a radiolabled PCR product from -440 to +53 relative to the translational start site of *nblA* and increasing concentrations of purified MBP-NblR. The result shows a complex that forms a smear rather than distinct bands indicating non-specific binding (Figure 4.5). This suggested there are additional requirements for optimal NblR/DNA interaction. The presence of two conserved cysteine residues in the receiver domain of NblR and all homologs (Figure 4.8) suggested that the thiol-redox state may play a role in binding. Upon addition of 100 mM DTT (a thiol-reducing agent) compared to normal levels usually used (1 mM) in mobility shift assays, the smears were decreased and two distinct defined bands of bound complex emerged. It is unlikely that the addition DTT caused dissociation of MBP-NblR aggregates because of the presence of non-specific DNA-binding and the increase in intensity of the two bands suggesting an increase in both dimeric and polymeric NblR binding. Thus NblR binding to *nblA* may be regulated by a thiol-group-dependent redox control mechanism, however other mechanisms cannot be excluded.

To identify regions upstream of *nblA* harboring the binding site of NblR, we performed DNase I footprinting assays using singly end-labeled PCR products consisting of the upstream region and part of the transcribed region of *nblA* with increasing amounts of MBP-NblR under high thiol-reducing conditions. A single protected region was detected upstream of *nblA* representing bases from -193/-183 to -141/-131 relative to the two transcriptional start sites (Figure 4.7B). We noted within this region an inverse repeat consisting of the half site: (T/C)CT(C/G)AGAAAGG with a six nucleotide spacing (Figure 4.7C) we have termed this motif reduced NblR binding 1 element (RNB1). The position of binding upstream of the promoter region suggest that NblR is involved in activation of *nblA* expression and this is supported by previous work (Schwarz and Grossman 1998).

4.4.5 Conclusions

We have shown in *S. elongatus* that RpaB, the putative response regulator for NblS, binds upstream of *hliA* and *nblA* at the HLR1 element overlapping their promoter regions, suggesting that it excludes RNA polymerase from binding their promoters to initiate transcription. In the case of *hliA*, RpaB appears to be the primary regulation as demonstrated by previous work looking for *cis*-acting elements upstream of *hliA* that identified the HLR1 (Kappell *et al.* 2006). In contrast, *nblA* can now be seen to be regulated through three *cis*-acting regulators, NtcA (Luque *et al.* 2001; Sauer *et al.* 1999), NblR (Luque *et al.* 2001; Schwarz and Grossman 1998), and now RpaB. While RpaB appears to be involved in repression of *nblA* expression, NblR and NtcA are activators (positive-acting regulators) of *nblA* expression. The activity of the *nblS-1* mutant acting through RpaB would suggest that RpaB repression of *nblA* expression has to be lifted before *nblA* expression activated by NtcA or NblR, may take place.

We have also shown *in vitro* that NblR has to be in a highly reducing environment to bind specifically to the RNB1 upstream of *nblA*. Under nitrogen and other nutrient limiting conditions it is possible for the cellular redox state to become reduced (Alfonso *et al.* 2001; Reyes and Florencio 1995), which may cause NblR to bind. However, further exploration into the redox regulation of NblR is needed because the conserved thiol group may be reactive to several different physiological signals based on diverse thiol modifications that are possible (Kim *et al.* 2002). Moreover, even though phosphorylation of the conserved Asp residue is not necessary for activity of NblR (Kato *et al.* 2008), regulation of NblR by a sensor kinase cannot be ruled out.

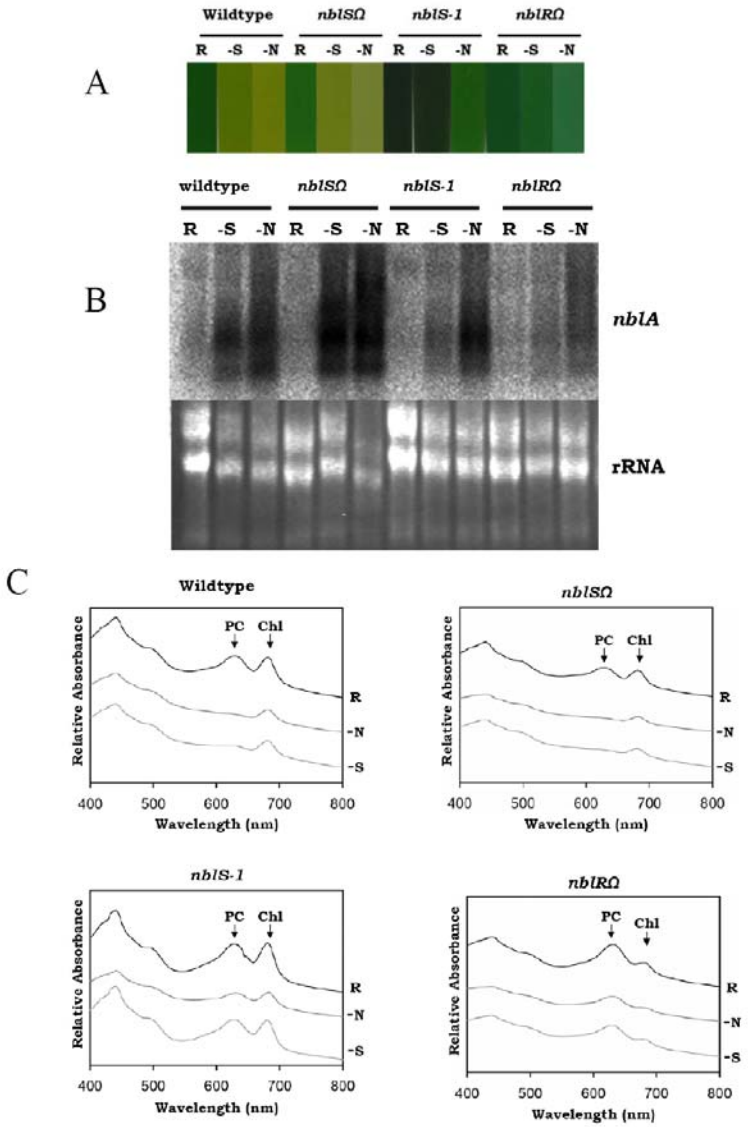

Figure 4.1 Characterization of the response to nutrient deprivation in wild type, in the *nblSΩ* mutant, and in other mutants. Liquid medium cultures (A) and whole cell spectra (C) of wild-type cells, the *nblS*-interrupted mutant (*nblSΩ*), a strain bearing two point mutations in *nblS* (*nblS-1*), and the *nblR*-interrupted mutant (*nblRΩ)* after 48 hours in replete medium (R) or medium lacking sulfur (-S) or nitrogen (-N). An arrow (PC) marks the 620 nm absorbance peak of phycocyanin and an arrow (Chl) marks the 680 nm absorbance peak of chlorophyll. Cultures were adjusted to the same A_{750} before determining the spectra. Spectra were offset along the y axis, and relative heights of absorbance peaks at 620 nm and 680 nm can be compared. (B) RNA blot hybridization of a probe specific for *nblA* to RNA isolated from wild type, *nblSΩ, nblS-1,* or *nblRΩ* strains grown for 48 h in replete medium (R) or media lacking sulfur (-S) or nitrogen (-N). For comparison, total RNA was stained with ethidium bromide (rRNA).

