

# Negative control of the high light-inducible *hliA* gene and implications for the activities of the NblS sensor kinase in the cyanobacterium *Synechococcus elongatus* strain PCC 7942

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Received: 27 April 2006 / Revised: 7 July 2006 / Accepted: 14 July 2006 / Published online: 9 August 2006  
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**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.

**Keywords** Cyanobacteria · High intensity light · *hliA* gene · HLR1 *cis* element · NblS sensor kinase

## Abbreviations

GUS  $\beta$ -glucuronidase  
HL High light  
HLR1 High light regulatory 1 sequence  
LL Low light

## 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; Noguchi 2002; Nishiyama et al. 2004). Therefore, photosynthetic cells have evolved various acclimation mechanisms to deal with excess light exposure, many of which necessitate alterations in gene expression (Demmig-Adams 1990; Demmig-Adams and Adams 1992; Chow 1994; 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

**Electronic Supplementary Material** Supplementary material is available to authorised users in the online version of this article at <http://dx.doi.org/10.1007/s00203-006-0154-0>.

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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; Pfannschmidt 2003; Chen et al. 2004), 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; He et al. 2001; Havaux et al. 2003; 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 (Funk and Vermaas 1999; Bhaya et al. 2002) 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; Suzuki et al. 2001; Huang et al. 2002; Kanesaki et al. 2002; Mikami et al. 2002; Li et al. 2004). 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<sub>6</sub>f* (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 (Suzuki et al. 2001; Mikami et al. 2002; Marin et al. 2003; Hsiao et al. 2004; 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.

## Materials and methods

### 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 ( $50 \mu\text{mol}$  of photons  $\text{m}^{-2} \text{s}^{-1}$ ), and the cultures ( $50 \text{ ml}$  in glass culture tubes ( $25 \text{ mm}$  diameter)) were bubbled with  $3\%$   $\text{CO}_2$  in air during growth and light treatments. Cultures larger than  $50 \text{ ml}$  were grown and low light (LL) adapted in glass flasks placed on an orbital shaker and bubbled with  $3\%$   $\text{CO}_2$  in air. HL ( $800 \mu\text{mol}$  of photons  $\text{m}^{-2} \text{s}^{-1}$ ) was supplied from incandescent white-light bulbs. UV-A light ( $350\text{--}400 \text{ nm}$  with a peak at  $366 \text{ nm}$ ) was supplied from black-light blue bulbs at  $27 \mu\text{mol}$  of photons  $\text{m}^{-2} \text{s}^{-1}$ . 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 ( $10 \mu\text{mol}$  of photons  $\text{m}^{-2} \text{s}^{-1}$ ) for 18 h before HL or UV-A light treatment (as  $50 \text{ ml}$  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  $\beta$ -glucuronidase (GUS) reporter gene. *Escherichia coli* bearing pHLIP-GUS constructs were grown in LB medium supplemented with  $50 \mu\text{g/ml}$  ampicillin, and *S. elongatus* bearing these plasmids were grown in BG-11 medium supplemented with  $2 \mu\text{g/ml}$  ampicillin or carbenicillin.

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

Unless stated otherwise, all molecular genetic techniques were performed according to standard protocols (Sambrook et al. 1989; Ausubel et al. 2002) or as previously reported (van Waasbergen et al. 2002). Nested deletions were performed on the plasmid pHLIP-GUS (following digestion with *SalI* and *SphI*) 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 *StuI* site, digesting the product with *SalI* and *StuI*, filling in of the *SalI* 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 ( $5'$ -CTATTCTCAATCTC-

CTGT) 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).

#### Formation of an NblS-disrupted strain

The *nblS* gene cloned in the plasmid pUC119 was interrupted by insertion of a spectinomycin ( $\Omega$ ) resistance gene (Elhai and Wolk 1988) at the *EcoNI* 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  $25 \mu\text{g/ml}$ ). Despite repeated transfer of strains in low light on media containing high levels ( $250 \mu\text{g/ml}$ ) of spectinomycin, PCR analysis of genomic DNA isolated from transformants always indicated the presence of both wild-type and interrupted copies of *nblS*.

#### RNA isolation, RNA blot hybridizations, 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  $4^\circ\text{C}$ . Cell pellets were stored at  $-80^\circ\text{C}$ . 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 *NcoI*-digested pTHL with SP6 RNA polymerase and the Strip-EZ RNA probe synthesis kit (Ambion) with  $[\alpha\text{-}^{32}\text{P}]\text{UTP}$  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 *NotI* fragment of pTRP and labeled by using the Strip-EZ DNA probe synthesis

kit (Ambion) with [ $\alpha$ - $^{32}$ 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'-CTGGTTCGATGGCAAAGTTGTTGAGACGGT; for *hliB*,

5'-GGGTTCAATGGCGAAGTTGTTGAGACGGT; for *hliC*,

5'-TTCGGCGAAAGCAGTGAATCCAAAT; for *hliD*,

5'-AAATTTGGGATCTTCCTGCACGGGGTTGGT.

