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The *rpoD1* gene of *Synechococcus* sp. strain PCC 7942 encodes the principal sigma factor of RNA polymerase

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RNA polymerase was purified from the unicellular cyanobacterium, *Synechococcus* sp. strain PCC 7942, and found to be associated with a 52 kilodalton (kDa) polypeptide. The determined N-terminal sequence of the polypeptide was identical to the predicted amino-acid sequence of the *rpoD1* gene product. Furthermore, the *rpoD1* gene is suggested to be indispensable for viability by the inability to disrupt the gene. These results indicate that the *rpoD1* gene product is the principal sigma factor of RNA polymerase.

Key Words——amino acid sequence; cyanobacterium; principal σ factor; purification; RNA polymerase; *rpoD1* gene; *Synechococcus* sp.

Cyanobacteria are a group of divergent eubacteria which are specified by their ability to perform oxygenic photosynthesis. Previously, cyanobacteria were regarded as a group of eukaryotic algae due to their plant-type photosynthesis. However, an analysis of basic cellular machinery including RNA polymerase clearly indicates that they belong to the eubacterial phyla.

RNA polymerases of eubacterial strains have been extensively purified, and found to share common subunit structures comprised of α, β, β′ and σ subunits (Burgess, 1975). The α, β and β′ subunits assemble into an $\alpha_2\beta\beta'$ tetramer, and this complex is responsible for the basic enzyme activity of RNA polymerase (core enzyme). The σ subunit donates the core enzyme promoter recognition specificity (Helmann and Chamberlin, 1988; Lonetto et al., 1992). Although multiple and heterogeneous σ species have been found to modulate transcriptional specificity, a predominant and essential σ subunit is comprised of a single molecular species, and is called the principal σ subunit.

The purification and analyses of RNA polymerases from cyanobacteria have revealed a characteristic subunit structure of the core enzyme (Curtis and Martin, 1994; Schneider and Haselkorn, 1988; Schneider et al., 1987). That is, a subunit corresponding to the β′ subunit of other eubacteria has split into two parts, thus forming γ and β′ subunits. Such a characteristic has also been found for the RNA polymerase of plant chloroplasts, indicating that these two genetic systems are closely related evolutionally (Bergsland and Haselkorn, 1991; Hu and Bogorad, 1990; Hu et al., 1991; Schneider and Haselkorn, 1988).

Thus far, sigma factor genes have been identified from at least four cyanobacterial strains. In *Anabaena* sp. strain PCC 7120, a gene (*sigA*) encoding the principal σ subunit was cloned and analyzed (Brahamsha and Haselkorn, 1991). Two other genes (*sigB* and *sigC*) encoding closely related sigma factors have also been identified in this strain (Brahamsha and Haselkorn, 1992). In *Synechococcus* sp. strain PCC 7002, cloning and analysis of the *sigA* gene encoding the principal σ subunit was recently reported (Caslake and Bryant, 1996). More recently, the genome analysis of *Synechocystis* sp. strain PCC 6803 identified several σ subunit genes on its genome (Kaneko and Tabata, pers. comm.). In a previous study, we identified four closely related sigma factor genes from the chromosome of *Synechococcus* sp. strain PCC 7942 (Tanaka et al., 1992a). Since one of the genes (*rpoD1*) shares great similarity with the *sigA* gene of *Anabaena* sp. strain PCC 7120, we have postulated that this gene encodes the principal σ subunit of this strain (Tanaka et al., 1992b). In this study, we purified the RNA polymerase from *Synechococcus* sp. strain PCC 7942, and present evidence that the *rpoD1* gene product is in fact the principal σ factor.

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Materials and Methods

Bacterial strains, plasmids and recombinant DNA. Synechococcus sp. strain PCC 7942 was provided by Dr. R. Rippka. Recombinant techniques were performed as described in a manual (Sambrook et al., 1989), and *Escherichia coli* XL1blue was used as the host strain. pNA1 was as described previously (Tanaka et al., 1992a).