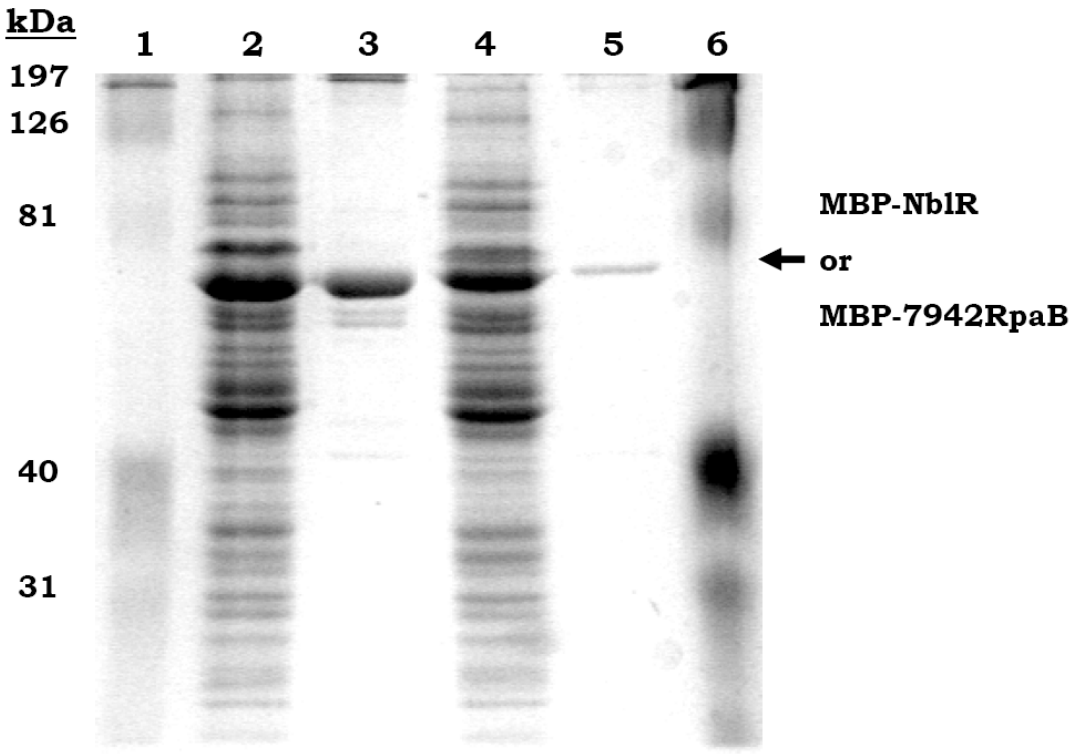

Figure 4.2 Purification of MBP-7942RpaB and MBP-NblR. Fractions from steps of the overexpression and purification were analyzed by SDS-PAGE (16% polyacrylamide gel), and the gel was stained with Commassie brilliant blue. The arrow marks the position of purified MBP-tagged proteins. Lane 1 (5 µL) and lane 6 (10 µL), Kaleidoscope Prestained Protein Standards, Broad Range (BioRad); lane 2 and 4, soluble crude lysates from cells induced for 3 hours overexpressing MBP-NblR and MBP-7942RpaB, respectively; lane 3 and 5, purified MBP-NblR (2.8 µg in 10 µL) and MBP-7942RpaB (0.597 µg in 10 µL), respectively, eluted from amylose resin.

