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Characterization of two carotenoid gene promoters in the cyanobacterium *Synechocystis* sp. PCC 6803

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Abstract

The in vivo promoter activity of the 5′-region of the genes encoding the carotenogenic enzymes phytoene desaturase (*crtP*) and phytoene synthase (*crtB*) from the cyanobacterium *Synechocystis* sp. PCC 6803 has been studied by deletion mapping, primer extension, Northern blot, and using the chloramphenicol acetyltransferase (*cat*) reporter gene. *crtP* and *crtB* are closely linked in the *Synechocystis* genome, but it is shown that both genes are independently transcribed. The level of transcription in both cases is very low. Their expression is affected in a similar way by light intensity: very little or no expression is observed in the dark compared with expression under illumination conditions. High light intensities induce a transient increase in transcription initiation as detected with the *cat* probe. DNA fragments containing 81 base pairs upstream of the *crtP* putative transcription initiation site or 192 base pairs upstream of the *crtB* putative transcription initiation site are enough to confer light sensitive expression to a reporter gene.

Keywords: Carotene biosynthesis; Cyanobacteria; Promoter; Phytoene desaturase; Phytoene synthase; *Synechocystis*

1. Introduction

Carotenoids are pigments that are synthesized by all photosynthetic organisms [1]. They participate in light harvesting and play a role in protecting from potential oxidative damage that may occur upon over excitation of the photosynthetic apparatus [2]. In plants and cyanobacteria, carotenoids are synthesized by a similar pathway from mevalonate, which is converted to the C_{40} precursor phytoene. Phytoene is converted to ζ-carotene by two sequential desaturations catalyzed by the enzyme phytoene desaturase. ζ-Carotene is further oxidized by ζ-carotene desaturase to give lycopene, which is converted to β-carotene by lycopene cyclase [3,4]. The biosynthesis of carotenoids is photoregulated in many organisms [5,6]. This has been best studied in non-photosynthetic bacteria and fungi, where a blue light receptor seems to be involved [7,8]. In photosynthetic microorganisms, the induction of carotene accumulation by light has been reported in algae [9,10]. There have been no reports on regulation by light of carotenogenic genes in cyanobacteria. In this respect, the high light induction of a carotene-binding protein from *Synechococcus* sp. PCC 7942 has been described [11].

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Genes coding for enzymes of the carotene biosynthesis pathway have been recently cloned from micro-organisms, fungi, and plants. In cyanobacteria, the genes coding for phytoene synthase [12,13], phytoene desaturase [14,15], ζ-carotene desaturase [16] and lycopene cyclase [17] have been identified. The gene coding for phytoene desaturase (*crtP*, previously named *pds*) in cyanobacteria is transcribed at very low levels [12,18], and phytoene desaturation seems to be the rate-limiting step in carotene biosynthesis [19]. Phytoene desaturase is the target of the herbicide norflurazon, which acts as a non-competitive inhibitor [20]. We have previously isolated *Synechocystis* mutants resistant to norflurazon, and have cloned the *crtP* gene from the mutant and wild-type strains [15]. The mutants contain a point mutation in the coding sequence of the *crtP* gene that is responsible for the resistance [21]. We have also identified the gene coding for phytoene synthase (*crtB*, previously named *pys*) in *Synechocystis* just downstream of *crtP* [13]. *crtP* and *crtB* correspond to open reading frames slr1254 and slr1255, respectively, in the entire genome sequence of *Synechocystis* [22]. In this work, we describe the 5′-sequences that are required for expression of the *crtP* and *crtB* genes in *Synechocystis* and analyze the effect of light on their expression by Northern blot. To study in more detail the promoter of the *crtP* and *crtB* genes, we have also used a promoter-fusion system that uses the chloramphenicol acetyltransferase gene (*cat*) as a reporter. We have used this system to show that expression from the *crtP* and *crtB* promoters is very low, but at significant levels in vivo under normal light conditions. However, the promoter activity is increased transiently when the cultures are illuminated at high light intensity.