Assay of RNA polymerase. The reaction mixture for RNA polymerase activity contained, in a total volume of 40 μl, 50 mM Tris–HCl (pH 7.9), 0.05 mM EDTA, 0.5 mM dithiothreitol (DTT), 10 mM $MgCl_2$, 0.5 mM each of ATP, CTP, GTP, and UTP, 0.65 μCi [5, 6-^{3}H]UTP (TRK412, Amersham, Buckinghamshire, U.K.), 4 μg poly[dAdT] (Sigma Chemical Co., St. Louis, MO, U.S.A.) and 5 μl enzyme solution. The mixture was incubated at 37°C for 10 min. The reactions were spotted onto Whatman GF/C filters and washed three times with 5 ml of 5% trichloroacetic acid (TCA). The filters were then dried and counted in toluene base scintillation fluid. One unit of RNA polymerase activity was defined as the activity corresponding to the incorporation of 1 nmol of UMP in 10 min at 37°C to the TCA insoluble fraction.

Purification of RNA polymerase. The purification procedures for RNA polymerase from *Synechococcus* sp. strain PCC 7942 were followed by the methods used for the RNA polymerases of *Anabaena* sp. strain PCC 7120 (Schneider et al., 1987) and *Synechococcus* sp. strain PCC 6301 (Kumano et al., 1986) with modifications. *Synechococcus* sp. strain PCC 7942 cells were cultivated in BG-11 medium at 30°C under continuous fluorescent lights and with aeration. Cells were harvested when the culture reached an OD_{750} of 0.8 by centrifugation, washed in buffer A (20 mM Tris–HCl (pH 7.9), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 1 mM phenylmethylsulfonyl fluoride and 5% glycerol) and frozen at −80°C until use. The frozen cells (40 g wet weight) were thawed and suspended in 120 ml buffer A. Lysozyme (30 mg) was added to the cell suspension and stirred for 30 min at room temperature. The suspension was cooled on ice and subsequent procedures were carried out at 4°C. After the addition of a 6 ml Brij solution (0.1 M $MgCl_2$, 1 mM DTT and 2% Brij-58), the cells were sonicated with a Branson sonifer (Cell Disrupter 200; total 3 min by 50% pulse). The lysate was centrifuged for 90 min at 100,000×*g* and the upper three-quarters of the supernatant was taken as the high-speed supernatant. The RNA polymerase was precipitated by the addition of polyethyleneimine (PEI, Sigma) to 0.05%, recovered by centrifugation (15 min at 10,000×*g*), washed three times with buffer A containing 0.1 M NH_4Cl and dissolved in 50 ml of buffer A containing 0.9 M NH_4Cl. After spinning down, proteins in the supernatant solution containing the RNA polymerase were precipitated by 60% saturation of $(NH_4)SO_4$ and recovered by centrifugation. The precipitate was resuspended in 10 ml of buffer A containing 0.6 M NH_4Cl and dialyzed against the same buffer (PEI eluate). Gel filtration was performed on a 2×90 cm Sephacryl S-300HR (Pharmacia, Uppsala, Sweden) column in the same buffer. The peak activity was pooled, concentrated to 1 ml using a Centricon-100 (Amicon, Gloucester, U.K.) and dialyzed against buffer A (Sephacryl pool). The protein solution was loaded onto a 1×6 cm heparin agarose (Sigma) column, washed with 20 ml of buffer A containing 0.1 M KCl and eluted with 5 ml of buffer A containing 0.5 M KCl. The eluate was concentrated to 300 μl using a Centricon-100 and dialyzed against buffer A containing 50% glycerol (heparin agarose pool) for storage at −20°C.

N-Terminal amino-acid sequencing. One milligram of the purified RNA polymerase was separated by 12.5% polyacrylamide gel electrophoresis and blotted onto a PVDF membrane (Immobilon-P, Millipore Waters, Watford, U.K.) as described previously (Dunn, 1989). A band containing the 52-kDa protein was excised, and the N-terminal amino-acid sequence was determined by the Applied Biosystem Japan Co. (Tokyo, Japan).

Gene disruption experiment. Oligonucleotide DNAs, 5′-ACTCAGGCCACCGAACTACTCGAC-3′ and 5′-GCCGATTTCTTCCAGCGTTTTCAT-3′, were synthesized on an Applied Biosystems Model 392 DNA synthesizer. Polymerase chain reaction (PCR) was carried out for 25 cycles with pNA1 (Tanaka et al., 1992a) as a template. Each cycle was 95°C for 1.5 min, 50°C for 2 min, and 72°C for 3 min. The amplified fragment was cloned in pCRII (TA cloning kit, Invitrogen), to make pTAD1-NC. Cyanobacterial transformation was performed as described previously (Golden et al., 1987), and selected on BG-11 plates containing 20 μg/ml kanamycin.