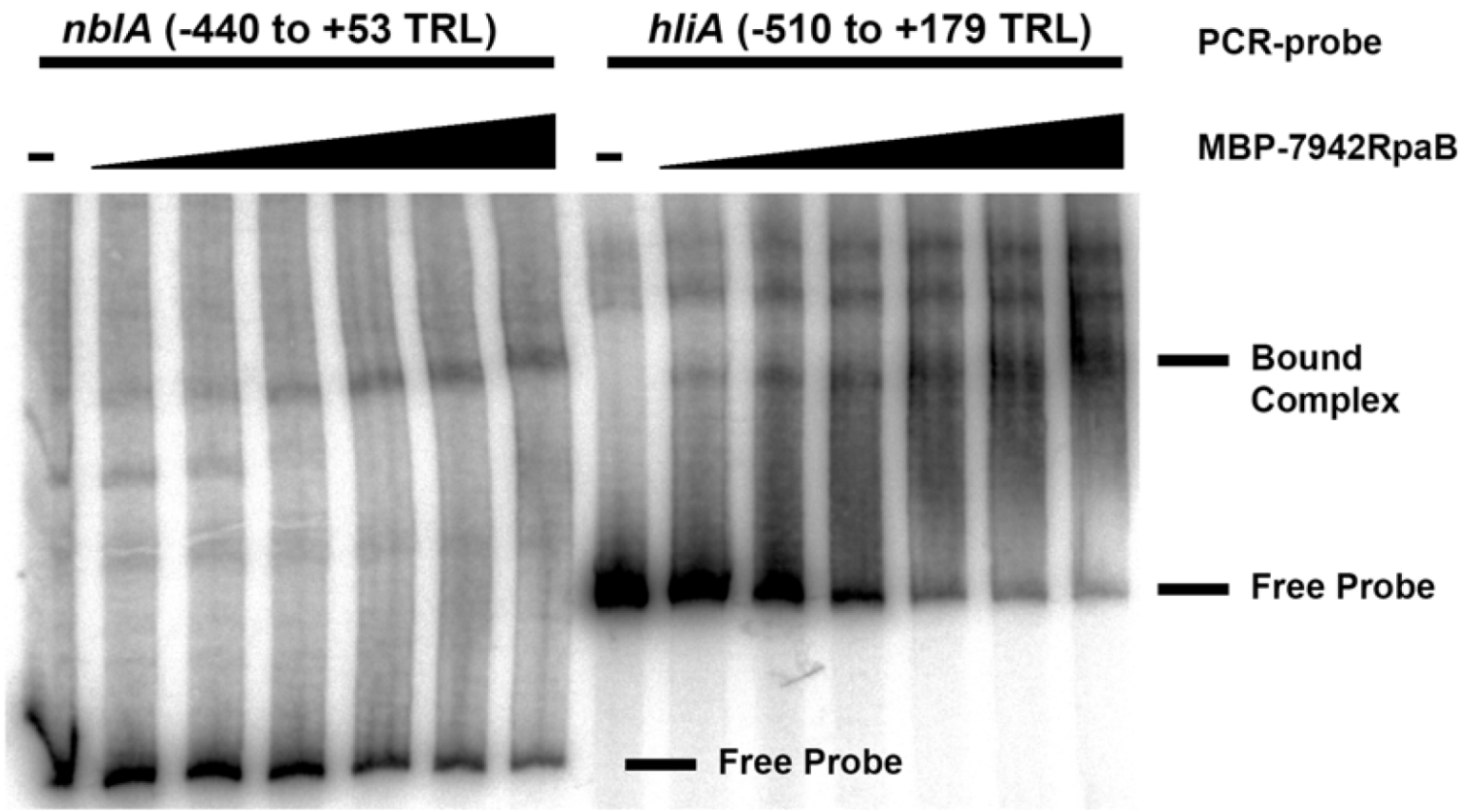

Figure 4.3 Electrophoretic mobility shift assay shows that RpaB binds upstream of *nblA* and *hliA* from *S. elongatus*. Assays were performed with increasing amounts of MBP-7942RpaB purified protein (indicated by the wedges) incubated with radiolabeled PCR fragments consisting of the region from -440 to +53 of *nblA* relative to the translational start site (TRL) or consisting of the region from -510 to +179 of *hliA* relative to the translational start site. The amounts of protein used in the binding reactions were: no protein (lanes 1 and 8), 28.9 nM (lanes 2 and 9), 57.8 nM (lanes 3 and 10), 115 nM (lanes 4 and 11), 231 nM (lanes 5 and 12) , 289 nM (lanes 6 and 13), and 404 nM (lanes 7 and 14). The position of the unbound or "free" probe (migration of the probe when no protein extract is added) is indicated, as is the position of the complexes that are unique in lanes containing MBP-7942RpaB.

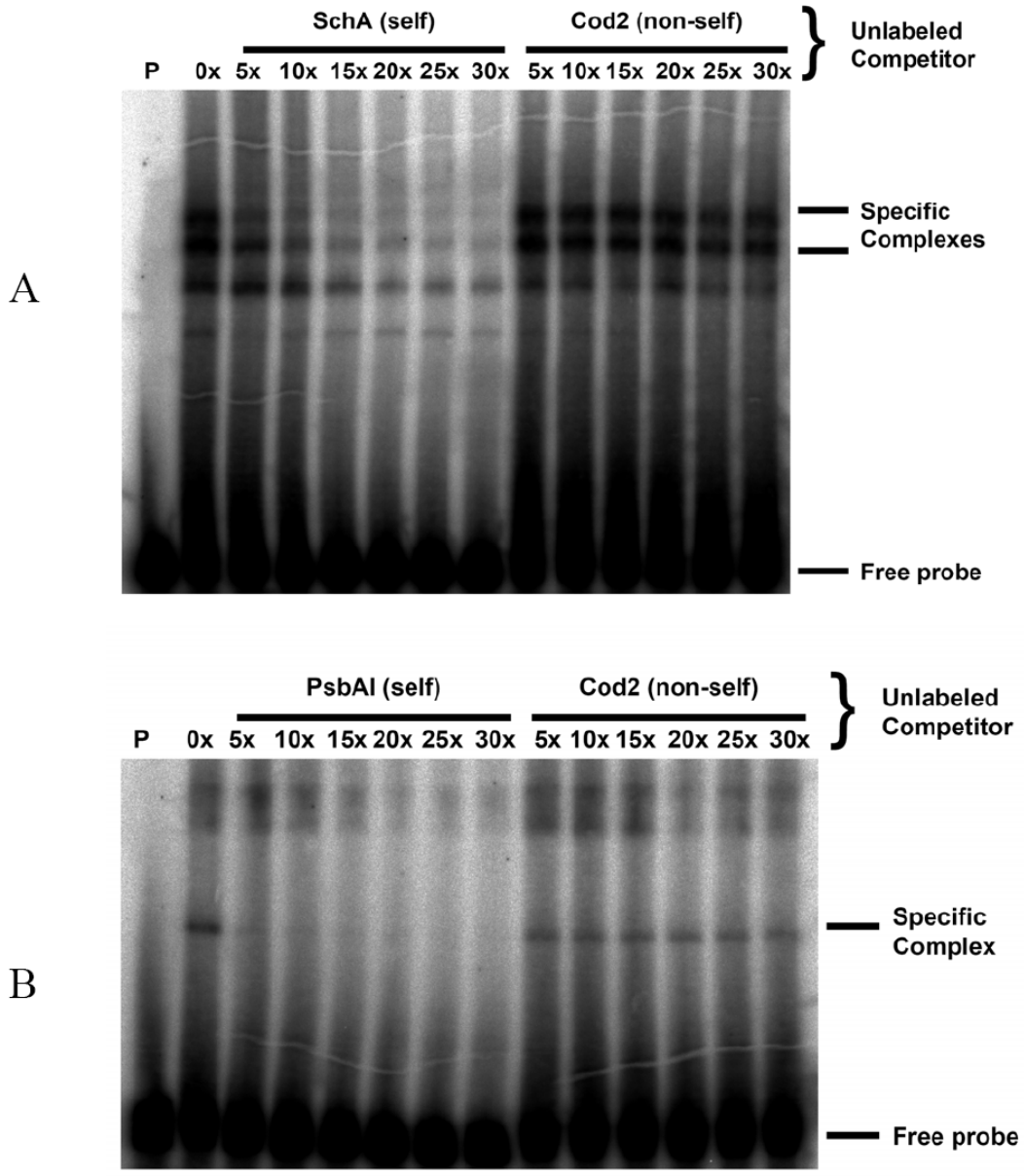

Figure 4.4 Electrophoretic mobility shift assay shows that RpaB specifically binds to the *hliA* HLR1-conatining region (probe SchA)(A) and the *psbAI* HLR1-containing region (probe PsbAI) (B). Competitive assays were performed with MBP-tagged, purified MBP-7942RpaB (151 nM) in the presence or absence of either an unlabeled self (SchA(A) or PsbAI(B)) competitor fragment or an unlabeled non-specific competitor fragment from the coding region of the *hliB* from *Synechocystis* (Cod2), at the indicated fold excesses of the probe concentration. The position of the unbound or "free" probe (migration of the probe when no protein extract is added) is indicated, as is the position of the complexes that are specific for the SchA(A) or PsbAI(B) fragment versus a non-specific competitor (Cod2).

