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The *sphR* Product, a Two-Component System Response Regulator Protein, Regulates Phosphate Assimilation in *Synechococcus* sp. Strain PCC 7942 by Binding to Two Sites Upstream from the *phoA* Promoter

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In the photosynthetic cyanobacterium *Synechococcus* sp. strain PCC 7942, the *sphS* and *sphR* genes were previously suggested to encode a typical pair of two-component signal transduction proteins. A deletion mutant strain lacking these genes failed to exhibit induction of alkaline phosphatase, the *phoA* gene product, in response to phosphate limitation in the medium. The SphR protein was overexpressed in *Escherichia coli* and then purified to near homogeneity. A truncated form of the SphS polypeptide (named SphS*) was also isolated. Here, we demonstrate that purified SphR is phosphorylated by phosphotransfer from SphS and binds to two distinct sites upstream from the *phoA* promoter. From these results, we conclude that the SphS and SphR proteins are directly involved in the regulation of *phoA* transcription in response to phosphate limitation in *Synechococcus* species.

The cyanobacteria would seem to be the organisms of choice for the study of fundamental processes such as oxygen-evolving photosynthesis, nitrogen fixation, and inorganic carbon and phosphate assimilation (for a review, see reference 6 and references therein). As is common in free-living microorganisms, the cyanobacterial cells must constantly monitor external conditions and adjust their structure and physiology accordingly. In this respect, intensive studies on other bacteria such as *Escherichia coli* and *Bacillus subtilis* revealed that they have evolved common and sophisticated signaling systems for eliciting responses adaptive to their environment (for reviews, see references 19 and 20 and references therein). These adaptive response systems, the so-called two-component systems, involve two families of signal transduction proteins, each of which displays striking sequence (or domain) similarity. One family (sensory kinase) monitors some environmental parameters, and the other (response regulator) mediates changes in gene expression or locomotion in response to external signals.

In the cyanobacteria, putative open reading frames that could encode proteins homologous with the bacterial response regulator family were noted previously for *Synechocystis* sp. strain PCC 6803 and *Synechococcus* sp. strain PCC 7002, respectively (4, 24). In another cyanobacterium, *Anabaena* sp. strain 7120, a mutation implicated in the control of heterocyst formation was reported to be mapped to the *patA* gene (11), whose product resembles an *E. coli* member of the response regulator family, CheY (19). In regard to this, we have recently reported the occurrence of a pair of signal transduction proteins in a unicellular cyanobacterium (*Synechococcus* sp. strain PCC 7942), in which the genes encoding a typical pair of sensory kinase and response regulator proteins have been identified (1). These gene products (named SphS and SphR) were suggested to be involved, either directly or indirectly, in the signal transduction mechanism underlying a response to aberrant phosphate limitation in this microorganism. Although, as in other prokaryotes, phosphate limitation significantly affects cyanobacterial cell growth, studies on phosphate regulation are still at an early stage for the cyanobacteria (3, 5, 8, 9). In this study, we demonstrate that the SphR protein has a direct role in the regulation of *phoA*, encoding alkaline phosphatase, by showing that SphR binds to two sites upstream from the *phoA* promoter in *Synechococcus* sp. strain PCC 7942.

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MATERIALS AND METHODS

Materials. DNA-manipulating enzymes were all purchased from Takara Shuzo Co. and Toyobo Ltd. [γ-^{32}P]ATP (222 TBq/mmol) and [α-^{32}P]dCTP (110 TBq/mmol) were obtained from Amersham International. All other materials were of reagent grade.

