Microbiology (1994), **140**, 1151–1159 Printed in Great Britain

Cloning, sequencing and transcriptional studies of the genes for cytochrome *c*-553 and plastocyanin from *Anabaena* sp. PCC 7120

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In some cyanobacteria and eukaryotic algae, cytochrome *c*-553 (*c*-552) and plastocyanin function as alternative electron carriers between the cytochrome b_6-*f* complex and Photosystem I. In these organisms plastocyanin is the electron carrier under copper-replete conditions, and cytochrome *c*-553 is the electron carrier during copper deprivation. In this paper we report the cloning, sequencing and transcriptional analysis of the genes for cytochrome *c*-553 and plastocyanin from *Anabaena* sp. PCC 7120. The gene for cytochrome *c*-553 encodes a preprotein containing 111 amino acids with a predicted N-terminal transit peptide sequence of 25 amino acids. The gene for plastocyanin encodes a preprotein containing 139 amino acids with a N-terminal transit peptide sequence of 34 amino acids. RNA transcript analyses indicate that the expression of the genes for cytochrome *c*-553 (*petJ*) and plastocyanin (*petE*) are regulated in reciprocal ways in response to copper concentration. In copper-replete conditions, *petJ* is expressed at very low levels, but is transcribed at high levels under copper deprivation; *petE* is down-regulated in the absence of copper, but is rapidly up-regulated when copper is added back to the medium.

Keywords: *Anabaena* sp. PCC 7120, cytochrome *c*-553, plastocyanin, gene regulation, algae, cyanobacteria

INTRODUCTION

Cytochrome *c*-553 (*c*-552) and plastocyanin are soluble photosynthetic electron transporters that ferry electrons between the membrane-bound cytochrome b_6-*f* complex and Photosystem I in cyanobacteria and many eukaryotic algae. Although cytochrome *c*-553 is the only electron carrier performing this function in some cyanobacteria, in others either plastocyanin or cytochrome *c*-553 are used, and their use appears to depend on the availability of environmental copper (Ho & Krogmann, 1984; Sandmann & Böger, 1980). Many eukaryotic algae have similar changes in response to copper availability (Sandmann *et al.*, 1983). In the green alga, *Chlamydomonas reinhardtii*, *in vitro* translation systems and antibodies to plastocyanin and cytochrome *c*-552 were used to show that the production of plastocyanin is controlled at the post-transcriptional level, while cytochrome *c*-552 expression is controlled at the transcriptional level (Merchant & Bogorad, 1986). Subsequently, the cloned gene for *C. reinhardtii* cytochrome *c*-552 was used to show that it is only expressed in copper-deficient cells (Merchant & Bogorad, 1987).

The genes for plastocyanin (*petE*) and cytochrome *c*-553 (*petJ*) have been cloned from *Anabaena* sp. PCC 7937 and *Synechocystis* sp. PCC 6803. Transcript analyses indicated that in *Anabaena* sp. PCC 7937 *petE* is strongly down-regulated in copper-deficient conditions (Van der Plas *et al.*, 1989); contradictory reports on the expression of *petE* in *Synechocystis* sp. PCC 6803 indicated that the gene is either constitutively expressed under all copper regimes (Briggs *et al.*, 1990) or is regulated in a manner similar to *Anabaena* sp. PCC 7937 (Zhang *et al.*, 1992). However, in both species *petJ* was shown to be expressed in the absence of copper and down-regulated when copper was added to the medium (Zhang *et al.*, 1992; Bovy *et al.*, 1992). *petJ* (called *cytA*) has been cloned in *Synechococcus* sp. PCC 7942

The GenBank accession numbers for the nucleotide sequence data reported in this paper are M97009 and L19417.

and shown to be constitutively expressed under copper-replete and copper-deficient conditions; however, this cyanobacterium does not appear to have a plastocyanin gene (Laudenbach *et al.*, 1990). In this paper we report the cloning and sequencing of *petJ* and *petE* from *Anabaena* sp. PCC 7120. We demonstrate that *petJ* shows very low levels of transcription in the presence of environmental copper, but is strongly expressed under conditions of copper deprivation; *petE* displayed the complementary pattern of transcriptional regulation. Hybridization experiments with total cellular RNA indicate that the message for plastocyanin reaches steady state levels within 1 h of adding copper to copper-deficient medium.

METHODS

Materials. All chemicals were reagent grade. Restriction endonucleases were obtained from BRL, New England Biolabs and Pharmacia. Alkaline phosphatase was obtained from Boehringer Mannheim; T4 polynucleotide kinase from New England Biolabs; T4 DNA ligase from BRL. [α-^{32}P]dCTP (3000 Ci mmol^{-1}) was obtained from ICN, and [γ-^{32}P]ATP (5000 Ci mmol^{-1}) and [^{35}S]dATP (1000 Ci mmol^{-1}) were obtained from Amersham (1 Ci = 37 GBq).

Culture conditions. *Anabaena* sp. PCC 7120 was grown in BG11 medium in liquid culture (Allen, 1968) at 29 °C. To prepare copper minus medium, copper was omitted from the micronutrient stock solution of BG11. All media were made in acid-washed glassware with deionized, ultrapure water from a Milli-Q Reagent Water System (Millipore).