2. Materials and methods

2.1. DNA manipulations

Enzymes were purchased from Boehringer Mannheim or Pharmacia. Radioactive nucleotides were obtained from Amersham or New England Nuclear. Routine DNA manipulations were done by standard procedures [23].

2.2. RNA analysis

Total RNA from *Synechocystis* was obtained as described [24] and used for primer extension using reverse transcriptase and primers 5′-GCGATCACAACGCGCATGGG-3′ (*crtP*) or 5′-TTTCGTAAGCTTCCTCAACAG-3′ (*crtB*). The same oligonucleotides were used to prime sequencing reactions that were used as size markers.

Northern RNA analysis was carried out as described [23] with 15 μg of total RNA. RNAs were separated on a horizontal formaldehyde agarose gel and blotted to Hybond N^+ membrane. Hybridization was done with internal probes corresponding to the coding sequences of *crtP* (a 611-bp *Eco*RV–*Bam*HI fragment), *crtB* (a 520 bp *Apa*I fragment) or *rnpB* [25].

2.3. Plasmid construction and Synechocystis transformation

Plasmid pKW1188 [26] was used to introduce in *Synechocystis* all the constructions used in this work. pKW1188 contains *Synechocystis* DNA sequences flanking a multiple cloning site and a kanamycin resistance marker. Any DNA sequence cloned within the cyanobacterial flanking sequences can be integrated in the chromosome by homologous recombination through these flanking sequences [26]. This integration occurs in a non-essential region of the *Synechocystis* chromosome. The promoterless *cat* gene was obtained from plasmid pJS133 [27]. In this plasmid, the *cat* gene is flanked by multiple restriction sites and contains translation stop signals just upstream of the initiation codon. *Synechocystis* transformation was done as described [28]. Transformants resistant to kanamycin were selected. The transformants were analyzed by Southern hybridization to confirm that the different constructions had integrated in the chromosome, were fully segregated, and that the endogenous wild-type *crtP* or *crtB* genes were intact (not shown).

Synechocystis strains were grown in 70 ml Pyrex tubes (2.5 cm of diameter) at 30°C in BG11 medium supplemented with 1.7 mM Na_2CO_3 and bubbled with 1.5% CO_2 in air as previously described [29]. When required, kanamycin (20 μg/ml) or chloram-

phenicol (10 μg/ml) were added to the media. Cultures were illuminated with white fluorescent light. For high light intensity experiments a Mazda mercury halide lamp was used. The irradiance of the cultures was modified by changing the distance of the culture tube from the lamp. Light intensity is always expressed as the photosynthetic photon flux density reaching the surface of the culture tube, measured with a LI-COR probe.

2.4. Chloramphenicol acetyltransferase assay

Extracts were prepared from the *Synechocystis* strains carrying different constructions with the *cat* gene inserted in the chromosome. Cultures were grown until absorbance at 580 nm was 1 and then subjected to the different treatments as specified in Section 3 and figure legends. After the treatments, the cultures were pelleted by centrifugation, resuspended in 5 ml of 50 mM Tris-HCl (pH 7.8) and disrupted by sonication (70 W, 3 min). The lysates were centrifuged for 15 min at 15000 rpm and aliquots of the supernatants were used to assay for chloramphenicol acetyltransferase (CAT) activity by the scintillation diffusion method [30]. The amount of extract used in the assay was adjusted to give linear kinetics during the time of the assay. The activity was estimated from the slope of the linear part of the reaction. All assays were done in duplicate with at least two extracts for each experimental condition. The protein concentration of the extracts was estimated by the Bradford assay [31] using ovalbumin as standard.

3. Results

The transcription initiation sites for the *crtP* and *crtB* have been determined by primer extension (Fig. 1A). A single extended product is observed in the case of *crtP*. The deduced transcription start site is 36 bp upstream of the initiation ATG and 11 bp downstream a sequence identical to *Escherichia coli* −10 consensus promoter sequence (Fig. 1B). Also, a sequence almost identical to *E. coli* −35 consensus sequence is present, but the distance between these hypothetical −35 and −10 elements is 30 base pairs, while the *E. coli* consensus distance is 17 base pairs.

