UV-A-induced expression of GroEL in the UV-A-resistant marine cyanobacterium Oscillatoria sp. NKBG 091600

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The authors have examined the response to UV-A irradiation of the UV-A-resistant marine cyanobacterium Oscillatoria sp. NKBG 091600, which produces the UV-A-absorbing compound biopterin glucoside. The expression of a 60 kDa protein was markedly induced at 500 min after UV-A irradiation. This protein was identified by N-terminal amino acid sequence analysis as GroEL. Northern blot analysis demonstrated that GroEL synthesis was controlled by UV-A at the transcriptional level. A CIRCE element and a putative SOS consensus sequence were found upstream of the groESL operon, overlapping two putative promoter sequences. Primer extension analysis revealed that groESL transcription in UV-A-induced cells starts from the proximal promoter overlapped by the SOS consensus sequence. This indicates that an SOS response regulation is instrumental in UV-A-induced GroEL expression of Oscillatoria sp. NKBG 091600. Furthermore, this UV-A-inducible GroEL may function to upregulate biopterin glucoside biosynthesis, thereby allowing growth under UV-A irradiation.

Keywords: stress response, UV-A resistance, biopterin glucoside, GroEL, marine cyanobacterium Oscillatoria sp.

INTRODUCTION

UV-A irradiation causes oxidative stress in several eukaryotes and prokaryotes (Bose et al., 1990; Cadet et al., 1993; Hu & Tappel, 1992; Parat et al., 1995; Savoure et al., 1996; Yamada et al., 1996). UV-absorbants have been found in several cyanobacteria (Matsunaga et al., 1993; Scherer et al., 1988; Shibata, 1969), and the biosynthesis of these pigments was found to be UV-responsive (Ehling-Schulz et al., 1997; Matsunaga et al., 1993; Scherer et al., 1988). We have investigated the physiological response of a UV-A-resistant marine cyanobacterium, Oscillatoria sp. NKBG 091600, to UV-A irradiation (Matsunaga et al., 1993). A UV-A-absorbing pigment was isolated and identified as biopterin glucoside, which is chemically related to pterin pigments of butterfly wings. UV-A was found to be a potent elicitor of biopterin glucoside biosynthesis. UV-A may cause significant damage to cyanobacteria by inhibiting energy acquisition by the photosynthetic reaction system (Hirosawa & Miyachi, 1982, 1983; Sinha et al., 1995). Wachi et al. (1995) investigated the effect of UV-A intensity on cell growth, biosynthesis of biopterin glucoside and photosynthetic activity in a UV-A-resistant Oscillatoria sp., and observed that the loss of photosynthetic activity following exposure to UV-A was attenuated by prior UV-A exposure. These observations support the hypothesis that biosynthesis of UV-absorbing pigments functions as an adaptive strategy for protection against the adverse effects of UV irradiation. To further elucidate the biochemical basis of cyanobacterial UV-A resistance, we have examined the effect of UV-A irradiation on protein synthesis in Oscillatoria sp. NKBG 091600.

METHODS

Cyanobacterial strains and culture conditions. The marine Oscillatoria sp. NKBG 091600 and the freshwater Synechococcus sp. PCC 7942 were used in this study. NKBG 091600 and PCC 7942 were cultured in BG-11 medium (Allen & Stanier, 1968), supplemented with and without 3% NaCl,"
respectively, under cool-white fluorescent light (500 \mu W cm\(^{-2}\)) at 28 °C. Initial cell density was 10\(^6\) cells ml\(^{-1}\). Growth was monitored by measuring OD\(_{750}\).

**UV-A treatment of cyanobacterial cells.** Cells grown to mid-log phase with 300 \mu W cm\(^{-2}\) of cool-white fluorescent light irradiation were harvested and then suspended in fresh medium (final cell density adjusted to OD\(_{750}\) 0.8). A disposable Petri dish (Iwaki; 8.5 mm diameter) containing 20 ml of the cell suspension was irradiated with a combination of cool-white fluorescent light (500 \mu W cm\(^{-2}\)) and UV-A light (900 \mu W cm\(^{-2}\)) from a UV-A lamp (Cosmo Bio; CSL-30A). Cells were harvested by centrifugation (3500 g for 10 min at room temperature) at 100 min intervals over the course of 15 h, frozen using liquid nitrogen, and stored at −80 °C until analysed.