#### 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  $\mu$ M 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'-AAAGATTAAGAAAAACGTCACAGAACTTTACGTTGTGTTACTTCAAACA;

Del2 (the sequence from pHG-del analogous to that in SchA on pHLIP-GUS),

5'-GAGCTTGCATGCCTGCAGGTCGACCTTACGTTGTGTTACTTCAAACA;

Cod2 (*Synechocystis* PCC 6803 *hliB* from within the coding region +17 to +67 relative to the initial ATG),

5'-TTCGCCTCGACCAAGACAACCGTCTCAACAACCTCGCCATTGAACCCCTG;

PsbAI (*S. elongatus psbAI* from positions -115 to -54 relative to the transcriptional start site),

5'-GATCGCTCTAAACATTACATAAATTCACAAAGTTTTTCGTTACATAAAAATAGTGTCTACTTA; 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'-CTTCCTGTTACAAAGCTTTACAAAACCTCATTAATCCTT.

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. (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  $\mu$ l 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  $\times$  g, 20 min). Proteins were precipitated from the supernatants at 80% ammonium sulfate saturation. Protein precipitates were pelleted by centrifugation (15,000  $\times$  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 [ $\gamma$ - $^{32}$ P]ATP per the manufacturers instructions, and labeled products were purified using a QIAquick Nucleotide Removal Kit (Qiagen). Partially purified protein extract (20  $\mu$ g) was incubated at room temperature for 20 min with 50 fmol of labeled DNA fragment in binding buffer (1 mM MgCl<sub>2</sub>, 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  $\mu$ l. 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× TBE, 2.5% glycerol). Electrophoresis was performed at 11°C in 0.5× 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).

### Bioinformatic analyses

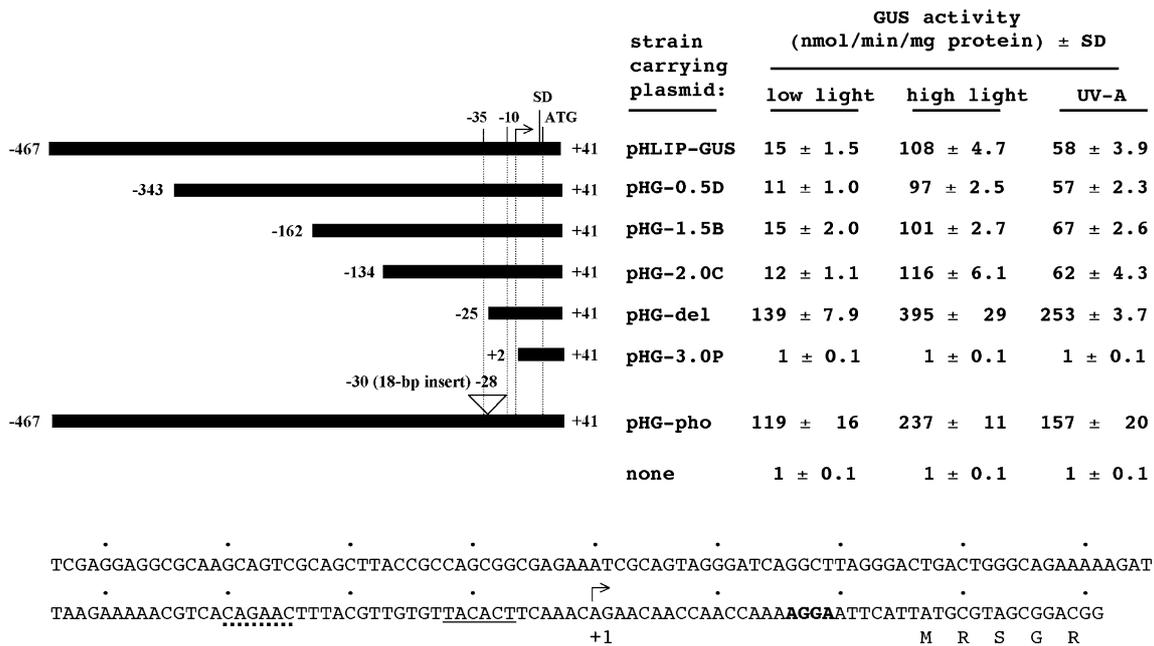
The motif-finding program ScanACE (Roth et al. 1998; Hughes et al. 2000) was used to scan genome sequences for sites within intergenic regions that were close matches to a given DNA motif (in the form of a weight matrix generated from a given set of input sites). A site thus discovered was then determined 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).

### 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* (Fig. 1). 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 11-fold increase in GUS activity in LL and an approximate fourfold 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 ninefold 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 (Fig. 1) 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 (Fig. 2). 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.