We had previously suggested that *crtB* transcription was independent of *crtP* [13]. Therefore we decided to study also *crtB* by primer extension. Several products are observed by primer extension of *crtB* mRNA. The longest band would indicate that transcription starts between two in frame ATG codons (Fig. 1C). Therefore, this result suggests that the actual initiation codon is the downstream ATG. This conclusion is supported by the fact that the *crtB* gene products from *Synechococcus* sp. PCC 7942 and from *Synechocystis*, which are more than 70% identical, can be aligned only from this second ATG (Fig. 1C). No promoter consensus sequences are observed upstream of the *crtB* transcription initiation site.

We have determined how much of the upstream sequences are required for expression of the *crtP* gene from *Synechocystis*. For this purpose we have constructed *Synechocystis* strains with the *crtP* coding sequence from the norflurazon resistant mutant AV4 [21] and containing different 5′-extensions. The fragments with 5′-extensions of 173, 81, or 10 bp were cloned in plasmid pKW1188 and then introduced in *Synechocystis* sp. PCC 6803 as described in Section 2. The different fragments used are shown in Fig. 2B. Recombinant cells resistant to kanamycin were selected and analyzed by Southern hybridization (not shown). In all cases, the *crtP* gene from strain AV4 had been integrated in the chromosome and the endogenous wild-type copy of the gene was intact, indicating that recombination had happened between the flanking regions of pKW1188 and the homologous region in the chromosome of *Synechocystis*, as expected. Thus, the recombinant strains had in each chromosome two copies for the *crtP* gene, carrying one complete wild-type copy and one copy from strain AV4 with different 5′-sequences. Because the *crtP* gene from strain AV4 contains a mutation that makes the protein resistant to norflurazon, the recombinants were analyzed for resistance to the herbicide as a test of the expression of the mutant copy of the gene. As shown in Fig. 2B, 81 nucleotides upstream of the transcription start point are sufficient to confer resistance to norflurazon, and thus are the only sequences required to express the *crtP* gene. When only 10 nucleotides upstream of the transcription start point are present, the strain is not resistant to norflurazon, indicating that the *crtP* gene from AV4 is not expressed in this case. The same