**Preparation of the water-soluble fraction from cyanobacterial cells and SDS-PAGE analysis.** Pelleted cells stored at −80 °C were thawed quickly, and suspended in disruption buffer containing 25 \mu l glycerol, 1 % SDS, 0.1 % β-mercaptoethanol and 5 % 200 mM phenylmethanesulfonyl fluoride, followed by the addition of 300 \mu l buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7.5). The cells were ruptured by sonication for 15 min at 4 °C, then centrifuged (25000 g, 30 min, 4 °C).

The supernatant was denatured for 10 min at 100 °C in 40 \mu l 0.0625 M Tris/HCl, pH 6.8, 1.25 % SDS, 25 % glycerol and 1.25 % 2-mercaptoethanol. The denatured solution was centrifuged for 10 min and a sample of the supernatant (20 \mu l containing 100 ng protein) was analysed by SDS-PAGE on a 1-0 mm thick slab gel (10 % acrylamide separating gel, 4 % acrylamide stacking gel) using the buffer system of Laemmli (1970). The gel was stained with Coomassie brilliant blue. Protein concentration in the supernatant was determined by spectrophotometry at 285 nm (Scopes, 1974).

**Protein purification and N-terminal amino acid sequencing.** The target band in the stained gel was excised, and the protein was extracted by electrodialysis. The solution of the target protein was concentrated, and spotted on a PVDF membrane (Millipore; Immobilon-P). The N-terminal amino acid sequence was determined using a poly peptide sequencer (Shimadzu; PPSQ-10).

**DNA sequencing of groEL and Northern analysis.** Genomic DNA was extracted from cyanobacterial cells by standard procedures (Porter, 1988). Two primers for amplifying groEL from Oscillatoria sp. NKBG 091600 were designed based on conserved DNA regions in groEL sequences from Synechococcus sp. PCC 7942 and Synechocystis sp. PCC 6803 (ES1, GAGTTCCGGAAMCCRYACT; ELR1, TCTTGGATAYTG-GCCAGGATTOT). The PCR product was cloned into pCRII vector (Invitrogen), and sequenced using an automatic DNA sequencing machine (Shimadzu; DSQ-1000L). A computer software package, LASERGENE (DNASTAR), was used for DNA sequence analysis.

Northern analysis was conducted using total RNA extracted from harvested cells according to the method of Chomczynski (1987). Digoxigenin (DIG)-labelled DNA probe for Northern hybridization was prepared by PCR amplification of a part of the groEL gene of Oscillatoria sp. NKBG 091600. Hybridization was performed using a DIG DNA Detection Kit (Boehringer Mannheim).

**Primer extension analysis.** Potential transcriptional start sites were identified by primer extension with RNA isolated from UV-A-irradiated cells. DNA-free total RNA (10 \mu g) and FITC-labelled primer (AGGGATACGTCTGCCATAGGAATAC-TCTCC: 40 pmol) were dissolved in 40 \mu l buffer [10 mM Tris/HCl (pH 8.3), 50 mM KCl, 5 mM MgCl\(_2\), 1 mM each dNTP, 40 \mu M human placenta ribonuclease inhibitor (Takara)] containing 10 U AMV [avian myeloblastosis virus] reverse transcriptase (Life Sciences). Extension reaction was carried out at 55 °C for 1 h, then incubation at 99 °C for 10 min to inactivate reverse transcriptase. The synthesized cDNA was ethanol-precipitated, washed with 70 % ethanol and dissolved in 1 \mu l nuclease-free water. After adding 1 \mu l formamide loading dye (Thermo sequence fluorescent labelled primer cycle sequence kit; Amersham), the reaction mixture was heat-denatured at 95 °C for 5 min and analysed by the DSQ-1000L automatic DNA sequencing machine in parallel with a dyeoxynucleotide sequencing reaction primed with the same oligonucleotide.

**RESULTS**

**Identification of a UV-A-inducible protein in Oscillatoria sp. NKBG 091600**

Fig. 1 shows the SDS-PAGE pattern of water-soluble proteins extracted from the UV-A-resistant Oscillatoria sp. NKBG 091600 and the UV-A-sensitive Synechococcus sp. PCC 7942 (Hirosawa & Miyachi, 1983) after 24 h UV-A irradiation. Induction of a 60 kDa protein was observed in NKBG 091600. In contrast, induction of a corresponding SDS-PAGE band was not observed in the UV-A-sensitive PCC 7942.