Library construction and screening. *Anabaena* sp. PCC 7120 genomic DNA was isolated as follows. Cultured cells were harvested by centrifugation, washed in 50 mM NET (50 mM NaCl, 50 mM EDTA, 50 mM Tris/HCl, pH 8·0) and resuspended in 48 ml 50 mM NET containing 5 mg lysozyme ml^{-1} per 5 g wet-packed pellet. The suspension was incubated for 1 h at 37 °C. After incubation, 6 ml 20% (v/v) Triton X-100 and 5 ml 10% (w/v) Sarkosyl were added; with this mixture of detergent the cells lysed rapidly to form a viscous solution. NaCl (5 M) was gently mixed in to give a final concentration of 0·4 M. The mixture was then sequentially extracted with phenol (saturated with 0·1 M Tris, pH 8·0) and then with $CHCl_3$–isoamyl alcohol (24:1, v/v) for 15 min each. Two volumes of ethanol were added and the precipitated DNA was spooled onto a glass rod. The DNA was redissolved in 20 ml TE (10 mM Tris/HCl, 1 mM EDTA, pH 8·0), RNase A was added to a final concentration of 40 μg ml^{-1} and the mixture was incubated at 37 °C for 30 min. The salt concentration was raised to 0·2 M NaCl by addition of 5 M NaCl. The mixture was carefully extracted once with an equal volume of phenol–$CHCl_3$ (1:1, v/v) and then extracted twice with an equal volume of $CHCl_3$–isoamyl alcohol (24:1, v/v). Finally, the DNA was precipitated and spooled as before and dissolved in 15 ml TE. This procedure yielded about 5 mg DNA from an initial 5 g of cellular pellet.

Partial plasmid libraries were constructed from restriction-enzyme-digested genomic DNA that had been separated by size on a low-melting-temperature agarose gel. Regions of the gel that contained the appropriate fragment size were excised, DNA was isolated from the gel by phenol extraction (Maniatis *et al.*, 1982), and the purified DNA was ligated into restriction-enzyme-digested pGEM-3Zf(+) (Promega) or pBluescript (Stratagene) for *petJ* and *petE*, respectively. DNA transformations of *Escherichia coli* were performed by the method of Hanahan (1985), and the libraries were screened by Southern blot hybridization (Southern, 1975) of restriction-enzyme-digested DNA isolated from pooled groups of 25 clones. Finally, individual clones were screened from the group that reacted with the radioactive probes. The probes for *petJ* are found in Fig. 1; the probe for *petE* was a 285 bp PCR fragment generated using oligonucleotides, 5′ AAACTAGGCAGCGATAAAGG 3′ and 5′ TTTACCGACCATACCAGC 3′ from the *petE* sequence of *Anabaena* sp. PCC 7937 (Van der Plas *et al.*, 1989). Selected clones were positively identified by double-stranded DNA sequencing and by comparing the translated products of open reading frames with published amino acid sequences of cytochrome *c*-553 from *Anabaena variabilis* (Aitken, 1976) and with

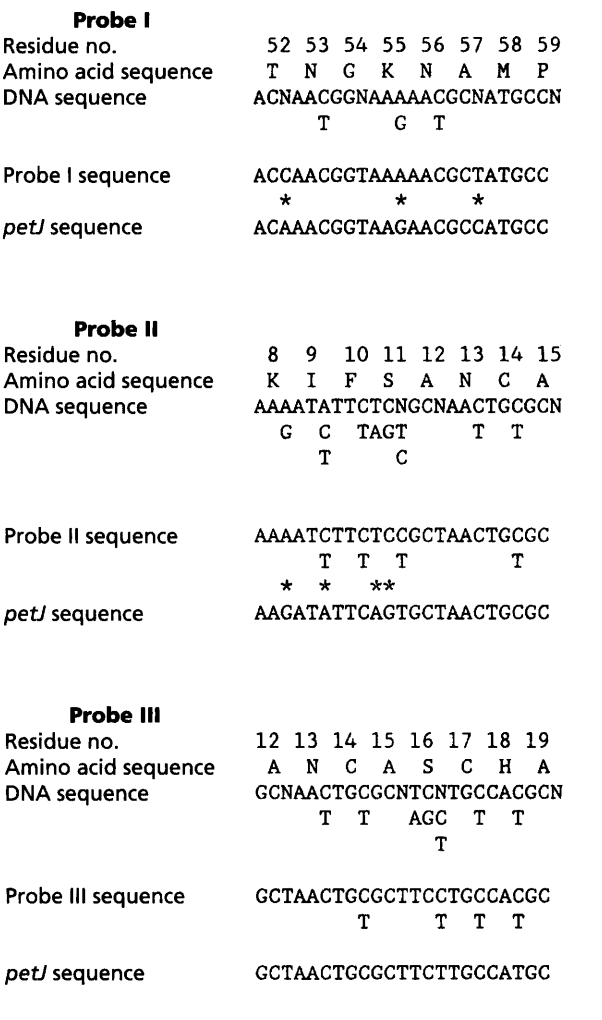

Fig. 1. Primers used to probe for *petJ* from *Anabaena* sp. PCC 7120. Three amino acid sequences were selected from conserved areas of cytochrome *c*-553 from *A. variabilis*. The amino acids are numbered according to Laudenbach *et al.* (1990). The DNA sequence with staggered bases allows for all the degeneracy of the codons at each position; N indicates the possibility of having any one of the four bases at that position. The sequence of the probes was arbitrarily selected after consulting the frequency of codon usage from a number of published *Anabaena* sp. PCC 7120 gene sequences. The actual sequence of *petJ* for the probe site in *Anabaena* sp. PCC 7120 (from this report) is placed below the sequence of each probe; asterisks indicate places where the probe differs from the actual sequence.