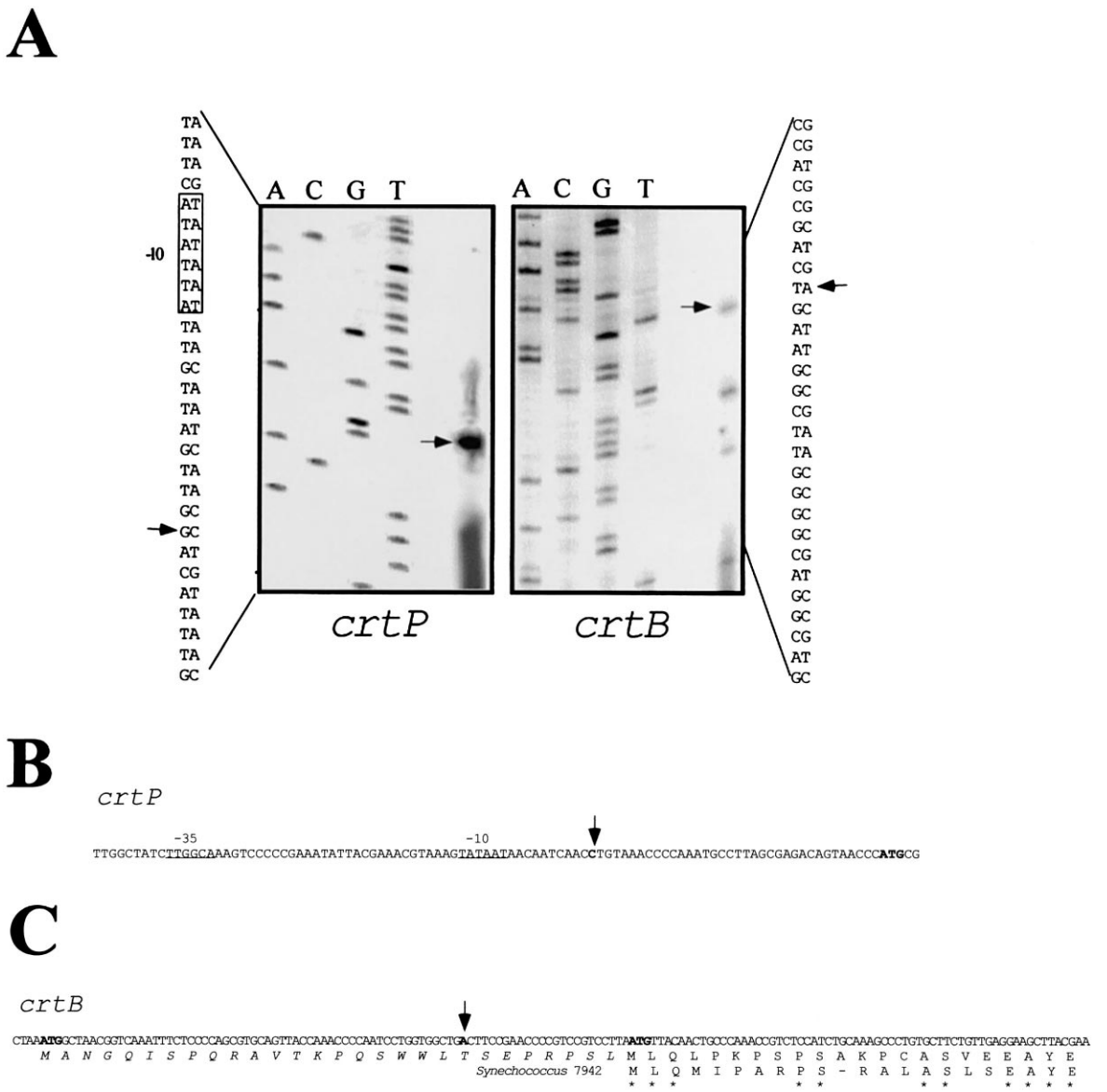

Fig. 1. (A) Identification of the transcription start points of *crtP* and *crtB* by primer extension. Possible transcription start points are indicated by arrows. (B) Nucleotide sequence around the transcription start point (arrow) of *crtP*. Sequences similar to *E. coli* consensus −35 and −10 boxes are underlined. The ATG start codon of *crtP* is shown in bold. (C) Nucleotide sequence around the transcription start point (arrow) of *crtB*. The two possible ATG start codons are shown in bold. The translated *crtB* sequence from *Synechocystis* is aligned with the homologous sequence from *Synechococcus* sp. PCC 7942.

results were obtained when the three constructions described were inserted in the chromosome in the opposite orientation. Thus, it can be concluded that sequences present between position −81 and position −10 are required for expression of the *crtP* gene.

A similar experiment was done with *crtB*. In this case, promoter activity was monitored by fusing the relevant DNA fragments to a promoterless *cat* gene. The *crtB–cat* fusions were cloned into pKW1188 and introduced in *Synechocystis* as before. Recombinants were analyzed for resistance to chloramphenicol. While a fragment containing 192 upstream of the transcription initiation site was able to confer resistance to chloramphenicol, a fragment containing only 71 bp was not (Fig. 2C).

crtP and *crtB* are expressed at low levels and it is difficult to detect their mRNAs. We have been able to analyze mRNA accumulation by Northern hy-