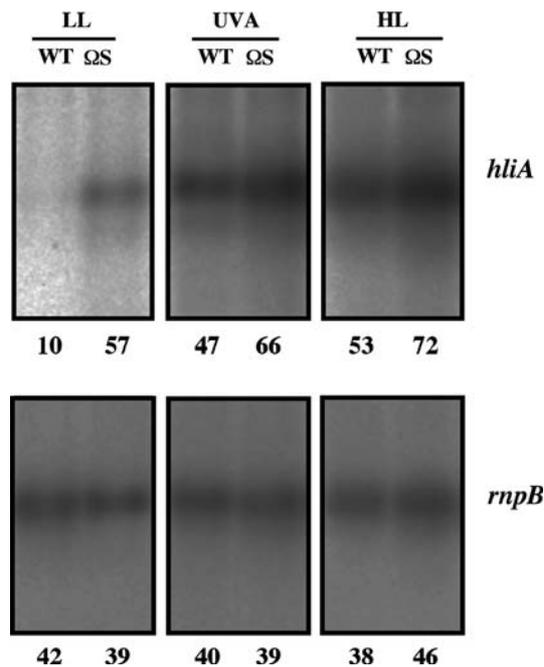
**Fig. 1**  $\beta$ -glucuronidase activities from *hliA*::GUS fusions under various light conditions. GUS assays were performed on *S. elongatus* carrying plasmids that bear various *hliA* promoter region fragments fused translationally to the GUS reporter gene. These promoter region fragments are diagrammed 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 3 h. Shown at the bottom of the figure is the sequence of 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 [data from (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

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 *hli2* and *hli3* genes) and homologous genes in *Synechocystis* PCC 6803 (Fig. 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 *hli2* and *hli3*); 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

(Fig. 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<sub>4</sub>-TTACAA, which they termed high light regulatory 1 (HLR1), 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 Fig. 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, red algal chloroplasts, and cyanophage) (Table S1 (supplementary material)). We also searched for the extended HLR1 motif upstream of all genes in the



**Fig. 2** RNA hybridization analysis of *hliA* transcripts following various light treatments in a wild-type or *nblS*-interrupted ( $\Delta S$ ) 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)

genome of *Synechocystis* PCC 6803. Of the 22 new matching sites discovered (i.e., sites other than those shown in Fig. 3 used to generate the input alignment), 12 (54%) are upstream of genes recognized through microarray analyses (Mikami et al. 2002; 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 S2 (supplementary material)], as are all those shown in Fig. 3 except *Synechocystis* PCC 6803 *nblA*. This further indicates the importance of the HLR1 in conveying NblS/DspA-mediated signals.

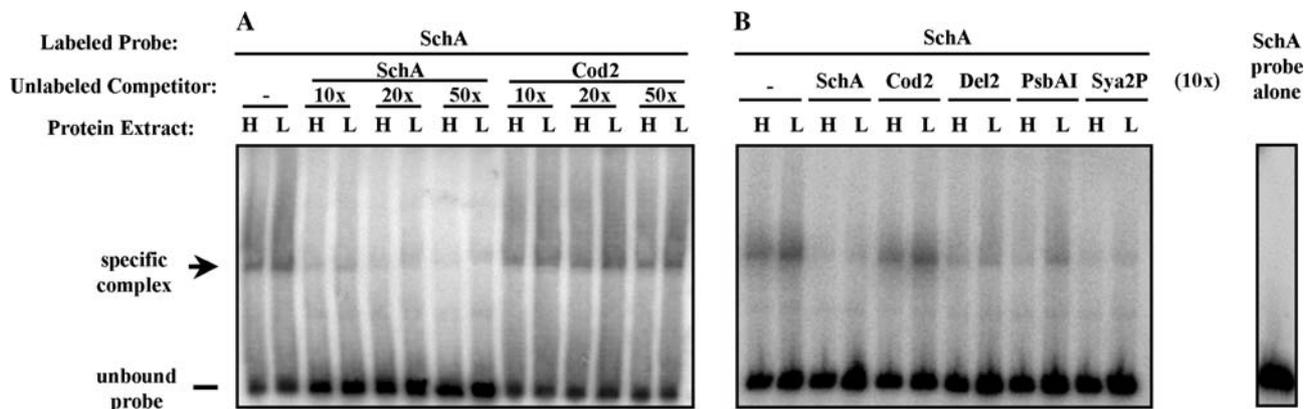
In order to explore the activity of DNA-binding proteins to the HLR1 of *S. elongatus hliA*, we performed gel electrophoresis mobility shift assays using a radiolabeled 51-bp DNA fragment from the upstream region surrounding the HLR1 (from  $-50$  to  $+1$ ; SchA) and partially purified protein extracts from LL adapted cells and LL-adapted cells that had been exposed to HL (30 min). The results (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 4b), underscoring the evolutionarily conserved nature of this element.

## 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; Fig. 1), 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 (Fig. 2). 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).





**Fig. 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). Electrophoretic 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 tenfold excess of the probe concentration). A control sample with the SchA radiolabeled 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*)

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 (Fig. 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 (Fig. 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 Fig. 3 (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 its 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 (Fig. 3 and Tables S1 and S2 (supplementary material)), 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.

**Acknowledgment** We thank Kavitha Salem for her help with RNA hybridizations and Kimberly Shahi for her help with GUS assays. This work was supported with funds to L.G.V. from The University of Texas at Arlington.

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