Bacterial strains and culture conditions. *Synechococcus* sp. strain PCC 7942 was kindly provided by T. Omata (Nagoya University). This bacterium was photoautotrophically grown at 30°C in BG-11 medium (liquid or solid containing 1.5% agar) containing 20 mM TES-KOH [N-Tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid] (pH 8.0) (22). For liquid cultivation, cultures were continuously aerated. When required, kanamycin was added at a concentration of 30 μg/ml. For phosphate deprivation experiments, cells were grown in BG-11 medium to the mid-logarithmic growth phase. The harvested cells were washed in phosphate-free BG-11 medium (K_2HPO_4 was replaced by KCl) and then resuspended and incubated in the same medium. *E. coli* K-12 strains used were as follows: JM83 (*ara Δlac pro rpsL thi* φ80d *ΔlacZ15*) (18) and DZ225 [F$^-$ *ΔenvZ ΔlacU169 araD139 rpsL relA flbB thiA* φ(*ompC-lacZ*$^+$)10-25] (17). These strains were grown in Luria broth unless otherwise indicated (16).

Measurement of phosphatase activity. Alkaline phosphatase activity in *Synechococcus* sp. strain PCC 7942 was measured as described previously (1). Alkaline phosphatase activity was expressed as arbitrary units.

DNA analysis. Genomic DNA was prepared from *Synechococcus* sp. strain PCC 7942 as described previously (28). Sequencing of double-stranded DNA on plasmid pUC19 was carried out by the dideoxy chain termination method with a commercially available sequencing kit (Takara Shuzo Co.)

(23). The generation of successively shortened DNA subclones was performed by using a Kilo-Sequence Deletion Kit (Takara Shuzo Co.). Other recombinant DNA techniques were carried out in accordance with a conventional laboratory manual (15).

Purification of SphR and SphS*. The SphR protein was purified as follows. *E. coli* JM83 transformed with plasmid pAS21 was grown until the late-logarithmic growth phase in the presence of ampicillin (50 μg/ml). To 10 g of cells was added 30 ml of sodium phosphate buffer (100 mM, pH 7.1) containing 5 mM $MgSO_4$ and 2 mg of DNase I. The suspension was passed through an AMINCO French pressure cell (FA 073) twice at 10,000 lb/in^2 and centrifuged at 100,000 × *g* for 3 h at 4°C to isolate the clear supernatant fraction. The volume of the suspension fraction was brought up to 150 ml by addition of 50 mM sodium phosphate buffer (pH 7.1) containing 2.5 mM $MgSO_4$. Solid ammonium sulfate was added to 45% saturation at 4°C. Precipitates formed were recovered by centrifugation and then dissolved in 10 ml of a buffer containing 20 mM Tris-HCl (pH 7.8) and 2 mM 2-mercaptoethanol. After dialysis against the same buffer, the sample was applied onto a DEAE column (1.5 by 20 cm) (Whatman DE52) previously equilibrated with the Tris-HCl buffer (pH 7.8). Proteins were eluted with a 150-ml linear NaCl gradient from 0 to 300 mM. SphR-containing fractions were eluted at around 100 mM NaCl. This sample was further applied onto a heparin column (1.5 by 20 cm) (Pharmacia CL-6B) under the same buffer conditions as described above. Proteins were eluted with a 150-ml linear NaCl gradient from 0 to 700 mM. SphR-containing fractions, which were essentially free from other proteins, were eluted at around 230 mM NaCl. The pooled peak fractions from this column were used directly as the purified SphR protein. During the purification, the SphR protein was monitored by means of sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Purification of a truncated form of SphS (named SphS*) was carried out as described previously (1). These purified proteins, SphR and SphS*, were stably stored at −20°C in Tris-HCl buffer (pH 7.8) containing 50% glycerol.

DNA-binding assay. DNA-binding assays by nondenaturing polyacrylamide gel electrophoresis were carried out as described previously (10).

Phosphorylation experiments. The procedures were essentially the same in detail as those described previously (1, 2, 18).

Footprinting experiments. DNase I footprinting was carried out essentially as described previously (7). A ^{32}P-labeled DNA fragment (10^5 cpm, 0.24 pmol) was mixed with the purified SphR protein (0.3 to 1.5 μg), before incubation at 25°C for 15 min in a buffer (35 μl) of 50 mM Tris-HCl (pH 7.8) containing 3 mM magnesium acetate, 5 mM $CaCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, and bovine serum albumin (0.0025%). Then, the reaction mixture was treated with DNase I (0.1 μg/ml) in the presence of 15 mM NaCl at 25°C for 1 min. The digests were precipitated with ethanol and then analyzed on an 8% polyacrylamide sequencing gel (15).