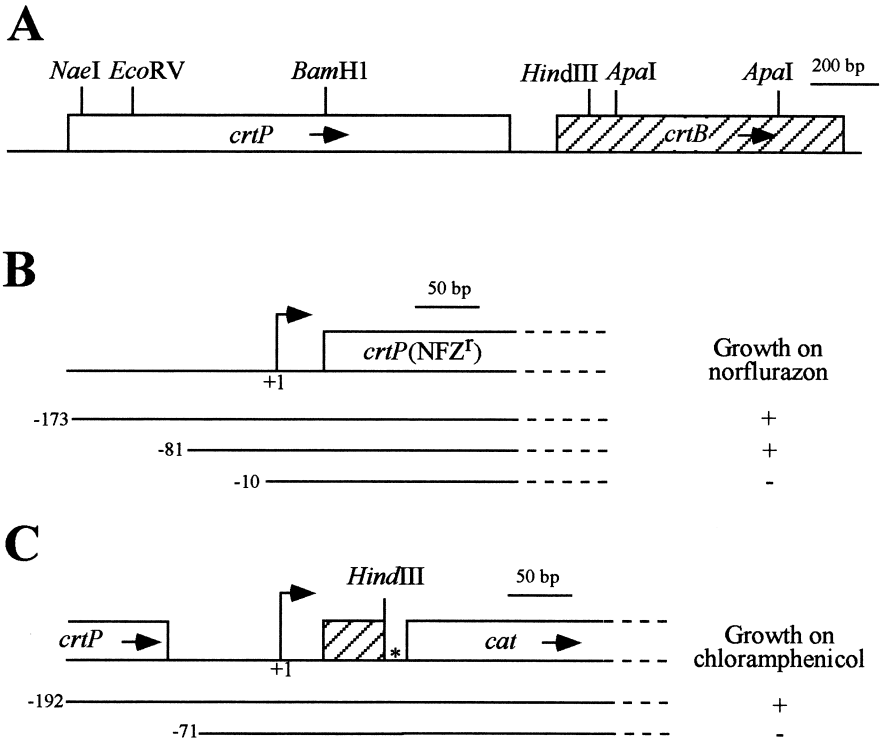

Fig. 2. (A) Diagram of the *crtP–crtB* region. Relevant restriction sites are shown. (B) Structure of the DNA constructions containing the coding sequence of *crtP* carrying mutation AV4, which confers resistance to norflurazon, that were introduced in *Synechocystis* and analyzed for growth in norflurazon. Numbering is relative to the putative transcription start point (+1). Resistance to norflurazon was determined by the ability to form colonies on plates containing 70 μM norflurazon at normal light intensity. (C) Structure of the DNA constructions containing portions of the 5′-region of *crtB* fused to a promoterless *cat* gene that were analyzed for growth in chloramphenicol. Numbering is as in B. The asterisk is to indicate that there are translation stop signals just upstream of the promoterless *cat* gene. Chloramphenicol resistance was determined by the ability to form colonies in 10 μg/ml of chloramphenicol at normal light intensity.

bridization (Fig. 3A) using a sensitive detection system (Instantimager). Instead of discrete size bands, a smear is observed, indicating that the mRNAs for *crtP* and *crtB* are degraded very fast. Control hybridization with a probe for the *glnA* gene (not shown) or with the *rnpB* gene (Fig. 3A) gives no indication of RNA degradation. Therefore the smear observed with the *crtP* and *crtB* probes are not due to poor quality of the RNA preparation, but is rather specific for these mRNAs.

The longest size detectable with the *crtP* probe is about 1.6 and 1.1 kb with the *crtB* probe. These sizes are in agreement with the sizes expected for monocistronic mRNAs but are incompatible with a single transcript containing the coding sequences for both genes.

mRNA accumulation is affected by light. Cultures maintained 12 h in the dark have little amount of *crtP* mRNA and barely detectable *crtB* mRNA. Five minutes of illumination are enough to increase significantly the level of mRNA accumulated and high light intensities are more effective in increasing the amount of mRNA (Fig. 3A,B). The inhibition of photosynthetic electronic flow by the addition of the herbicide DCMU at a concentration of 15 μM does not affect the light activation described (data not shown).