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                                                                      ACGATTG

          -170      -160      -150      -140      -130
GCGAATTTGCCGCGTTACAGAAGACGCAATGAGCCAATTAGCCAAGGGGAAGTGAAGAAG

          -110      -100       -90       -80       -70
GCAAGGGAGCAGGGAGCAGGTGAGCAGAGGGGCAGGGGAAGCAAAGAAGAAGAACCAATG

           -50       -40       -30       -20       -10        -1
ACAACTGACAACTGACAACCGACTATTAAAGTTTGACAGCTTGTCATTTGACTATGACAG

            10        20        30        40        50        60
CAGCTCAACTGAAAATAATTGAGGATGATATTGGTCTCAATGAGCCTTATTTGCAGAGAG

            70        80        90       100       110       120
AACGCGACATGAAAAAGATTTTTCCCTAGTACTGTTAGGCATAGCACTCTTCACTTTTG
         M  K  K  I  F  S  L  V  L  L  G  I  A  L  F  T  F

           130       140       150       160       170       180
CCTTCAGTAGCCCTGCATTAGCAGCAGACAGTGTAAATGGAGCCAAGATATTCAGTGCTA
A  F  S  S  P  A  L  A ▲A  D  S  V  N  G  A  K  I  F  S  A

           190       200       210       220       230       240
ACTGCGCTTCTTGCCATGCAGGTGGCAAGAATTTGGTTCAAGCACAGAAAACTCTGAAGA
N  C  A  S  C  H  A  G  G  K  N  L  V  Q  A  Q  K  T  L  K

           250       260       270       280       290       300
AAGCCGATTTGGAAAAATATGGGATGTACTCAGCAGAGGCAATCATTGCCCAGGTAACAA
K  A  D  L  E  K  Y  G  M  Y  S  A  E  A  I  I  A  Q  V  T

           310       320       330       340       350       360
ACGGTAAGAACGCCATGCCTGCTTTCAAAGGTCGCTTAAAACCTGAACAAATTGAAGATG
N  G  K  N  A  M  P  A  F  K  G  R  L  K  P  E  Q  I  E  D

           370       380       390       400       410       420
TAGCTGCTTACGTGCTAGGAAAAGCCGATGCAGATTGGAAGTAAATTCCACTTCAAGAGC
V  A  A  Y  V  L  G  K  A  D  A  D  W  K

           430       440       450       460       470       480
AAAGCTAACTAACAGATAGCGGGAAATAATCCCGCTATTTTTATCATGAGAGAGATGAAG
         ------------->     <--------------

           490       500       510       520       530       540
TAATAAGTGGAAAGCGCAAATTAAATTAGATAATCTTCCACATTTGGCACATCTACCCAG

           550       560       570
CTAGCAGTTATATAAGATTGATGAGATAAATCCATT
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Fig. 2. Nucleotide sequence of *petJ* and flanking regions. The numbering designates the first transcribed nucleotide as number 1; this position was determined by primer extension experiments. The single-letter abbreviations of the deduced amino acid sequence are shown below the nucleotide sequence. A putative transcriptional stop site is underlined with inverted arrows. The putative polypeptide cleavage site is marked with a solid triangle.

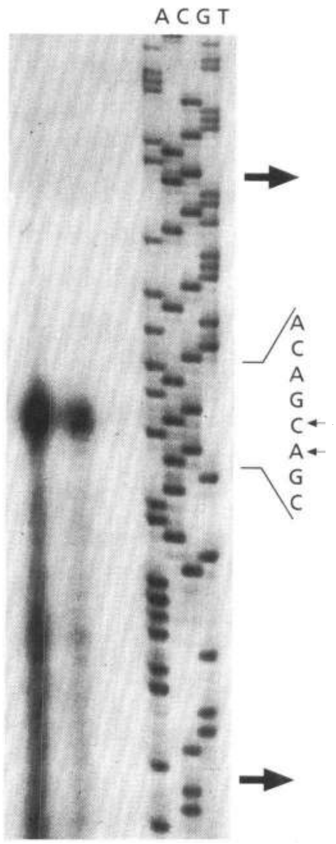

Fig. 3. Transcriptional start site for *petJ*. The two extension products are marked on the parallel sequence ladder; the arrows indicate the position of the putative transcriptional start sites in the DNA sequence. The sequence ladder was generated using the same oligonucleotide primer (a 24-mer) as that used for primer extension. The lanes of the sequence ladder have been labelled with the bases that complement the dideoxynucleotides of each reaction, so that the sequence read from the ladder corresponds to the sequence of Fig. 2.

published amino acid sequences of plastocyanin from *Anabaena* sp. PCC 7937 (Van der Plas *et al.*, 1989).

RNA isolation and DNA:RNA blot analysis. Total cellular RNA was isolated by the method of Golden *et al.* (1987). The RNA was denatured with glyoxal and dimethyl sulphoxide, and electrophoresed on a 1·1% (w/v) agarose gel according to Sambrook *et al.* (1989). RNA from the gel was transferred to nylon membranes (Nytran, Schleicher and Schuell) with the Vacugene transfer system (Pharmacia) and a transfer buffer of 7·5 mM NaOH. DNA:RNA blot hybridizations were carried out at 65 °C as described by Sambrook *et al.* (1989). The probes for these hybridizations were PCR fragments that were generated using sequence oligonucleotides that border the open reading frames of *petE* and *petJ*.