Amino acid sequencing. Amino acid sequencing was performed with an automated amino acid sequencer (Applied Biosystems model 447A).

RESULTS AND DISCUSSION

A pair of sensory kinase and response regulator (SphS and SphR) proteins is involved in inducible production of alkaline phosphatase in response to phosphate limitation. We previously provided evidence for the occurrence of a bacterial two-component regulatory system that is implicated in the phosphotransfer signaling in *Synechococcus* sp. (1). This regulatory system consists of two regulatory genes (*sphR* and *sphS*), which possibly constitute a single transcriptional unit (or operon), as schematically shown in Fig. 1. The upstream gene (*sphR*) encodes a protein of 29,012 Da, the amino acid sequence of which shows a high degree of homology to those of members of the response regulator family, and the downstream one (*sphS*) encodes a protein of 46,389 Da, which appears to belong to the sensory-kinase family. Our preliminary results with regard to the possible physiological function of *sphR-sphS* suggested that these genes may be involved in an adaptive response to limitation of external phosphate (1). To confirm this assumption, we measured alkaline phosphatase (PhoA) activity expressed in cells grown in a phosphate-limited medium, since this particular enzyme was reported to be induced in response to phosphate limitation in *Synechococcus* sp. strain PCC 7942 (3, 21). As shown in Fig. 2, PhoA activity was indeed induced in the wild-type cells in response to phosphate limitation, while an *sphR-sphS* deletion strain (HAI140) exhibited a very low level of PhoA activity even after prolonged incubation in the phosphate-limited medium. In HAI140, a chromosomal region encompassing both the *sphR* and *sphS* genes was replaced by the *neo* (kanamycin resistance) gene (Fig. 1). However, when an *E. coli-Synechococcus* sp. shuttle vector carrying both the *sphR* and *sphS* genes was introduced into HAI140, PhoA activity was normally induced (Fig. 2), as in the case of the wild-type cells. It was thus reasonable to assume that the transcription of *phoA* is regulated through the functions of the *sphR-sphS* gene products.

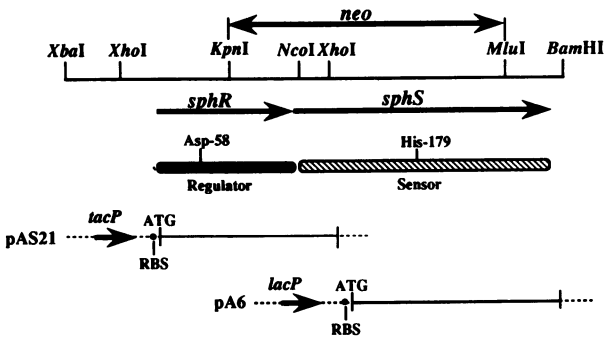

FIG. 1. Restriction map of the *Synechococcus* sp. strain PCC 7942 chromosomal DNA encompassing the *sphR* and *sphS* genes. The coding regions of *sphR* and *sphS* are indicated by arrows. Their predicted products, SphR (response regulator) and SphS (sensory kinase), are also schematically shown, and relevant amino acid residues are indicated. In a mutant strain (HAI140), the region encompassing both the *sphR* and *sphS* genes was replaced by the *neo* (kanamycin resistance) gene, as indicated. The structures of *E. coli* plasmids (pAS21 and pA6) are schematically shown. These were used for isolation of the corresponding SphR and SphS polypeptides in *E. coli.* RBS, ribosome binding site.