To study more accurately the effect of light on the expression of *crtP* and *crtB*, the minimal DNA sequences required for expression of both genes were fused to the promoterless *cat* gene and introduced in *Synechocystis* with the pKW1188 vector. A sensitive CAT assay was used to monitor transcription activity. As a control of transcription that could occur from chromosomal or pKW1188 sequences, the promoterless *cat* gene was also introduced in the *Syne-*

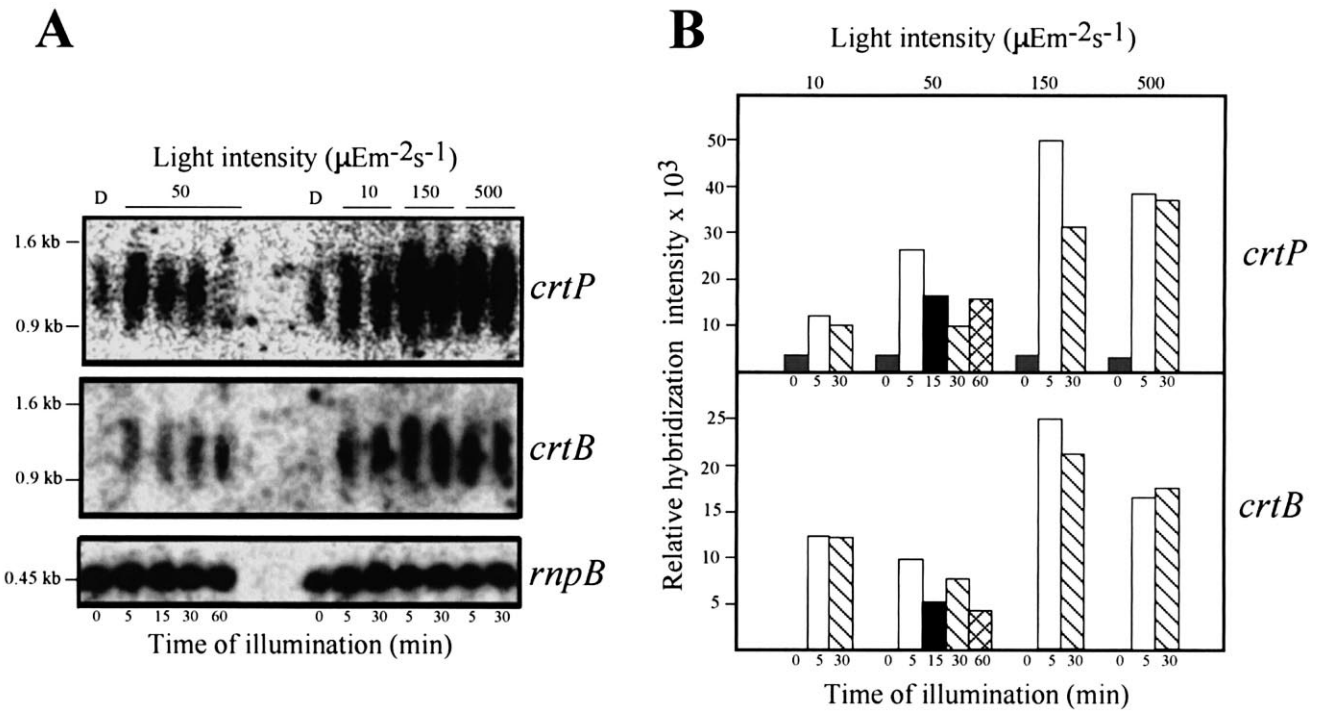

Fig. 3. (A) Northern blot analysis of the expression of *crtP* and *crtB* under different illumination conditions. Cells were incubated in the dark for 12 h (D) and shifted to different light intensities for the indicated times before extraction of the RNAs. Filters were hybridized sequentially with probes corresponding to the *crtP*, *crtB* and *rnpB* genes and analysed in an Instantimager (Packard). The exposure time of the hybridization with the *crtP* and *crtB* probes was twenty times longer than with the *rnpB* probe. (B) Quantitative analysis of the blots shown in A. The amount of hybridization for each probe was determined in the Instantimager and normalized with respect to the radioactivity corresponding to probe *rnpB*.