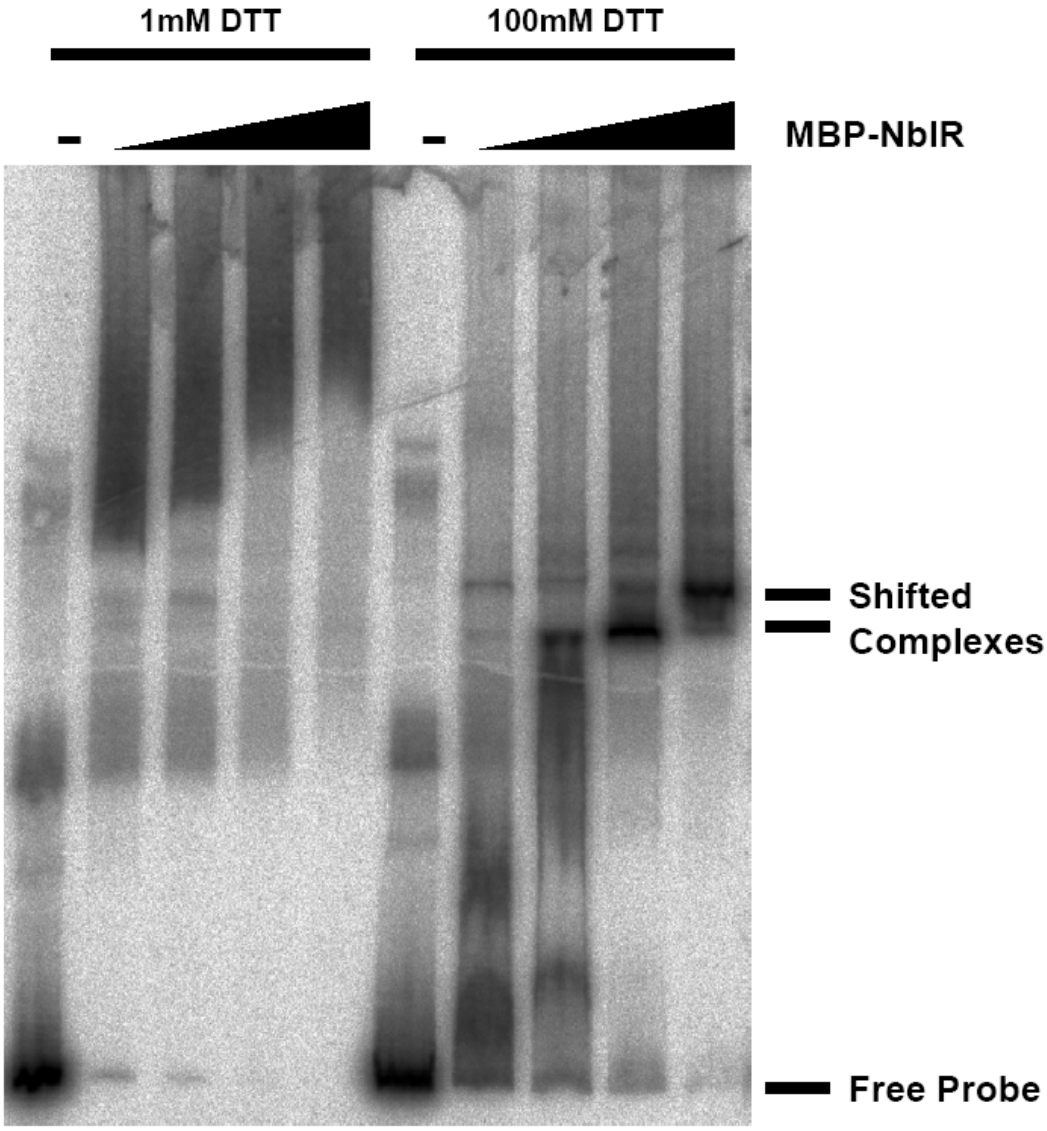

Figure 4.5 Electrophoretic mobility shift assay shows that NblR binds upstream of *nblA* from *S. elongatus* and does so in a redox dependent manner. Assays were performed with increasing amounts of MBP-NblR purified protein (indicated by the wedges) incubated with radiolabeled PCR fragment consisting of the region from -440 to +53 of *nblA* relative to the translational start site. The amounts of protein used in the binding reactions were: no protein (lanes 1 and 6), 0.439 μM (lanes 2 and 7), 0.908 μM (lanes 3 and 8), 1.815 μM (lanes 4 and 9), 3.630 μM (lanes 5 and 10). Lanes 1 thru 5 contain 1 mM DTT and lanes 6 thru 10 contain 100 mM DTT. The position of the unbound or "free" probe (migration of the probe when no protein extract is added) is indicated, as is the position of the complexes that have formed smears and complexes that indicate specific binding of MBP-NblR to the PCR probe.