Nucleotide sequencing and sequence analysis. Both strands of cloned DNA fragments containing *petE* or *petJ* were sequenced by the double-stranded sequencing technique of Zagursky *et al.* (1985) employing the commercially available Sequenase kit (USB). All sequence reactions were primed by oligonucleotides synthesized on a Cyclone DNA synthesizer (Biosearch). DNA sequence analyses, protein sequence alignments, and protein similarity comparisons were performed with Microgenie version 6.0 (Beckman Instruments).

Primer extension analysis. Primer extension was carried out using the method of Kjems & Garrett (1988), developed for reverse transcriptase sequencing. However, in these reactions the dideoxynucleotides were omitted from the extension mixture, the labelled primer was hybridized overnight to the total RNA, and the extension reaction was carried out at 42 °C.

RESULTS AND DISCUSSION

Cloning and sequence analysis of *petJ*

Comparative lists of cytochrome *c*-553 amino acid sequences from eukaryotic algae and cyanobacteria indicate that a significant number of amino acids have been conserved in evolution (Laudenbach *et al.*, 1990; Sprinkle *et al.*, 1986; Aitken, 1976). Two areas that showed a substantial amount of conservation in the cyanobacteria include the haem binding site and the region between residues 50 and 60 (Laudenbach *et al.*, 1990). Fig. 1 contains the sequences of three oligonucleotide probes that were constructed to these two areas. When these probes were hybridized to Southern blots of restriction-enzyme-digested DNA from *Anabaena* sp. PCC 7120, relatively few bands hybridized to Probe I whereas

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AVAR  ADSVNGAKIFSANCASCHAGGK(     )TLKKADLEKY(              )NAMPAFKGRLKPEEIZBVAAYVLGKADADWK
            10        20        30        40        50        60        70        80
      *   *     **** **  *      *** *  *         *   **  ** *** *   *    *  ** *       *
7120  ADSVNGAKIFSANCASCHAGGKNLVQAQKTLKKADLEKYGMYSAEAIIAQVTNGKNAMPAFKGRLKPEQIEDVAAYVLGKADADWK
7937  ..VA.............................E....F.............................D..........Q..KS..
7942  ..LAH.GQV......A..L..R.V.N.....Q....DQ...A.I...TT......G.....GSK.SADD.A...S...DQSEKG.QG
ANID  ..LAH.GQV.........L..R.V.N.....E....DE...A.I...TT......G.....GAK.SADD..G..S.A.DQSGKE.-
6312  ..IAD...V......A..M....V.M.N.....EA..QF..N..D..MY..Q.........G...SEA...N......DQSSNK.AG
AFLO  ..T.S..AL.K....Q..V..G...NKA.....EA.G..N....K.......H..G.......-K..A....N......EQ..NG..K
PBOR  ..AAA.G.V.N....A...S.GGQINGA.....NA.TAN.KDTV...V.......G.........SDD..QS..L...D..EKG.-
SMAX  G.VAA..SV......A..M..R.VIV.N...S.S..A..KGFDDDAVVA.........G.N...S.K.........VDQ.EKG.-
                                           L       AA Y
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Fig. 4. Comparative alignments of cyanobacterial cytochrome *c*-553. The alignments were generated by the similarity algorithm resident in Microgenie. The asterisks above the sequences represent the positions of amino acids that are absolutely conserved for all known sequences. Parentheses in the amino acid sequence of *A. variabilis* reflect incomplete sequence in this region. Strains are designated as follows: AVAR, *A. variabilis* (Aitken, 1976); 7120, *Anabaena* sp. PCC 7120; 7942, *Synechococcus* sp. PCC 7942 (Laudenbach *et al.*, 1990); ANID, *Anacystis nidulans* (Ludwig *et al.*, 1982); 6312, *Synechococcus* sp. PCC 6312 (Aitken, 1979); AFLO, *Aphanizomenon flos-aquae* (Ulrich *et al.*, 1982); PBOR, *Plectonema boryanum* (Aitken, 1979); SMAX, *Spirulina maxima* (Aitken, 1979). Letters in aligned sequences represent the presence of different amino acids at the position in question; dots indicate that no change has occurred at that position; dashes indicate a postulated deletion; the arrowheads below the sequence of *Spirulina maxima* indicate the position of inserted amino acids.

multiple bands hybridized to Probes II and III. A series of restriction enzyme fragments were identified which hybridized to all three oligonucleotide probes and to a 0·9 kb *Hin*dIII–*Pst*I fragment of *petJ* from *Synechococcus* sp. PCC 7942. Partial plasmid libraries were constructed to these fragments, and DNA from each library was screened sequentially with all four probes. Pooled clones, and subsequently individual clones, were screened with Probe I. Finally, two independent recombinant plasmids were isolated that contained a 2·2 kb *Hin*dIII–*Hin*cII fragment which hybridized to all the probes. This fragment was shown to contain *petJ* by double-stranded DNA sequencing using oligonucleotide Probe I as the initial sequencing primer. The sequence of both strands of the entire gene was obtained by double-stranded DNA sequencing using oligonucleotide primers whose sequences were generated from previously run sequencing reactions.

Fig. 2 contains the sequence of the gene for cytochrome *c*-553 from *Anabaena* sp. PCC 7120. Primer extension analysis (Fig. 3) yielded two major extension products differing by a single nucleotide; the longest extension product indicated a 5′ leader sequence 68 nucleotides long. The gene encodes a polypeptide of 111 amino acids. On the basis of the published partial amino acid sequence of cytochrome *c*-553 from *A. variabilis* (Aitken, 1976), the mature protein begins at amino acid 26 (alanine) and contains 86 amino acids. The first 25 amino acids form the N-terminal signal sequence required to transport cytochrome *c*-553 across thylakoid and cytoplasmic membranes (Serrano *et al.*, 1990). A potential transcriptional termination structure, with a thermal stability of $\Delta G = -13{\cdot}4$ kcal mol^{-1} ($-56{\cdot}0$ kJ mol^{-1}), is located between nucleotide positions 430 and 463, 28 bases downstream from the last codon of *petJ*.