Purification of SphR and SphS. To facilitate biochemical study of SphR and SphS, these proteins were purified to near homogeneity in large quantities, as judged by SDS-polyacrylamide gel electrophoresis (data not shown). This was achieved by employing a set of recombinant plasmids as well as *E. coli* as hosts (Fig. 1). Plasmid pAS21 was constructed, in which the entire *sphR*-coding sequence was placed under the control of the *E. coli tac* promoter and an ideal ribosome-binding sequence. Another plasmid, pA6, carried a *lac* promoter-controlled gene encoding a fusion protein, of which the N-terminal 16 amino acids from *E. coli* β-galactosidase are followed by the SphS sequence extending from Asp-53 to the C-terminal

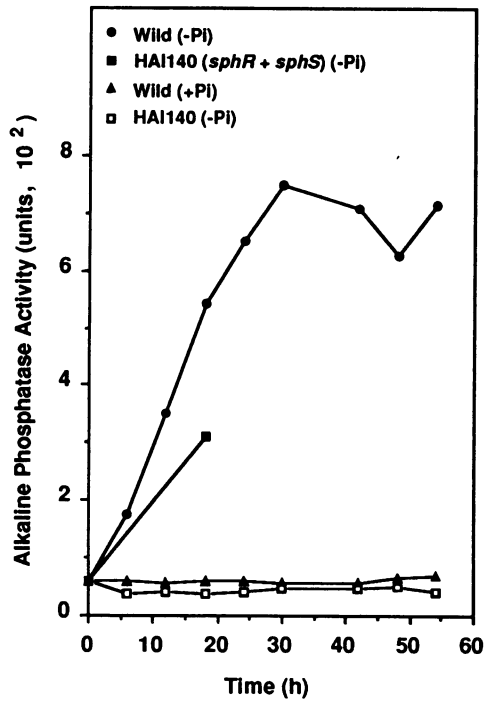

FIG. 2. Induction of alkaline phosphatase activity. The wild-type strain of *Synechococcus* sp. strain PCC 7942 and its *sphR-sphS* deletion derivative (HAI140) were incubated in BG-11 medium in the presence (+Pi) and absence (−Pi) of a phosphate source. HAI140 harboring a plasmid carrying both the *sphR* and *sphS* genes (1) was also examined. The time course of expression of alkaline phosphatase was measured, as described previously (1).

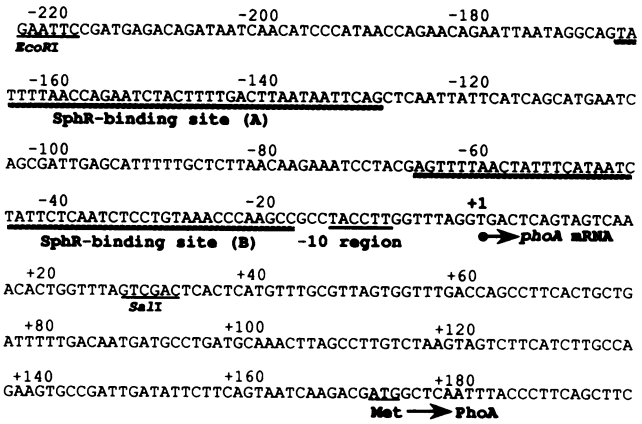

FIG. 3. The structure of the *phoA* promoter region. Characteristic features revealed for the *phoA* promoter in which an extended nucleotide sequence for the upstream region was newly determined in this study are summarized. The transcription start site of *phoA* was adopted from the results of Ray et al. (21). The −10 region was tentatively assigned, as indicated. The region upstream of the −10 region contains two separate SphR-binding sites (A and B), as indicated. The translation initiation codon (ATG) for PhoA is shown.

Pro-415. The pA6 plasmid was constructed previously to produce a truncated form of SphS (named SphS*) in *E. coli* (1) (Fig. 1). It should be noted that the recombinant *sphR* gene used was inferred to encode the SphR polypeptide, in which the second amino acid (Thr-2) from the N-terminal Met-1 was replaced by Asn. This replacement was due to the introduction of a unique restriction site (*Eco*RI) into the *sphR*-coding sequence. In any case, these purified polypeptides were confirmed to correspond to SphR and SphS* by means of amino acid sequencing of their N-terminal regions.