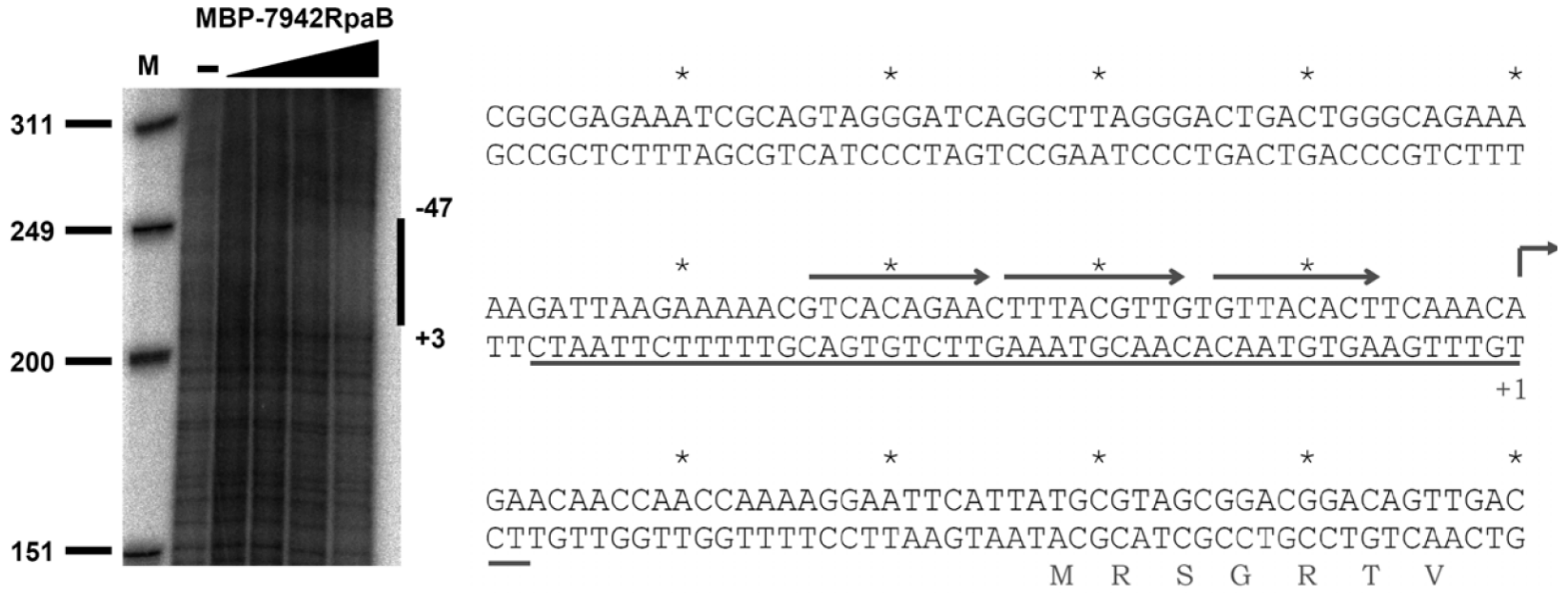

Figure 4.6 DNase I footprinting assays of the *hliA* upstream region bound by RpaB. MBP-tagged RpaB was added at increasing concentrations (indicated by the wedge) to DNase I footprinting reaction mixtures containing the radiolabeled *hliA* upstream fragment consisting of the region from -510 to +179 relative to the translational start site. The bold line indicates the location of the RpaB protected region with the positions (relative to the transcriptional start site) marked. Lane 1, radiolabeled marker (M) with band sizes indicated (in bp); lane 2, no protein; lanes 3-6, increasing amounts of MBP-7942RpaB protein included in the binding reaction with the *hliA* upstream region probe before DNase I digestion: 28.9 nM , 57.8 nM, 115 nM, 404 nM. Also shown is the sequence of the promoter region of *hliA* with the transcriptional start site marked (+1, bent arrow), the region protected by MBP-7942RpaB from the DNase I footprinting assay (solid line under sequence) and the HLR1 sites (arrows indicating half sites, above sequence). The top strand of the sequence is 5' to 3' and marked by a star every 10 bp.

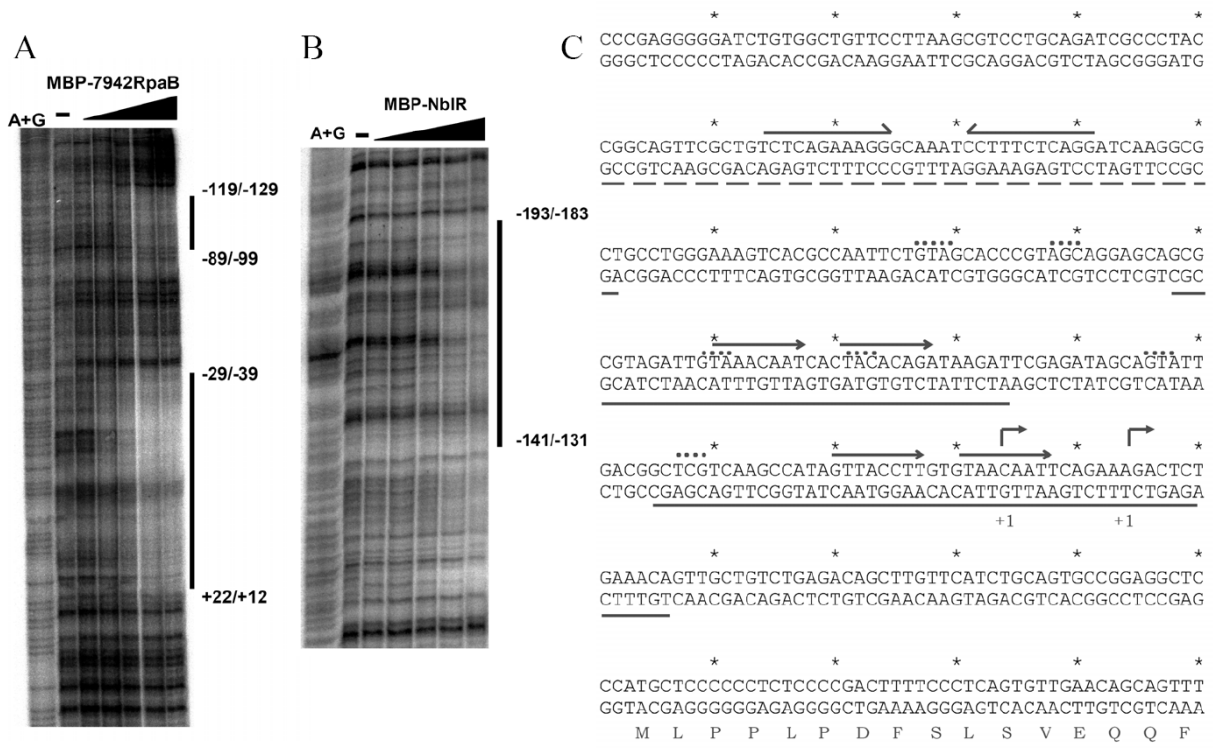

Figure 4.7 DNase I footprinting assays of the *nblA* upstream region bound by RpaB and NblR. (A) MBP-tagged RpaB was added at increasing concentrations (indicated by the wedge) to DNase I footprinting reaction mixtures containing the radiolabeled *nblA* upstream fragment consisting of the region from -440 to +53 relative to the translational start site. The bold lines indicate the location of the RpaB protected region with the positions (relative to the transcriptional start sites) marked. Lane 1, A+G sequence by Maxim-Gilbert chemical based sequencing of the *nblA* fragment as a marker; lane 2, no protein; lanes 3-7, increasing amounts of MBP-7942RpaB protein included in the binding reaction with the *nblA* upstream region probe before DNase digestion: 144 nM , 289 nM, 578 nM, 1.156 μM and 2.312 μM. (B) MBP-tagged NblR was added at increasing concentrations (indicated by the wedge) to DNase I footprinting reaction mixtures containing the radiolabeled *nblA* upstream fragment consisting of the region from -440 to +53 relative to the translational start site. The bold lines indicate the location of the NblR protected region with the positions (relative to the transcriptional start sites) marked. Lane 1, A+G sequence by Maxim-Gilbert chemical based sequencing of the *nblA* fragment; lane 2, no protein; lanes 3-7, increasing amounts of MBP-NblR protein included in the binding reaction with the *nblA* upstream region probe before DNase digestion: 366 nM , 732 nM, 1.098 μM, 1.464 μM and 2.928 μM. (C) Shown is the sequence of the promoter region of *nblA* with the transcriptional start sites marked (+1, bent arrow), the region protected by MBP-7942RpaB binding during DNase I footprinting (solid line under sequence), the region protected by MBP-NblR binding during DNase I footprinting (dashed line under sequence), an inverted repeat that may be the binding site of NblR (half arrows indicating the half sites, above sequence) and the HLR1 sites (arrows indicating half sites, above sequence). The three putative NtcA binding sites as in Luque *et al.* (2001) are also indicated (dotted lines, above sequence). The top strand of the sequence is 5' to 3' and marked by a star every 10 bp.