Fig. 4 shows a comparison of the amino acid sequence of the mature translated protein of *petJ* from *Anabaena* sp. PCC 7120 with the known amino acid sequences of cytochrome *c*-553 from other cyanobacteria. The sequence of the mature protein differs in eight positions from the sequence published for *Anabaena* sp. PCC 7937 (Bovy *et al.*, 1992). However, our sequence is almost identical to the partial amino acid sequence determined from isolated cytochrome *c*-553 of *A. variabilis* (Aitken, 1976). The two sequences differ in only one amino acid at position 65 where *Anabaena* sp. PCC 7120 contains a glutamine, whilst *A. variabilis* was reported to have a glutamic acid. The close match between these sequences indicates that these two *Anabaena* cultures are either the same strain or very closely related strains. Our sequence fills in the two gaps in the sequence from *A. variabilis*, amino acids 19–25 and 40–55, and identifies the amino acids at positions 67 and 68 as glutamic acid and aspartic acid, respectively (Fig. 4). Preliminary data citing unpublished results have been reported for these two gaps (Ulrich *et al.*, 1982); our sequence is identical for the first gap (19–25) but differs in 3 of the 16 amino acids in the second gap (40–55, GMYSAEAIIAQVTNGK instead of GAYSAMAIGAQVTNGK). Since our sequence is also identical to that of *Anabaena* sp. PCC 7937 in this region it seems reasonable to assume that the preliminary report was in error for these three amino acids.

Alignment of the different mature protein sequences shows a substantial number of strictly conserved sites (Fig. 4). Within these conserved sites are H-18 and M-58 which are the iron ligands, F-61 which is thought to lie near the top of the haem, F-10 and Y-76 which compare to the two aromatic residues forming the right channel in mitochondrial cytochromes *c* and, of course, C-14 and C-17 that covalently bind the porphyrin (Ulrich *et al.*, 1982).

Cloning and sequence analysis of *petE*

When hybridized with genomic DNA from *Anabaena* sp. PCC 7120, the PCR-generated probe for *petE* hybridizes with a 2·3 kb *Eco*RI–*Cla*I fragment. Two independently isolated plasmids containing this fragment were isolated from partial plasmid libraries and sequenced (Fig. 5).

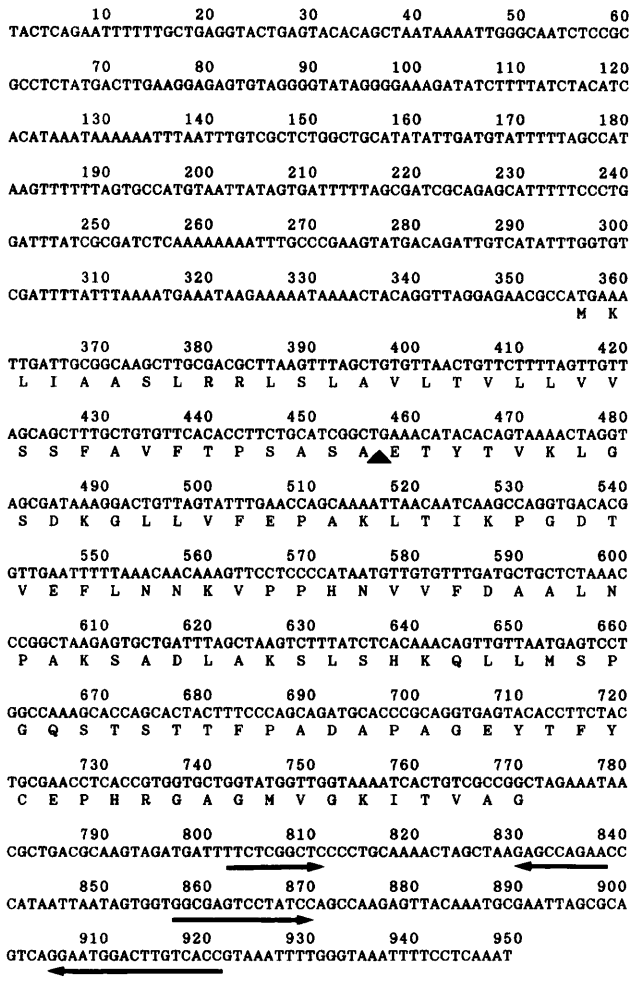

Fig. 5. Nucleotide sequence of *petE* and flanking regions. The single-letter abbreviations of the deduced amino acid sequence are shown below the nucleotide sequence. Putative transcriptional stop sites are underlined with inverted arrows. The putative polypeptide cleavage site is marked with a solid triangle.