Twenty-four N-terminal amino acid residues (without the first two amino acids) of the UV-A-responsive 60 kDa protein were determined. Homology searching using protein databases indicated that the 60 kDa UV-A-responsive protein sequence displayed similarity to cyanobacterial GroEL chaperonins (96 % identity to Synechococcus sp. PCC 6301 and PCC 7942, and 84 % to Synechocystis sp. PCC 6803). The 60 kDa protein cross-

**Fig. 1.** SDS-PAGE of water-soluble proteins extracted from Synechococcus sp. PCC 7942 (lanes 1 and 2) and Oscillatoria sp. NKBG 091600 (lanes 3 and 4) irradiated with UV-A. Lanes 1 and 3, cells were irradiated with a cool-white fluorescent light; lanes 2 and 4, cells were irradiated with a combination of cool-white fluorescent light and UV-A light for 15 h. M, size markers. The induced 60 kDa protein in lane 4 is indicated by an arrowhead.
UV-A-induced expression of GroEL in cyanobacteria

The sequence of upstream region

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The sequence of upstream region

<table>
<thead>
<tr>
<th>GroESL operon of Oscillatoria sp. NKBG 091600</th>
<th>GroESL operon of Synechococcus sp. PCC 7942</th>
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<tr>
<td>-35 GAGTTGGGA AACCAYACTG ATCAGTAAGT TGGGAGGCC TACCACAGTC GTTTAAATTA 60</td>
<td>-35 GCACTACAGA GGTGAGAGTG CTAATCCCTC ATTCCTCACT GCCCTGCTT CACTACGA GT 120</td>
</tr>
<tr>
<td>CIRCE</td>
<td></td>
</tr>
<tr>
<td>-35 GAAAGTGGGAG AGTATTCCCT RBS</td>
<td>-35 ATG GCA GCA GTA TCC GTG AGC GTT TCA ACC GTT</td>
</tr>
<tr>
<td>294</td>
<td>M AAA V S L S V S T V</td>
</tr>
</tbody>
</table>

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**Fig. 2.** The groESL operon from Oscillatoria sp. NKBG 091600. Two putative −35 and −10 sequences, and the putative RBS sequence, within the upstream region of groES are underlined. The CIRCE and a putative SOS box are marked with bold underlining. The amino acid sequence with shaded background shows the deduced GroEL amino acid sequence matching that obtained from N-terminal sequencing of the 60 kDa protein.

**Figure 2:** The groESL operon from Oscillatoria sp. NKBG 091600. Two putative −35 and −10 sequences, and the putative RBS sequence, within the upstream region of groES are underlined. The CIRCE and a putative SOS box are marked with bold underlining. The amino acid sequence with shaded background shows the deduced GroEL amino acid sequence matching that obtained from N-terminal sequencing of the 60 kDa protein.

reacted with antibody raised against GroEL of Synechococcus sp. PCC 7942 (data not shown).

**Cloning and sequencing of the groESL operon of Oscillatoria sp. NKBG 091600**

The groESL operon was amplified from genomic DNA of Oscillatoria sp. NKBG 091600 using PCR primers designed from conserved sequences in groESL operons of Synechococcus sp. PCC 7942 and Synechocystis sp. PCC 6803. The DNA sequence of the amplified fragment (1.7 kbp) shows that the amplified fragment includes groES with 260 bp of upstream region and 1059 bp of the groEL 5′ region (Fig. 2; GenBank AF054630). Part of the deduced GroEL amino acid sequence matched that obtained from N-terminal sequencing of the 60 kDa protein (Fig. 2, bottom). DNA sequence analysis revealed that groES and partial groEL of NKBG 091600 showed high similarity with the corresponding sequence from Synechococcus sp. PCC 7942 (74% identity at the nucleotide level). In contrast, the upstream region of groES, and the region between groES and groEL, from NKBG 091600 exhibited poor similarity to corresponding sequences from Synechococcus sp. PCC 7942. A perfect inverted repeat (IR) of 9 bp (TTAGCAGTCA-N_9-GAGTGCCTAA) is located 175 bp upstream of the groES translation initiation codon. This IR corresponds to the CIRCE (controlling inverted repeat of chaperone expression) element observed upstream of the groESL operon of Bacillus subtilis (Mogk et al., 1997). The CIRCE in the other cyanobacterial strains Synechococcus PCC 7942 and Synechocystis PCC 6803, is located 55 bp and 57 bp upstream of the translation initiation codon, respectively, i.e. closer to the translation initiation codon than the CIRCE of NKBG 091600. Putative −35 and −10 hexamers were identified according to similarity with the −10 and −35 consensus region of the groESL operon of Synechococcus sp. PCC 7942 (−35, GTTGCC; −10, TACATT) (Webb et al., 1990). The sequence CTGCTCAATCAACCGA (nt 141 to 156; the common motif is underlined), which resembles the SOS box consensus sequence of Escher-
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The putative groESL operon transcriptional start site determined by primer extension of RNA from cells irradiated with UV-A. An oligonucleotide complementary to groESL mRNA was used for both reverse transcriptase primer extension (far-right lane) and nucleotide sequencing. The DNA sequence of the putative SOS box is side-lined. +1, putative transcriptional start site.

ichia coli (Walker 1984), is located 104 bp upstream of the translation initiation codon within a second putative promoter sequence (−35 and −10).