SphR is a DNA-binding protein. Considering the fact that SphR has an amino acid sequence highly homologous to those of members of the response regulator family (e.g., *E. coli* PhoB and OmpR) (25), it was supposed to be a DNA-binding transcriptional regulator. To extend these lines of study at the molecular level, the *phoA* gene needed to be cloned from *Synechococcus* sp. strain PCC 7942, since this gene is the only cyanobacterial gene involved in phosphate metabolism characterized so far (21). An extensive study of the *phoA* gene, carried out by Ray et al. (21), revealed the following: (i) a high level of transcript of *phoA* accumulates after 8 to 12 h of phosphate deprivation, (ii) the nucleotide sequence of *phoA* was determined for its 5′ noncoding region as well as its coding sequence, and (iii) the putative transcription start site was also assigned in the 5′ noncoding sequence determined. In this study, the *phoA* gene was cloned with the aid of the restriction map which was reported previously (21). Analysis of its DNA sequence as well as its restriction map revealed that a 1.9-kb *Eco*RI-*Xba*I fragment, thus cloned, indeed encompasses the 5′ region of the *phoA*-coding sequence and its upstream sequence (data not shown). Only a 134-bp sequence has been reported for the upstream region of the putative transcription start site of *phoA* (21). As demonstrated below, however, a relatively long upstream sequence was suspected to be required for *phoA* to be fully regulated in response to an environmental stimulus. Thus, an extended nucleotide sequence was newly determined for its further upstream region (Fig. 3). It is also worth mentioning that our sequence of the *phoA* promoter region differs from the one reported by Ray et al. (21); i.e., only a single G nucleotide, instead of GG nucleotides, was found at position −67. (The nucleotide sequence data will appear in the DDBJ, EMBL, and GenBank nucleotide sequence data bases under the accession number D26162.) In any case, to demonstrate that SphR is able to specifically bind somewhere around the *phoA* promoter, a DNase I footprinting analysis with purified SphR was carried out (Fig. 4). The results revealed that SphR protected, against DNase I digestion, relatively large portions of the *phoA* promoter sequence, which were located immediately upstream of the transcription start site of *phoA*. Apparently, two distinct sites (Fig. 4, sites A and B), each consisting of 40 to 50 nucleotides (Fig. 3), were protected. It was thus concluded that the SphR protein binds directly to the extended upstream region of the *phoA* promoter, suggesting that this region serves as a *cis*-acting regulatory element for *phoA* transcription. SphR appears to be a positive regulator for the *phoA* gene, since a lack of this protein in cells results in the disappearance of *phoA* expression.

As previously noted by Ray et al. (21), no typical *E. coli*-like promoter consensus sequences have been found around the putative transcription start site of the *Synechococcus phoA* gene (Fig. 3). On the basis of the results presented here, however, the structural features of the *phoA* promoter were clarified. First of all, although the canonical −35 region could not be assigned in the *phoA* promoter, the sequence TACCAT can be assigned as an *E. coli*-like −10 consensus sequence (TATAAT). Second, the results of DNase I footprinting indicate that SphR covers two relatively long sequences located upstream of the −10 region. When the transcription start site is taken as nucleotide position +1, one SphR-binding sequence (site A) extends from −167 to −130 and the other

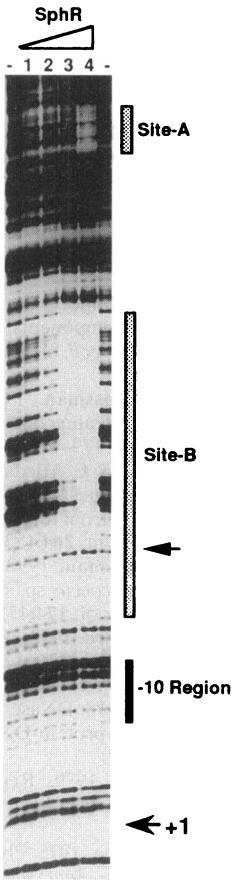

FIG. 4. DNase I footprinting of the *phoA* promoter region with the SphR protein. The *Eco*RI-*Sal*I fragment encompassing the *phoA* promoter region (positions −223 to +27 in Fig. 3) was isolated and then end labeled by ^{32}P at the 3′ end of the *Sal*I site (the nontranscribed strand in Fig. 3 was radioactively labeled). Using this labeled fragment, a DNase I footprinting experiment with the purified SphR protein was carried out. The amounts of SphR used were as follows: none (−); or 0.3, 1.5, 6, and 15 μg (lanes 1 to 4, respectively). To determine the position of nucleotides, products of the Maxam-Gilbert G cleavage reaction with the same labeled fragment were also electrophoresed side by side (data not shown). The relevant regions in the *phoA* promoter are schematically indicated (Fig. 3). An enhanced band, observed in site B, is also indicated by an arrow.