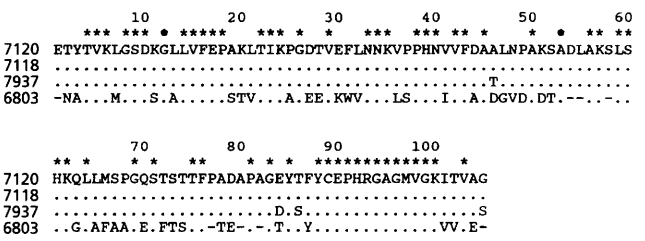

Fig. 6. Comparative alignments of cyanobacterial plastocyanins. 7120, *Anabaena* sp. PCC 7120; 7118, *Anabaena* sp. PCC 7118; 7937, *Anabaena* sp. PCC 7937; 6803, *Synechocystis* sp. PCC 6803. Letters in aligned sequences represent the presence of different amino acids at the position in question; dots indicate that no change has occurred at that position; dashes indicate a postulated deletion.

Two potential transcriptional stop structures are located down stream from the last codon of *petE*; these are nucleotides 802–838 with a thermal stability of $\Delta G = -13{\cdot}2$ kcal mol^{-1} ($-55{\cdot}2$ kJ mol^{-1}) and nucleotides 857–921 with a thermal stability of $\Delta G = -20{\cdot}2$ kcal mol^{-1} ($-84{\cdot}4$ kJ mol^{-1}). The open reading frame for *petE* encodes a preprotein containing 139 amino acids. By comparing this sequence with that published for *Anabaena* sp. PCC 7937 (Van der Plas *et al.*, 1989), the preprotein is assumed to have a N-terminal signal sequence containing 34 amino acids and a mature protein sequence of 105 amino acids. Four independent attempts to position the transcriptional start site proved unsuccessful; for these attempts, four different primers were synthesized to hybridize to different locations in the 5′ end of the message (three that were 30 nucleotides long and one that was 24 nucleotides long). Failure to detect an extension product could indicate that the leader sequence contains a stable secondary structure that impedes reverse transcriptase movement along the message; alternatively, lack of an extension product could result from a message abundance that is too low to produce detectable product. However, since RNA transcript analyses (see Figs 7–9) indicate that the message for *petE* is at least as abundant as the message for *petJ*, the former of the two explanations appears to be the more plausible.

In Fig. 6, the sequence of plastocyanin from *Anabaena* sp. PCC 7120 is compared with plastocyanins from other cyanobacteria. The sequence is identical to that of plastocyanin from *Anabaena* sp. PCC 7118 (Aitken, 1975), but differs in four positions from the sequence of plastocyanin from *Anabaena* sp. PCC 7937; only 51% of the sequence is the same as plastocyanin from *Synechocystis* sp. PCC 6803.

A comparison of the nucleotide sequences of *petE* and surrounding regions for *Anabaena* sp. PCC 7120 and *Anabaena* sp. PCC 7937 (Van der Plas *et al.*, 1989) indicates that the sequences upstream from the coding region show close homology up to nucleotide position 103 which is 252 nucleotides away from the start codon. After this point, the sequences appear to be totally unrelated. The sudden divergence in sequence homology at this point probably establishes the upper boundary for potential regulatory sequences in the promoter region of this gene. Comparisons of the sequences downstream from the coding region are very similar for the available data. Although a single base difference occurs in the inverted repeated sequences of each of the potential transcriptional termination structures (Fig. 5), the changes do not appear to destabilize either of the structures, since these differences are located in areas of local base mismatching.

N-terminal signal sequences

On the basis of the reported amino acid sequence for cytochrome *c*-553 from *A. variabilis* (Aitken, 1976), the first 25 amino acids of the translated open reading frame are not part of the mature protein (Fig. 2). This N-terminal domain of the precytochrome *c*-553 polypeptide has structural characteristics that are similar to those found in prokaryotic signal sequences (von Heijne, 1986;

Gierasch, 1989). These features include a basic N-terminal region containing two lysines (n-region), a central hydrophobic region (h-region) and a C-terminal region that follows the '−3, −1 rule' (c-region). The c-region of this signal sequence contains alanines in both the −1 and the −3 positions, which is the most frequent configuration for prokaryotic cleavage sites (von Heijne, 1986). The presence of this signal sequence is consistent with evidence that cytochrome *c*-553 in cyanobacteria or its functional equivalent in *Rhodobacter sphaeroides* is transported into the periplasmic space (Laudenbach *et al.*, 1990; Serrano *et al.*, 1990; Kiley & Kaplan, 1988). The basic features that characterize N-terminal signal sequences of prokaryotes are also found in the first 34 amino acids of the translated open reading frame of plastocyanin from *Anabaena* sp. PCC 7120, although this sequence has a substantially longer n-region containing two arginines and one lysine in ten amino acids (Fig. 5). Comparisons between the signal sequences of precytochrome *c*-553 and preplastocyanin show very little relationship, which is surprising because these proteins are thought to be alternative electron donors to P700 (Ho & Krogmann, 1984) and, thus, would be expected to be targeted to the same region of the cell. However, since signal sequences are notoriously variable (Gierasch, 1989), it would be premature to assume that the differences reported in this paper indicate different targeting sites or even independent transport mechanisms for cytochrome *c*-553 and plastocyanin in *Anabaena* spp.