Results and Discussion

Purification and characterization of RNA polymerase from Synechococcus sp. strain PCC 7942

In a previous study, we cloned a putative principal sigma factor gene (*rpoD1*) from the chromosome of *Synechococcus* sp. strain PCC 7942 using an oligonucleotide probe designed from the conserved amino-acid sequence motif among principal σ factors (*rpoD* box) (Tanaka et al., 1988, 1992a, 1992b). To obtain direct evidence that the *rpoD1* gene product is the principal σ subunit of this bacteria, we purified DNA-dependent RNA polymerase from cyanobacterial cells. The procedure used to purify the *Synechococ-*

cus RNA polymerase consisted of techniques of PEI precipitation and elution of DNA protein complex, gel filtration through a Sephacryl S-300HR column and heparin agarose affinity chromatography. The yields of enzyme activity and purification factors at each step are summarized in Table 1. RNA polymerase Activity in the high-speed supernatant fraction was considered not to be assayed precisely because of the presence of nuclease activity or inhibitory substances. Finally, we obtained 2 mg of RNA polymerase enzyme purified to 1,375-fold starting from 40 g of wet cells. The overall yield was estimated to be 19% given the activity in PEI eluate as 100%. The optimal salt concentration of the purified RNA polymerase was examined by assaying the activity at various KCl concentrations (from 0 to 125 mM). It was found that 50 mM KCl in the assay mixture is optimal for the activity when poly[dAdT] is used as a template (data not shown).

The purified RNA polymerase holoenzyme contained the rpoD1 gene product

The purified enzyme was analyzed using SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). By analogy to RNA polymerases from other cyanobacterial strains, the β′, β, γ, and α subunits comprising the core enzyme were identified, and the molecular

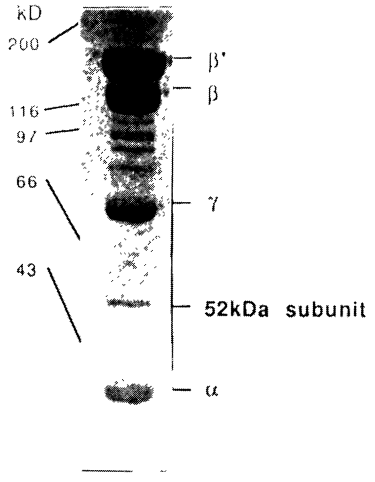
Table 1. Purification of *Synechococcus* RNA polymerase.

Purification step	Volume (ml)	Total protein[a] (mg)	Total units[b]	Specific activity (units/mg)	Yield[c] (%)	Purification factor (fold)
High-speed supernatant	74	1,751	654	0.4	—	1
PEI eluate	10	24	5,905	246	100	615
Sephacryl pool	1	3.6	1,448	406	25	1,005
Heparin agarose pool	0.3	2.0	1,100	550	19	1,375

[a] Protein amounts were estimated using a protein assay kit (Bio-Rad Laboratories, Richmond, CA, U.S.A.). Bovine serum albumin was used as the standard.

[b] One unit of RNA polymerase activity was defined as the activity corresponding to the incorporation of 1 nmol of UMP in 10 min at 37°C to the TCA insoluble fractions.

[c] Activity in the PEI eluate was assumed to be 100%.



(A)

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52 kDa Subunit     x  Gln Ala Thr Glu Leu  x  Asp  x  Ala Leu Lys
RpoD1         Met Thr Gln Ala Thr Glu Leu Leu Asp Pro Ala Leu Lys
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(B)

Fig. 1. Association of the *rpoD1* gene product with the purified RNA polymerase.