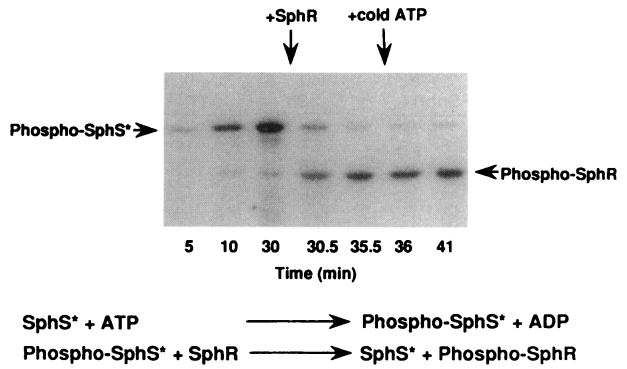

FIG. 5. Phosphotransfer between SphS* and SphR. The purified SphS* polypeptide (3 μg) was incubated with 0.05 mM [γ-^{32}P]ATP in a buffer for the indicated times under the same conditions as those described previously (2). SphR (3 μg) and subsequently cold ATP (1 mM) were added at the times indicated. At intervals, samples were taken and analyzed by SDS-polyacrylamide gel electrophoresis, followed by autoradiography. Inferred reactions are also schematically shown.

(site B) extends from −66 to −18. Interestingly, the sequence of site B seems to cover the region corresponding to the possible −35 region. There is the precedent of a similar situation in the case of a number of promoters belonging to the *E. coli pho* regulon. The *E. coli* PhoB-binding sites (called Pho-boxes) usually overlap with the possible −35 regions (14). In any case, it is reasonable to assume that SphR molecules bind immediately upstream of the −10 region and help RNA polymerase to trigger *phoA* transcription efficiently.

In vitro phosphotransfer between SphS and SphR. It is known that a histidine residue comprises the site of autophosphorylation in members of the sensory kinase family, whereas it is believed that an aspartate residue comprises the phosphoacceptor site in members of the response regulator family (for a review, see reference 26). In this regard, it was previously suggested that the isolated SphS* polypeptide exhibits autophosphorylation (or autokinase) activity in vitro (1) (Fig. 5). Here, it was further demonstrated that upon the addition of the purified SphR protein to the in vitro reaction mixture, the radiolabeled SphS* disappeared rapidly, whereas SphR was concomitantly radiolabeled. The radiolabeled SphR was rather stable even after prolonged incubation in the presence of excess nonradioactive ATP. When SphR alone was incubated in the presence of [γ-^{32}P]ATP, it was not radiolabeled (data not shown). These in vitro reactions observed for SphS* and SphR were interpreted as meaning that SphS* has autophosphorylation activity and is capable of phosphorylating SphR. These in vitro reactions are inferred to be as shown schematically in Fig. 5. Then, to examine the respective phosphorylation sites of SphS and SphR, radioactively phosphorylated SphS* and SphR were prepared in vitro under the conditions described above. They were subjected to acid or alkali treatment (data not shown). SphS*-phosphate was stable in both neutral and alkaline media, but was labile in acidic conditions, whereas SphR-phosphate was labile in alkaline conditions. The acid lability and alkaline stability observed for SphS*-phosphate are consistent with the view that it contains a histidinyl phosphate, and the opposite phenomena observed for SphR-phosphate suggest that it contains an acyl phosphate (26). More specifically, SphR appears to acquire a phosphoryl group at an aspartate residue (probably at Asp-58) from SphS, which is capable of undergoing autophosphorylation at a histidine residue (probably at His-179) (Fig. 1).