chocystis genome without any additional sequences in the 5′-region and in the same orientation. Very little or undetectable CAT activity was observed, under any of the conditions used, in this control (Fig. 4A). Furthermore, the *Synechocystis* strain carrying the promoterless *cat* gene was not able to grow on plates containing chloramphenicol. For the *crtP* and *crtB* promoter fusions, a significant increase in CAT activity was observed 15 min after shifting the cultures to high light (Fig. 4B,C), suggesting that high light induces an increase in transcription initiation from both promoters. This increase is transitory. For the *crtP* construction, there is no significant difference between normal and high light after 3 h. For the *crtB* construction, there is no significant difference between normal and high light after only 1 h.

4. Discussion

Phytoene synthase and phytoene desaturase are key enzymes involved in the initial steps of carotenoid biosynthesis. In cyanobacteria, phytoene desaturation seems to be the limiting step in carotenoid biosynthesis [19]. Both genes are linked in the cyanobacterial genome, with the *crtB* gene placed just downstream of *crtP*. From this gene organization it would be expected that both genes are cotranscribed in a single mRNA. However, we had previously observed that insertions in the *crtP* gene had no polar effect on the expression of *crtB* [13], suggesting that *crtB* is transcribed independently of *crtP*. Here we confirm that result by showing that: (1) there are primer extension stops just upstream of the *crtB* coding sequence; (2) short DNA fragments from the 5′-region of *crtB* have promoter activity with a reporter gene; and (3) the maximum sizes observed for the mRNAs of *crtP* and *crtB* are smaller than the minimal size expected for a dicistronic mRNA (2.5 kb). These results do not exclude that there is some degree of cotranscription of both genes. No obvious transcription termination structure is observed in

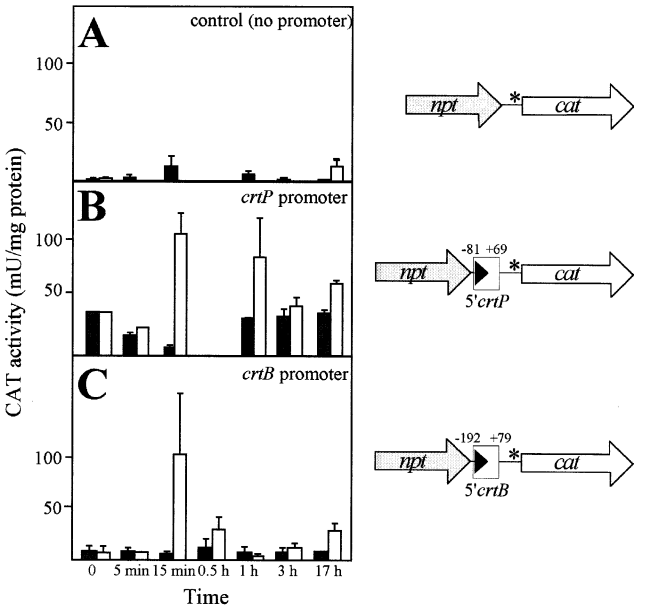

Fig. 4. Effect of light intensity on the CAT activity of extracts from *Synechocystis* strains carrying the *cat* gene without promoter (A), the *cat* gene fused with the promoter region of *crtP* (B), or the *cat* gene fused with the promoter region of *crtB* (C). Cells were grown at 50 μE/m^2/s until A_{580} was 1 and then half of the culture was kept in the same illumination conditions (dark bars) and half shifted to a light intensity of 500 μE/m^2/s (white bars) for the indicated times. No 0.5-h time point was taken for the control and *crtP* samples. The average and error of two independent experiments are shown. A diagram (not to scale) of the constructions introduced into the *Synechocystis* chromosome is shown on the right. The asterisk is to indicate that there are translation stop signals just upstream of the promoterless *cat* gene.

the intergenic region. But the amount of *crtB* expression obtained by transcription from the *crtP* promoter must be low, compared with expression from the *crtB* promoter.