(A) SDS-polyacrylamide gel electrophoresis of the purified enzyme. The positions of the molecular weight markers and the RNA polymerase subunits are indicated. (B) N-end sequence analysis of the 52-kDa protein. The determined amino-acid sequence of the 52-kDa protein is aligned with that deduced from the nucleotide sequence of the *rpoD1* gene. x denotes the amino-acid residue unidentified by the sequencing analysis.

weights were estimated to be 190, 126, 68, and 39.5-kDa, respectively, consistent with previous reports. In addition to these bands, several minor bands were detected. Among them, one band of 52-kDa was assumed to be the *rpoD1* gene product (Fig. 1A), which was expected to correspond to the principal σ subunit (Schneider and Haselkorn, 1988; Schneider et al., 1987; Tanaka et al., 1992b). Other bands between the β and γ subunits of more than 80-kDa were certainly not the *rpoD1* gene product because of their sizes. Furthermore, it is highly unlikely that any one of them is σ-like protein because they are significantly larger than the σ^{70} of *E. coli*, which has an exceptionally large size among eubacterial principal σ factors. Therefore, we assumed they were degradation products of either β′ or β subunits.

To characterize the 52-kDa protein, the N-terminal sequence of the protein was determined by an automated gas-phase protein sequencer. Since the deduced amino-acid sequence was identical to the predicted *rpoD1* gene product except for the N-terminal methionine (Fig. 1B), we concluded that the active RNA polymerase preparation from *Synechococcus* sp. strain PCC 7942 contains a significant amount of the *rpoD1* gene product, and is the principal σ subunit. The calculated molecular weight of the *rpoD1* gene product is 44,045 (Tanaka et al., 1992b), and is smaller than the mobility determined by SDS-polyacrylamide gel electrophoresis, as is usually observed with various σ factor proteins. Since the molar ratio of the *rpoD1* gene product to the core enzyme was less than one, the purified RNA polymerase appeared to be a mixture of core and holoenzyme.

Disruption experiment of the rpoD1 gene

Given the *rpoD1* gene product to be the principal σ subunit, this gene must be essential for viability. To examine the indispensability of the *rpoD1* gene, gene disruption experiments were performed using an integration vector pCRII, which can be replicated in *E. coli* but not in cyanobacteria. As shown in Fig. 2, pCRII plasmid has two antibiotic-resistant genes, *kan*^{r} and *amp*^{r}. A cyanobacterial DNA fragment cloned in pCRII is expected to be used as the homologous sequence to integrate into the cyanobacterial chromosome via Campbell-type integration. When pCRII derivatives having cyanobacterial sequences were transformed to cyanobacteria, Kan^r clones, which integrated pCRII into the homologous chromosomal region, were recovered on the selection plates. We have already known that this plasmid integrates at a significant frequency into dispensable genes and causes an expected disruption of the genes (data not shown).

To examine if the *rpoD1* gene can be disrupted by pCRII integration, a DNA fragment (1,044-bp) corresponding to the internal sequence of the *rpoD1* open reading frame was PCR-amplified, and cloned in an *E. coli* plasmid, pCRII (Fig. 2). This plasmid, pTAD1-NC, was used to transform the cyanobacteria. Since pCRII does not replicate in cyanobacteria, only recombinants via a Campbell-type integration within the *rpoD1* sequence can survive as Kan^r. Such an integration was expected to disrupt the intact gene. No stable transformant was obtained on selection plates. Although colonies as small as pin holes appeared occasionally, we failed to isolate them by replica plating. Therefore, the *rpoD1* gene was assumed not to be used as the integration site of the plasmid, which would result in the disruption of the *rpoD1* gene. This result, suggesting the indispensability of the *rpoD1* gene, supports our belief that the gene product is the RNA polymerase principal σ subunit.

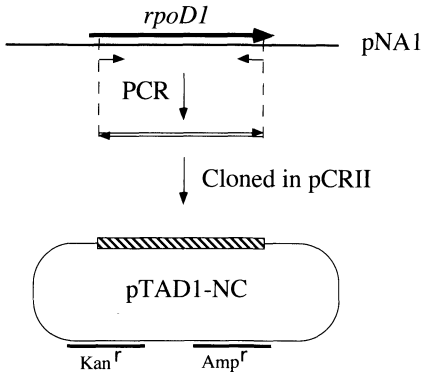

Fig. 2. Construction of pTAD1-NC used for the gene disruption experiment.

(A) A DNA fragment corresponding to the internal sequence of the *rpoD1* open reading frame was PCR-amplified, and cloned in pCRII. This plasmid contains both kanamycin and ampicillin resistance markers.

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