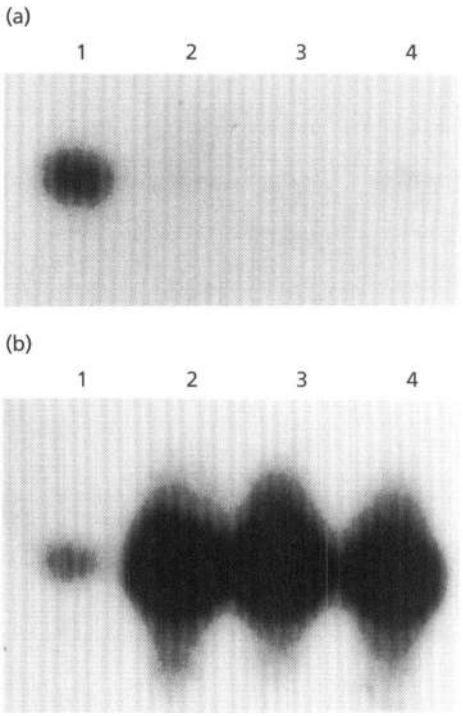

Fig. 7. Transcriptional analysis of *petJ* and *petE*. DNA:RNA blot analysis of RNA from cells grown in different copper regimes. (a) Hybridization results for a probe from *petJ*; (b) results for *petE*. Lanes 1–4 of each panel represent a sample of 13 μg RNA from cells grown in 0, 0·3, 0·6 and 0·9 μM copper, respectively.

Gene copy number

Some cyanobacteria have been shown to contain a series of closely related genes capable of producing physiologically equivalent proteins for specific functions in photosynthesis. For example three separate loci, *psbAI*, *psbAII* and *psbAIII*, encode the D1 protein and two distinct loci, *psbDI* and *psbDII*, encode D2 in species of *Synechococcus* and *Synechocystis* (Golden *et al.*, 1986; Jansson *et al.*, 1987; Vermaas *et al.*, 1988; Gingrich *et al.*, 1990; Bustos & Golden, 1991; Kulkarni *et al.*, 1992). Electrophoretically distinct species of cytochromes *c*-553 reported for a number of naturally isolated cyanobacteria indicate that there may be more than one copy of this gene in some cyanobacteria (Ho & Krogmann, 1984). In *Synechococcus* sp. PCC 7942 Southern blot hybridization failed to detect more than one copy of *petJ*, and insertional inactivation of this copy produced a mutant with no detectable cytochrome *c*-553. However, *Synechococcus* sp. PCC 7942 does not contain plastocyanin and the single *petJ* gene is not differentially expressed under different copper regimes (Laudenbach *et al.*, 1990).

To examine whether *Anabaena* sp. PCC 7120 contains related multiple copies of *petJ* or *petE*, total genomic DNA that was digested with a series of different restriction enzymes was reacted with PCR-generated probes for these genes. The Southern blot hybridization at high stringency (65 °C, 2 × SSC; not shown) showed that in each case only one fragment reacted to each probe; under these conditions we found that a probe for *psbDI* would cross-react with DNA fragments from *Anabaena* sp. PCC 7120 containing *psbDII*. This evidence indicates that there is only one copy of *petJ* or *petE* per genome in *Anabaena* sp. PCC 7120.

Transcript analysis of *petJ* and *petE*

To examine how *petJ* and *petE* are expressed in response to environmental copper concentration, total RNA was extracted from cells grown in normal medium containing different amounts of copper (Fig. 7). These RNAs were reacted by DNA:RNA blot hybridization to radioactive probes for *petJ* and *petE*. The patterns of hybridization indicated that the genes are transcribed in a reciprocal manner in response to copper concentration. However, both genes seem to retain low rates of transcription after their expression is down-regulated (this is obvious in Fig. 7 for *petE*, but requires longer exposure for *petJ* than shown). The sizes of the transcripts in relation to known single-stranded DNA markers indicate that the message for *petE* contains 740 nucleotides and the message for *petJ* contains 480 nucleotides. The length of the transcript of *petJ* agrees quite well with the predicted transcriptional start and stop sites of the gene. The predicted lengths also indicate that in each case the messenger RNA is just large enough to accommodate the open reading frames, the 5′ leader sequences and the 3′ trailing sequences of the respective genes. Therefore, both *petE* and *petJ* form

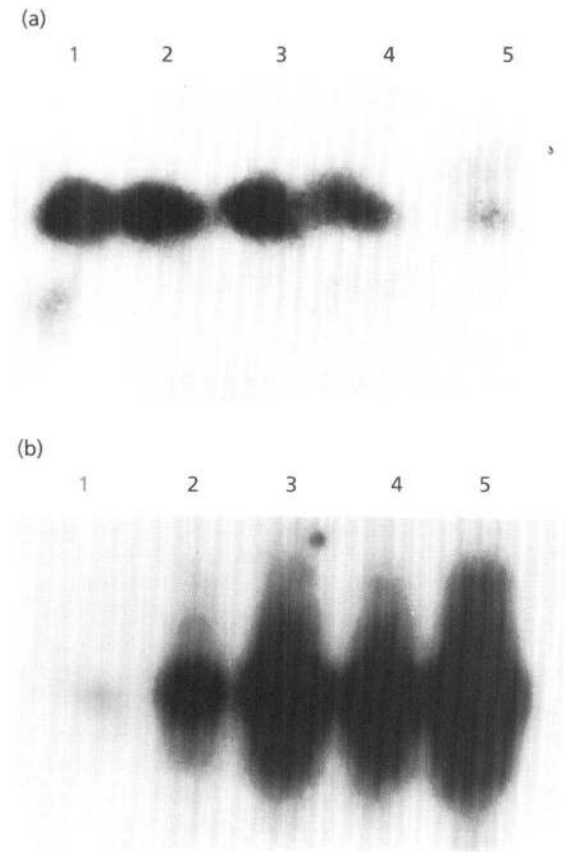

Fig. 8. Temporal studies of transcriptional response to the addition of copper to the medium. RNA was extracted at different time intervals after the addition of 1·0 μM copper to a culture of *Anabaena* sp. PCC 7120 that was grown in the absence of copper. (a) DNA:RNA blot hybridization results for an 18 μg sample of RNA reacted with a probe for *petJ*; (b) results for 12 μg RNA reacted with a probe for *petE*. In (a), lanes 1–5 correspond to times of 0, 15, 30, 60 and 90 min after the addition of copper, respectively. In (b), lanes 1–5 correspond to times of 0, 30, 60, 90 and 120 min after the addition of copper, respectively.