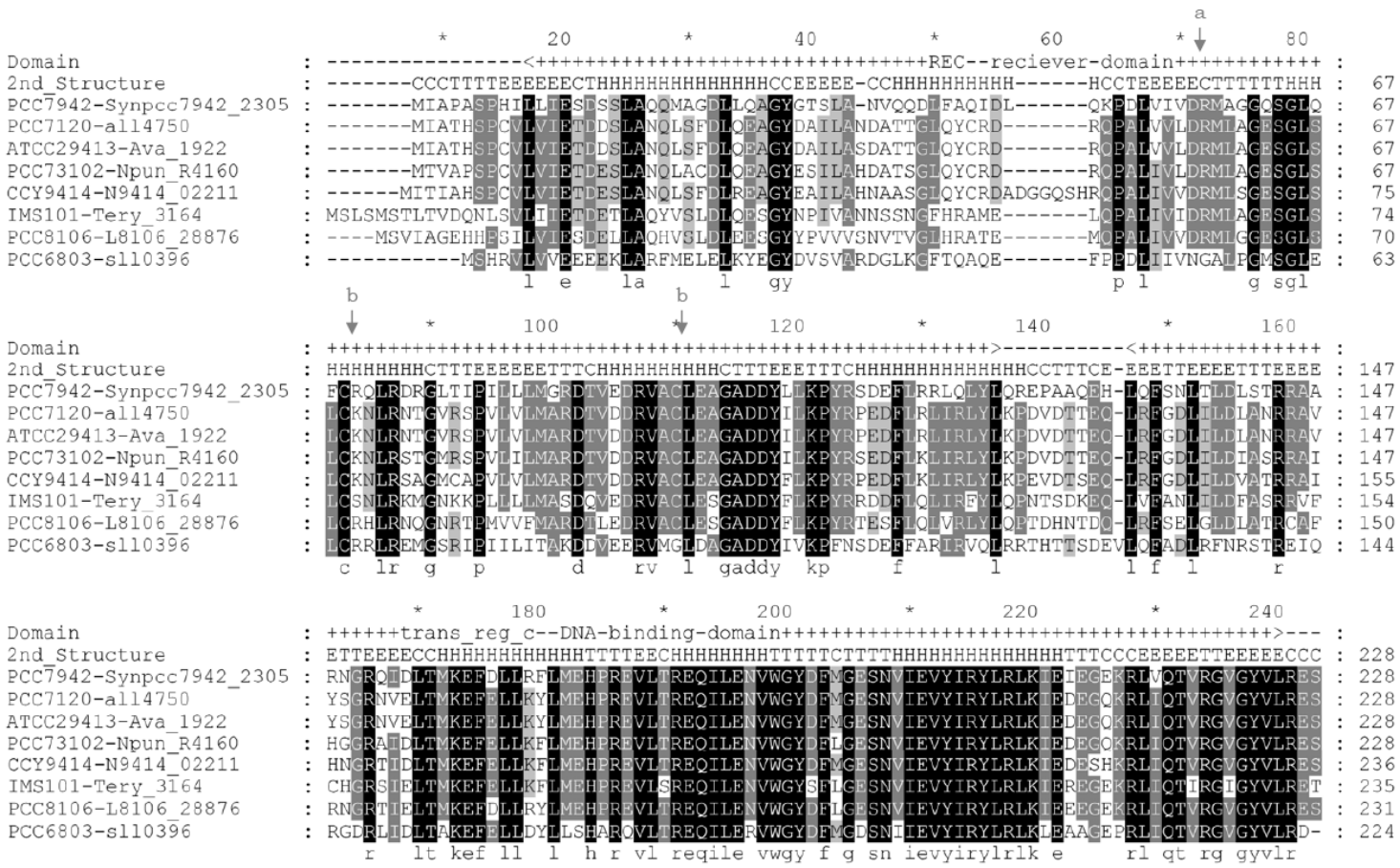

Figure 4.8 Alignment of NblR and homologs in cyanobacteria showing two conserved and potentially redox-active cysteine residues. The alignment was created using the program DAMBE [cite] to run a multiple alignment under default conditions and visualized using GeneDoc [cite]. The conserved Asp residue assumed to be the phosphorylation site (Kato *et al.* 2008) is shown (arrow, a) and the conserved Cys residue is shows (arrow, b) The homologs shown are from Ashby and Houmard (2006), except for the *Synechocystis* NblR-like protein which is from Zabulon *et al*. (2007). Listed are strain names followed by locus tag. Also shown is the domain (Domain) (Marchler-Bauer *et al.* 2007) and secondary structure predictions (2nd_Structure) (Karplus *et al.* 2003) of NblR based on *Synechococcus elongatus* PCC7942 (PCC7942). Shown are sequences from *Nostoc* sp. PCC 7120 (PCC7120), *Nostoc punctiforme* PCC 73102 (PCC73102), *Anabaena variabilis* ATCC 29413 (ATCC29413), *Nodularia spumigena* CCY9414 (CCY9414), *Lyngbya* sp. PCC 8106 (PCC8106), *Trichodesmium erythraeum* IMS101 (IMS101), and *Synechocystis* sp. PCC 6803 (PCC6803).

CHAPTER 5

FINAL CONCLUSIONS

5.1 Final conclusions and summary

The work presented within this dissertation provides considerable additional insight into the regulation of genes controlled by light and nutrient levels in cyanobacteria by the probable two-component signal transduction system, NblS-RpaB, and the putative one component regulator, NblR.

The histidine sensor kinase NblS and homologs are known to regulate the high light-inducible genes and other genes controlled by light intensity, nutrient limitation, redox stress, and a number of other stresses within cyanobacteria (Hsiao *et al.* 2004; Kanesaki *et al.* 2007; Suzuki *et al.* 2001; Tu *et al.* 2004). A number of these stresses limit photosynthetic carbon metabolism (Long *et al.* 1994) causing overreduction in pools of the terminal electron acceptor of photosynthesis, NADP, causing overexcitation of the photosynthetic apparatus similar to that of high light (Aro *et al.* 1993; Noguchi 2002; Nishiyama *et al.* 2004). This suggests that NblS is able to detect the photosynthetic redox state of the photosystems directly or indirectly, possibly through the detection of the redox state of a photosynthetic electron carrier, such as NADP (Salem and van Waasbergen 2004b). Previous works suggested that NblS regulated a number of genes including *hliA*, *nblA*, and *psbAI* genes of *Synechococcus elongatus* PCC 7942 (van Waasbergen *et al.* 2002). If we consider the expression of these genes in the point mutant *nblS-1*, which we now know is a gain-of-function mutant (with *hliA* expression decreased) and the results of the partial deletion mutant *nblSΩ* (with *hliA* expression increased), perhaps the simplest explanation would be that the NblS sensor kinase's cognate response regulator would then directly repress the expression of *hliA*. We explored the upstream region of the high light-inducible gene, *hliA,* from *Synechococcus elongatus* PCC 7942 for *cis*-acting elements involved