DNA-binding ability of phospho-SphR. Then, we examined the possible biochemical consequences of SphR phosphorylation. In particular, we compared the in vitro DNA-binding abilities of SphR under conditions where SphR was preincubated in the presence of SphS* with and without ATP. A 250-bp DNA fragment end labeled with ^{32}P, which encompasses the *phoA* promoter region and its upstream sequence, was incubated with these SphR preparations, and then subjected to a DNA-binding gel shift assay (Fig. 6). As expected, the purified SphR protein bound to the *phoA* promoter fragment. Even upon incubation of SphR with SphS* in the presence of ATP, however, the DNA-binding ability of SphR with respect to the *phoA* promoter sequence did not significantly change. Therefore, as far as the DNA-binding ability of SphR is concerned, we failed to detect a significant difference in nature between the in vitro-prepared phospho-SphR and the originally purified SphR protein. However, it should be

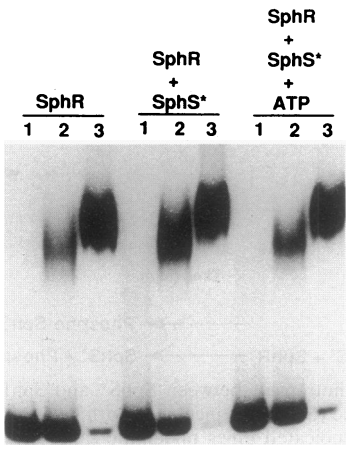

FIG. 6. DNA-binding analysis with phospho-SphR. The *Eco*RI-*Sal*I DNA fragment was end labeled with ^{32}P (0.024 pmol), which encompasses the *phoA* promoter region as shown in Fig. 3. This labeled fragment was incubated with SphR and SphS* (1.5 μg) in the absence and presence of ATP (0.1 mM). The amounts of SphR used are as follows: lane 1, none; lane 2, 1.5 μg; and lane 3, 6 μg. These samples were analyzed by nondenaturing polyacrylamide gel electrophoresis, followed by autoradiography.

considered that it is not certain at present whether the SphR protein we purified from *E. coli* represents the nonphosphorylated form of SphR, because there is in vitro and in vivo evidence that some response regulators can be phosphorylated rather nonspecifically by a low-molecular-weight phospho-donor (acetyl phosphate) in *E. coli* (12, 27). A portion of the purified SphR molecules should be in nonphosphorylated form, because they were radiolabeled in the presence of ATP by SphS* in vitro. Nevertheless, the majority of purified SphR molecules overexpressed in *E. coli* might have already been phosphorylated in vivo by the physiological phospho-donor. This possibility could not be ruled out. Clarification of the possible functional consequence of SphR phosphorylation must await further experiments.

Conclusion. In *E. coli*, the results of intensive studies on the phosphate regulon have established the importance of phosphotransfer signal transduction mediated by PhoR (sensory kinase) and PhoB (response regulator) in phosphate regulatory circuits (for a review, see reference 25). Through analogy with this *E. coli* system, together with the results presented in this paper, one can reasonably envision the following scenario. In *Synechococcus* sp. strain PCC 7942, SphS somehow monitors the availability of external phosphate. This putative phosphate sensor is capable of phosphorylating SphR, and consequently the function of SphR is modulated by the modification. Accordingly, SphR can directly regulate the level of *phoA* transcription in response to phosphate limitation. In this context, it is worth mentioning that the *Synechococcus* sensor (SphS) appears to be located in the cytoplasm, since this sensor lacks stretches of hydrophobic amino acids in its primary sequence (1). On the contrary, the corresponding *E. coli* sensor (PhoR) is in the cytoplasmic membrane (13). Therefore, despite an apparent analogy between the *E. coli* and *Synechococcus* phosphate regulatory circuits, the mechanisms underlying sensing of phosphate limitation in these cells may not necessarily be the same. This particular issue remains to be addressed.

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