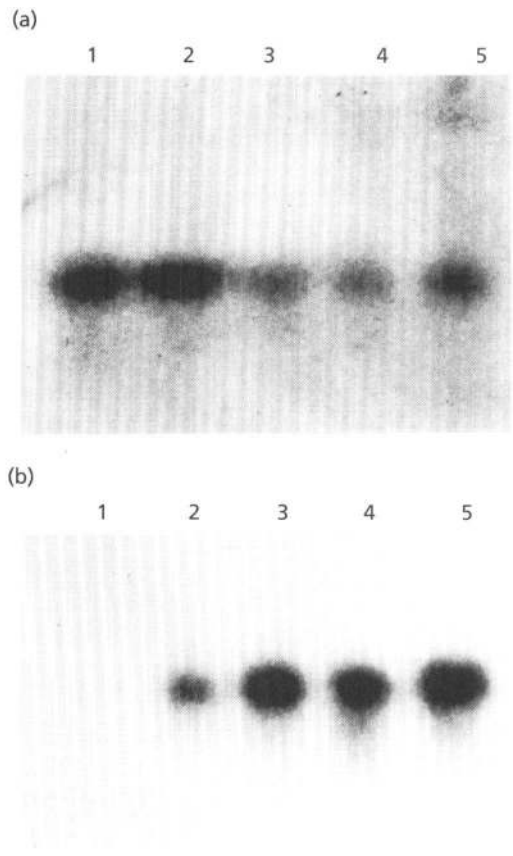

Fig. 9. Transcriptional regulation in response to copper concentration. RNA was extracted 3 h after the addition of various concentrations of copper to a culture of *Anabaena* sp. PCC 7120 that was grown in the absence of copper. (a) DNA:RNA blot hybridization results for a probe from *petJ*; (b) results for *petE*. Lanes 1–5 correspond to added copper concentrations of 0, 0·05, 0·1, 0·4 and 0·8 μM, respectively. Each lane represents the reaction of 15 μg total cellular RNA.

monocistronic operons whose expression is controlled by copper availability.

To examine the temporal aspects of transcriptional regulation, RNA was extracted at different times after the addition of copper to a culture of *Anabaena* sp. PCC 7120 that was grown in the absence of copper. The DNA:RNA hybridization reactions show that the expression of *petE* rapidly increased within a period of 30 min after the addition of copper (Fig. 8). The abundance of RNA from *petJ* decreased after 30 min, but only showed substantial reduction after 90 min; however, this temporal decrease in message abundance resulted from two factors, the down-regulation of the gene and the stability of the message. To better define the copper concentration responsible for the change in transcription of these two genes, different amounts of copper were added to cultures that were in mid-exponential phase growth in a medium lacking copper. RNA was isolated from each culture 3 h after the addition of copper and hybridized to each gene probe. The experiment was designed in this way to avoid changes in copper concentration in the medium that could result from long periods of cellular growth. Fig. 9 shows that the transition in expression of *petJ* occurred quite sharply between 0·05 and 0·10 μM copper; however, the expression of plastocyanin seemed to come on more gradually.

Concluding remarks

The presence of an alternative electron carrier for plastocyanin in many cyanobacteria and green algae suggests that copper deprivation may be a fairly common environmental phenomenon. Copper deficiency may be a simple result of low abundance of copper in the environment; however, it may also result from secondary effects of iron deprivation. Under the frequently occurring conditions of iron deprivation, bacteria and fungi produce siderophores to scavenge iron from the environment. However, siderophores also bind copper and exclude it from the cell. This enables iron-stressed cyanobacteria to

grow in environments of copper that would normally be toxic to cellular growth (Boyer *et al.*, 1987). In fact, the copper–siderophore complex appears to be the major copper species present in iron-stressed cyanobacterial blooms (McKnight & Morel, 1980). Because of this, the relationship between iron stress and copper availability should be re-examined as one of a number of factors that may have led to the loss of plastocyanin from many strains of cyanobacteria (Sandmann, 1986).

However, in other cyanobacteria and many eukaryotic algae, cytochrome *c*-553 (*c*-552) is an alternative electron donor to P-700, which replaces plastocyanin under conditions of copper deprivation. This report shows that the expressions of both cytochrome *c*-553 and plastocyanin in *Anabaena* sp. PCC 7120 are regulated at the transcriptional level, as has been reported for *Anabaena* sp. PCC 7937 (Bovy *et al.*, 1992), *Synechocystis* sp. PCC 6803 (Zhang *et al.*, 1992), and for the green alga, *Pediastrum boryanum* (Nakamura *et al.*, 1992). However, since the expression of *petE* has also been reported to be constitutive in *Synechocystis* sp. PCC 6803, it appears that more work is required in this organism to resolve the contradictory evidence (Briggs *et al.*, 1990).

ACKNOWLEDGEMENTS

This work was supported by a grant to N. A. Straus from the Natural Sciences and Engineering Council of Canada. We would like to thank Junyoung Park for helping in the preparation of this manuscript.

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Received 6 July 1993; revised 10 November 1993; accepted 24 November 1993.