in its regulation. We analyzed the transcription/translation of *hliA* as monitored by GUS activity in a strain bearing a plasmid with the *hliA* promoter and upstream region translationally fused to a GUS reporter gene. When alterations were made to the region surrounding the -27 upstream of the transcriptional start site, *hliA* expression was constitutively elevated, suggesting negative control by the binding of a repressor protein at this site. We identified direct repeats within the DNA sequence around this region that we also identified upstream of a number of genes controlled by NblS (and homologs), including the *hliA*, *nblA*, and *psbAI* genes of *Synechococcus elongatus* PCC 7942 and the *hliB* gene of *Synechocystis* PCC 6803. This turned out to be an extended version of a previously discovered DNA element known as the HLR1, with a consensus of two direct repeats of (G/T)TTACA(T/A)(T/A) separated by two nucleotides. We then showed that a protein element within crude protein extracts of high light- and low light-grown *S. elongatus* had specific binding for a DNA fragment containing the HLR1 element. This protein component was more abundant, or had greater affinity for the fragment, in the low light extracts than the high light grown extracts. This also suggested a repressor protein with greater affinity for the HLR1 element in low light than high light. A similar analysis using crude protein extracts of low light- and high light-grown *Synechocystis* cells with a DNA fragment containing the putative HLR1 elements upstream of the high light-inducible *hliB* gene also showed specific binding of a protein to the HLR1-containing element which had greater affinity or was more abundant in low light-grown extract than high light-grown extracts. This suggested a conserved mechanism for the factor among cyanobacteria. Since, HLR1-sequences occur upstream of many genes controlled by the NblS sensor kinase, we then proceeded to identify the factor that bound the HLR1-containing region, keeping in mind that the factor may be the cognate response regulator to NblS. Using suggestions from the literature as to which response regulator may be that to NblS, we found in *Synechocystis* that RpaB binds the upstream region of *hliB* at the HLR1 element overlapping the promoter. Similarly we have shown in *S. elongatus* that RpaB binds upstream of *hliA* and *nblA* at the HLR1 element overlapping the promoter

region, and as in the case of *hliB*, suggests that it excludes RNA polymerase from binding the promoter to initiate transcription. In the case of *hliA* and possibly *hliB*, RpaB appears to be the primary (possibly sole) regulation as demonstrated by previous work that identified the HLR1. The light controlled *psbAI* gene from *S. elongatus,* which shows increased expression in low light conditions and decreased transcript accumulation at high light (Bustos *et al.* 1990), showed specific binding of RpaB of the HLR1-containing element upstream of the promoter, suggesting RpaB may activate the expression of *psbAI* by interacting and stabilizing RNA polymerase binding. The nutrient stress-responsive gene, *nblA,* can now be seen to be regulated through three cis-acting regulators, NtcA (Luque *et al.* 2001; Sauer *et al.* 1999), NblR (Luque *et al.* 2001; Schwarz and Grossman 1998), and now RpaB. While RpaB appears to be involved in repression of *nblA* expression, NblR and NtcA are activators (positive acting regulators) of *nblA* expression. The activity of the *nblS-1* mutant acting through RpaB would suggest that RpaB repression of *nblA* expression has to be lifted before *nblA* expression, activated by NtcA or NblR. We have also shown *in vitro* that NblR has to be in a highly reducing environment to bind specifically to the RNB1 upstream of *nblA*. Under nitrogen and other nutrient limiting conditions it is possible for the cellular redox state to become reduced (Alfonso *et al.* 2001; Reyes and Florencio 1995), which may cause NblR to bind. However, further exploration into the redox regulation of NblR is needed because the conserved thiol groups may be reactive to several different physiological signals based on diverse thiol modifications that are possible (Kim *et al.* 2002). Moreover, even though phosphorylation of the conserved Asp residue is not necessary for activity of NblR (Kato *et al.* 2008), regulation of NblR by phosphorylation by a sensor kinase cannot be ruled out. Since *nblA* appears to be under positive control through the response regulator NblR binding to the upstream region, but under negative control through NblS, and *hliA* is regulated by NblS and RpaB, but not NblR, NblS and NblR are not likely to form a direct cognate response regulatory pair. We hypothesize that NblS and RpaB do form a cognate two-component regulatory pair.

5.2 The Model of NblS-Control of Gene Expression in *Synechococcus elongatus* PCC 7942 and Avenues of Future Research

Figure 5.1 shows our current model of the regulation of gene expression mediated by NblS in the cyanobacterium, *Synechococcus elongatus* PCC 7942. The regulation of *hli* genes and *nblA* in other cyanobacteria (including those without NblR) the NblS-RpaB putative two-component system would presumably be similar to that shown for *hliA*. The regulation of *nblA* by NblR within this model may also take place within other cyanobacteria that contain the response regulator NblR. In this model, in conditions where the amount of light energy absorbed is balanced with the amount of energy utilized, such as in nutrient replete and normal light conditions (Figure 5.1A), NblS presumably detects the lack of photosynthetic redox stress, directly or indirectly, and actively phosphorylates its putative cognate response regulator, RpaB. However, it should be noted that direct phospho-transfer from NblS to RpaB has yet to be shown experimentally. The phosphorylation of the receiver domain of RpaB causes a conformational change that activates its DNA-binding domain. Phosphorylation of the receiver domain has been traditionally shown to cause a decrease in negative protein-protein interactions between the receiver domain and the DNA-binding domain that suppresses DNA-binding activity (West and Stock 2001), however, the effect of phosphorylation of RpaB on DNA-binding activity is as of yet unknown. Once methods are developed to phosphorylate RpaB *in vitro* by phospho-transfer using a promiscuous histidine kinase or NblS, the affect of phosphorylation and binding efficiency can be determined. Presumably then, phosphorylated RpaB actively binds the HLR1 elements upstream of a number of genes. Depending on the interaction with the promoter region, other regulator factors, and RNA polymerase, RpaB represses the expression of genes including *hliA* and *nblA*, while activating others such as *psbAI*. In future, it would be interesting to identify other binding sites for RpaB by either ChIP-on-chip or Dip-chip methods and comparing them with expression data. In our model, upon an increase in light intensity (high light), perhaps through an increase in blue/UV-A, or during nutrient deprivation (or other photosynthetic redox stresses) (Figure 5.1B), NblS presumably

detects photosynthetic redox stress on the cell, and causes a conformational change that inactivates the kinase domain. RpaB is no longer phosphorylated by NblS and the binding affinity for the HLR1 sequences is decreased. The decrease in binding of RpaB to the HLR1 element causes activation of genes repressed primarily by RpaB such as *hliA,* while decreasing the activation of others, leading to adjustments in the cell that aid in high light adaptation. In the case of *nblA* during nutrient stress, repression is lifted with the decrease binding of RpaB to the HLR1 element; however, this does not seem to be enough to activate gene expression. In our model during nutrient deprivation the cytoplasm of the cell becomes highly reduced, causing modification of one or both conserved thiol groups of the NblR receiver domain, which causes conformational changes that activate the response domain. NblR then actively binds the RNB1 sites upstream of *nblA* and (along with NtcA in the case of nitrogen deprivation) then activates gene expression, presumably by a positive interaction with RNA polymerase triggering phycobilisome degradation. The thiol states that activate NblR are not known; through controlled modification of the thiols of NblR one may experimentally show different affinity of binding. NblR has only been shown to regulate *nblA* in *S. elongatus*, and identification of other NblR-binding sites upstream of other genes would lead to a better consensus of the RNB1 binding site as well as the nature of NblR binding.