We have studied the activity of the *crtP* and *crtB* gene promoters from *Synechocystis* 6803 by deletion analysis, Northern hybridization, and the use of fusions with the *cat* reporter gene. We have used an accurate and sensitive assay for chloramphenicol acetyltransferase, which has allowed us to analyze the activity of the promoter quantitatively, despite its low normal activity. In addition, the use of promoter fusions allows us to isolate the study of the regulation of the promoter from other factors such as differential mRNA stability.

We have constructed *Synechocystis* strains carrying one wild-type copy of the *crtP* gene and one copy with a mutation (AV4) which confers resistance to the herbicide norflurazon. These strains are resistant to norflurazon, provided that the mutant copy is expressed; thus, resistance is dominant over sensitivity, as expected. The norflurazon resistant *crtP* gene could be useful as a marker for cyanobacterial transformation besides the currently used antibiotic resistance markers.

The DNA fragment between −81 and +67 of *crtP* is enough to confer light activated expression to the CAT gene. In the case of *crtB*, a fragment of 271 bp (from −192 to +79) contains the light activated promoter activity. We have not detected the consensus motif described recently for light-regulated promoters in *Synechocystis* [32] in either *crtB* or *crtP*. However, only five light-regulated promoters were analyzed in that work. Light-responsible promoters have been studied in detail in *Synechococcus* sp. PCC 7942 [33,34]. The promoter sequences of *crtB* or *crtP* do not show homology with the *cis*-acting elements identified in *Synechococcus*.

The experiments described in this paper show that the promoter of the *crtP* gene is contained within 116 bp upstream of the ATG initiation codon. In this region, we can identify sequences similar to −10 and −35 consensus promoter sequences (Fig. 1). In *Synechocystis*, there are many promoters described which depart significantly from consensus promoters and there are instances where a −35 region can not be identified [35]. In the *crtP* gene, the sequence more similar to a −35 consensus sequence is 30 bp from the −10 sequence, and thus its significance is doubtful. This could explain the low level of transcription of this gene. In the case of *crtB*, where no promoter consensus sequences are detected, the level of transcription is still lower as observed by Northern blot and CAT activity.

The stimulation of carotene biosynthesis by light has been described in a number of systems. At a gene expression level, it has been shown that the mRNA for a carotenoid binding protein is induced by high light in the cyanobacterium *Synechococcus* sp. PCC 7942 [11]. Also in tomato, the mRNA for the *crtP* gene accumulates under light stress [36]. A direct relationship between carotene content and photosynthesis has been established in *Anacystis*, implying that carotene content is limiting photosynthesis [37]. The consideration of all these data makes the

crtP gene a prime target for the study of the regulation of carotene biosynthesis in cyanobacteria. The data presented in this paper show that the *crtP* promoter and the *crtB* promoter are in fact activated by light. The effect of light is not mediated directly by photosynthesis because there is no effect of the photosynthetic inhibitor DCMU. Therefore, our results are consistent with the hypothesis that transcription of *crtB* and *crtP* is induced by photooxidation produced by high light.

Additional regulation at other levels, such as differential mRNA stability, cannot be ruled out from our experiments. The difficulty in identifying a full-length *crtP* or *crtB* mRNA suggests that it has a low half-life and thus is a possible target for regulation by differential stability.

The work shown in this paper is the first description of the function of carotenogenic promoters in cyanobacteria. We have defined the DNA region involved in promoter activity of two carotenogenic genes and show that light affects the level of expression from these promoters.

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