**Time course of groEL gene expression and mRNA synthesis during UV-A irradiation**

The induction profile of GroEL was determined by SDS-PAGE (Fig. 3a). Induction of GroEL began at 400 min and reached a maximum at 500 min after the start of UV-A irradiation. This induction correlated with that of a UV-A-absorbing pigment, biopterin glucoside, which rapidly increases at approximately 9 h after UV-A irradiation (Matsunaga et al., 1993).

Northern blot analysis using a groEL probe (from nt 875 to nt 1767) demonstrated an accumulation of groEL mRNA at 300 min after UV-A irradiation, which persisted over 300 min (Fig. 3b). This result confirmed that the UV-A-dependent expression of GroEL is controlled at the transcriptional level.

**Determination of the transcription initiation site**

We determined the transcription start site of the groESL operon by primer extension analysis with an oligonucleotide, complementary to positions nt 247 to nt 276, on RNA isolated from a culture irradiated by UV-A. A single transcription start site was identified 89 bp upstream from the groES translation initiation codon and 15 bp downstream from the putative SOS box sequence (Fig. 4).

**DISCUSSION**

Various environmental stresses are known to induce GroEL, including heat, acid, radicals and near-UV (Hartke et al., 1997; Hendrix, 1979; Kilstrup et al., 1997; Mecsas et al., 1991; Segal & Ron, 1996; Sherman & Goldberg, 1992). Krueger & Walker (1984) showed that UV light and nalidixic acid activated the SOS response and the expression of GroEL by different regulation mechanisms in E. coli. Shibata et al. (1991) also demonstrated by using 14C labelling that near-UV irradiation (295–390 nm) induced synthesis of 16 proteins in Synechococcus sp. PCC 7942, several of which were also induced by heat shock, and five of which were induced by treatment with methyl viologen (a source of free oxygen radicals). In the present study, UV-A strongly induced transcription and synthesis of GroEL.
in the UV-A-resistant Oscillatoria sp. NKBG 091600 but not in the UV-A sensitive Synechococcus sp. PCC 7942.

Two putative promoters and two operator sequences were found upstream of groES in NKBG 091600. A 9 bp IR corresponds to CIRCE elements, which have been shown to play an important role in the heat induction of the groESL operons of B. subtilis and other bacterial species including cyanobacteria (Hecker et al., 1996; Mogk et al., 1997). Compared to previously characterized CIRCEs, the CIRCE of NKBG 091600 is located further upstream of the predicted transcriptional start site of the groESL operon. A putative promoter overlapping the CIRCE motif was apparently not used during UV-A-induced transcription of the operon. However, a transcript starting from a promoter located more proximal to the translation start site was identified by primer extension analysis of mRNA extracted from UV-A-induced cells. The promoter of this transcript overlaps with an SOS consensus sequence. As near-UV light is known to generate O$_2^*$ and H$_2$O$_2$ (Sammartano et al., 1985; Shibata et al., 1991; Tyrrell, 1985), it is plausible to assume that the SOS box is involved in the control of GroEL expression.

Hirosawa & Miyachi (1982) reported that cyanobacterial cells exposed to UV-A contained less chlorophyll and phycocyanin, and more carotenoid, than nonirradiated cells. We have also observed a decrease in the content of photosynthetic pigments, especially phycoantin, in Oscillatoria sp. NKBG 091600 after UV-A irradiation; however, a recovery of phycocyanin content correlating with GroEL and biopterin glucoside induction was observed (data not shown). The temporal relationship of GroEL induction in NKBG 091600 to biopterin glucoside induction was observed (data not shown). The temporal relationship of GroEL induction in NKBG 091600 to biopterin glucoside biosynthesis and the reconstitution of photosynthetic pigments suggests that this UV-A-inducible GroEL might function to upregulate biopterin glucoside biosynthesis, thereby allowing growth under UV-A irradiation.

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