It is still not known how NblS detects changes within the photosynthetic redox state of the cell. In the *nblS-1* mutant, one of the two point mutations changed a conserved amino acid in the PAS domain, suggesting that the mutant's phenotype was due to this change in the PAS domain. It would be interesting to identify other changes of the amino acid sequence within the PAS domain that may affect regulation by NblS and identify the possible ligand (if any) that binds the PAS domain. Within the sensory domain the presence of a conserved domain between the two transmembrane domains and a conserved HAMP domain also suggest that the molecule may detect a signal outside the cell or within the thylakoid lumen. In this regard, it

would be interesting to localize NblS to the thylakoid or cytoplasmic membranes as well as to disrupt these parts of the sensory domain to identify functionality.

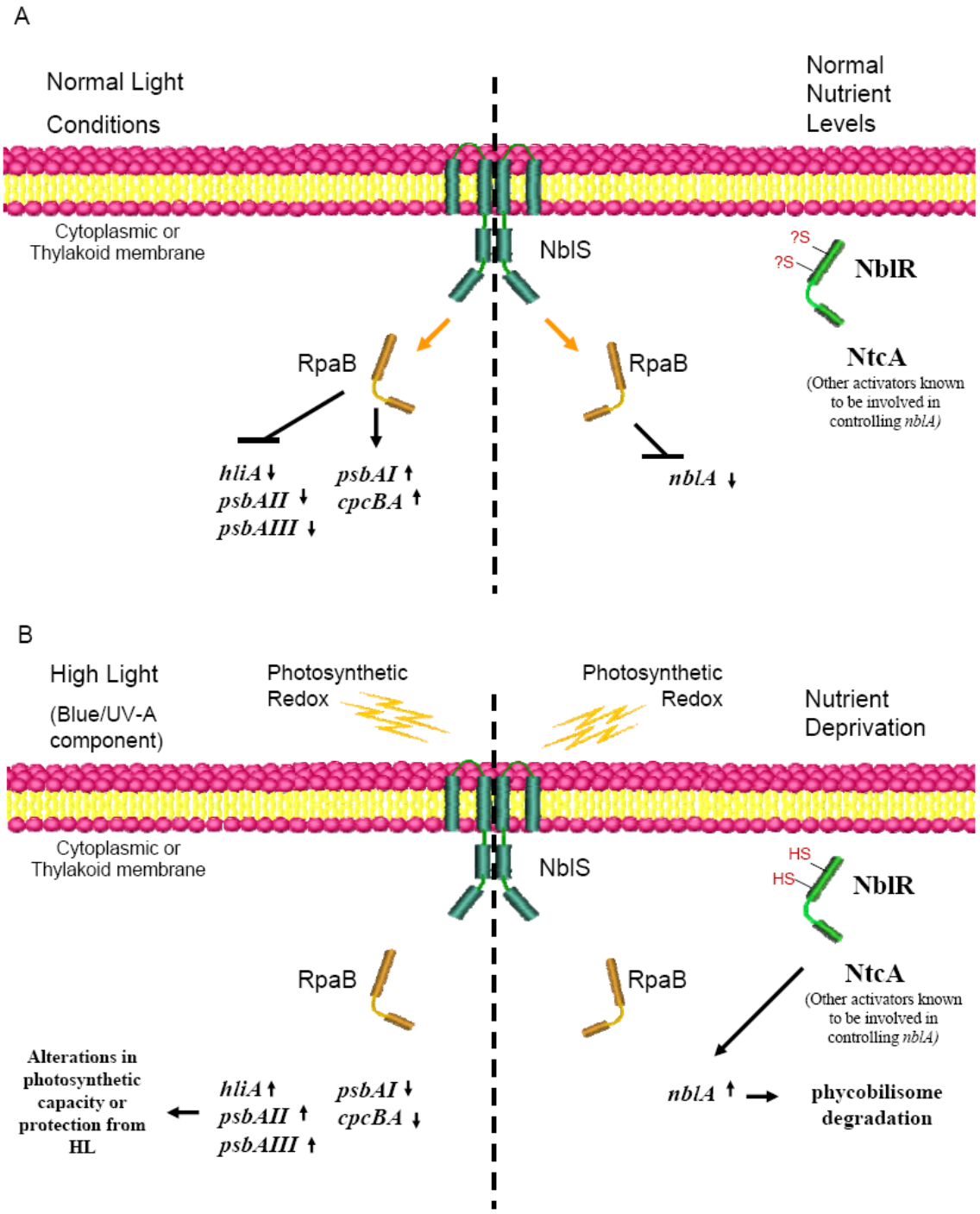

Figure 5.1 Regulation through NblS in *Synechococcus elongatus* PCC 7942 under normal, non-photosynthetic redox stress conditions (A) and during photosynthetic redox stress (B). (Refer to the text for details.)

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BIOGRAPHICAL INFORMATION

Anthony Kappell graduated from the University of Texas at Arlington (UTA) with a Bachelors of Science in Microbiology and Biology in 2003. He proceeded to enter the graduate program in the biology department of UTA were he performed the research contained in this dissertation. During that time he taught undergraduate students in the microbiology laboratory and performed maintenance on equipment with in the Genomic Core Laboratory. Anthony also met his wife, Guimel Molina, while she was working toward her Masters in Biology. He received his Ph. D. in Quantitative Biology in August of 2008.

Anthony Kappell's interest in research is in the regulation and mechanisms of photoadaptation, photoprotection, photoinhibition, and photoactivation of the photosynthetic apparatus and the integration of signals and overlapping of regulation. He wishes to continue to focus on this research in cyanobacteria.

Anthony's career goals include remaining in academia as a professor at a research institution. He is looking for a postdoctoral position and hopes to expand his knowledge of integration and regulation of carbon metabolism and photosynthesis, and develop new research skills that will allow him to develop his own research program. His abilities to write research grants and conduct a research project will certainly be honed by his interactions with scientists at his postdoc. He hopes that the experiences that he will gain will allow him to become a successful molecular cyanobacteriologist, scientist, academic instructor, researcher, and mentor.