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Phosphorylation of a bacterial activator protein, OmpR, by a protein kinase, EnvZ, stimulates the transcription of the *ompF* and *ompC* genes in *Escherichia coli*

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The OmpR of *Escherichia coli* is a positive regulator specific for the *ompF* and *ompC* genes, which encode outer membrane proteins OmpF and OmpC, respectively. The EnvZ protein is a protein kinase which specifically phosphorylates the OmpR protein. In this study, the results of in vitro transcription experiments revealed that the phosphorylation of the OmpR protein by the EnvZ protein stimulates the transcription of both the *ompF* and *ompC* genes.

Activator protein; OmpR; Kinase; EnvZ; Phosphorylation; Transcription activation; (*E. coli* outer membrane)

1. INTRODUCTION

Expression of the *ompF* and *ompC* genes, which encode *Escherichia coli* outer membrane proteins OmpF and OmpC, respectively, is affected in a reciprocal manner by the osmolarity of the growth medium [1]. At least two protein factors, OmpR and EnvZ, are known to be involved in the transcriptional regulation of *ompF* and *ompC* expression [2,3]. The OmpR protein is a positive regulator specific for the *ompF* and *ompC* promoters [4]. On the other hand, the EnvZ protein is believed to sense an environmental osmotic signal and then to modulate the functioning of the OmpR protein. Recently, it was demonstrated that the EnvZ protein is autophosphorylated in vitro in the presence of ATP, its phosphoryl group being subsequently transferred to the OmpR protein [5–7]. The phosphorylated OmpR protein then undergoes dephosphorylation, the process of which is also mediated by the EnvZ protein [8]. This EnvZ-OmpR phosphotransfer reaction, observed in vitro, was suggested to play a crucial role in the signal transduction and the consequent regulation of *ompF* and *ompC* expression in response to an environmental osmotic stimulus [8]. We addressed the main issue of what is the biochemical consequence of the phosphorylation of OmpR. In this respect, we previously demonstrated that the phosphorylation of the OmpR protein results in stimulation of its in vitro DNA-binding ability to both the *ompF* and *ompC* promoter DNAs [9]. In the present study, we further demonstrate that the phosphorylation of the OmpR protein results in stimulation of the transcription of both the *ompF* and *ompC* genes.

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Abbreviations: bp, base pairs; SDS, sodium dodecyl sulfate

2. MATERIALS AND METHODS

2.1. *Materials*

[γ-^{32}P]ATP (30 Ci/mmol) and [α-^{32}P]UTP (800 Ci/mmol) were purchased from Amersham International, and NEN Research Products, respectively. *E. coli* RNA polymerase was obtained from Boehringer Mannheim.

2.2. *Purification of the EnvZ and OmpR proteins*

Truncated forms of the wild-type and a mutant-type of the EnvZ protein, which were respectively designated as EnvZ* and EnvZ11*, were purified as described previously [5,8]. The OmpR protein was purified as described previously [4], using plasmid pFN108 [4] and host strain SG480Δ76 [10].

2.3. *Purification of radioactively phosphorylated EnvZ**

The purified EnvZ* or EnvZ11* was incubated with 0.4 mM [γ-^{32}P]ATP (500 cpm/pmol), 5 mM $MgCl_2$ and 200 mM KCl in TEDG buffer (50 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, 2 mM dithiothreitol, 10% glycerol (v/v)) for 30 min at 37°C. The reaction mixture was then immediately applied onto a Sephadex G-75 column previously equilibrated with TEDG buffer. The fractions containing the radioactively phosphorylated EnvZ* or EnvZ11*, which were free from ATP, were collected. The amounts of phosphate incorporated into EnvZ* and EnvZ11* were estimated to be 0.48 and 0.65 mol/mol protein, respectively.

2.4. *Preparation of template DNAs*

Plasmids pBOY-OH [11], pUCI-OL [12] and pHS1 (gifts from Fujita and Ishihama) were used to prepare template DNAs for in vitro transcription experiments; a 463-bp *Eco*RI-*Hin*dIII fragment from

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pBOY-OH encompassing the *ompF* promoter, a 516-bp *Hin*dIII fragment from pUCI-OL encompassing the *ompC* promoter and a 205-bp *Eco*RI fragment from pHS1 encompassing the *lacUV5* promoter. The predicted lengths of the run-off transcripts derived from these template DNAs are 125, 98 and 63 nucleotides, respectively.

2.5. *In vitro transcription experiment*

The three template DNAs described above (0.3 pmol each) and the OmpR protein (10 pmol) were mixed on ice in a buffer (27 μl) comprising 65 mM Tris-HCl (pH 7.8), 3.9 mM magnesium acetate, 0.13 mM EDTA, 0.13 mM dithiothreitol, 0.006% Nonidet P40 and 32.4 μg/ml bovine serum albumin. Phosphorylation of OmpR was initiated by adding various amounts of phosphorylated EnvZ* or EnvZ11* (1–25 pmol in 5 μl) at 37°C. After 1 min, RNA polymerase (0.35 units in 3 μl) was added to the mixtures. The mixtures were incubated for 10 min at 37°C to form open complexes under the final conditions of 50 mM Tris-HCl (pH 7.8), 3 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.005% Nonidet P40 and 25 μg/ml bovine serum albumin. The phosphorylated OmpR was analyzed by SDS-polyacrylamide gel electrophoresis [13]. RNA synthesis was initiated by adding the following substrates (15 μl); ATP, GTP, CTP (0.53 mM each), [α-^{32}P]UTP (0.17 mM) and heparin (0.67 μg/μl). The buffer conditions of the final mixture (50 μl) were the same as described above. After incubation for 5 min at 37°C, RNA synthesis was terminated by adding 50 μl of 40 mM EDTA containing 300 μg/ml of yeast tRNAs as a carrier. RNA transcripts were precipitated in 75% ethanol and 0.3 M sodium acetate and subjected to urea-polyacrylamide gel electrophoresis [14].

3. RESULTS

3.1. *Experimental design*

To determine the biochemical consequence of the phosphorylation of OmpR, we designed an in vitro transcription experiment, involving phosphorylated OmpR, and the *ompF* and *ompC* promoter DNAs as templates. OmpR purified in this study was reasonably assumed to represent the non-phosphorylated form of OmpR, since it was purified from cells lacking the *envZ* gene. However, as described previously [9], it is possible to practically prepare the phosphorylated form of OmpR in vitro. Briefly, after incubating purified EnvZ* with ATP, the autophosphorylated EnvZ* was purified free from ATP by gel filtration. Upon incubation of the phosphorylated EnvZ* with purified OmpR, the phosphoryl group on EnvZ* is rapidly transferred to OmpR, and the phosphorylated form of OmpR is rather stably accumulated in vitro. Taking advantage of this fact, we designed an in vitro transcription experiment involving mediation by the phosphorylated form of OmpR, as schematically shown in fig.1.

The non-phosphorylated form of OmpR was mixed with three kinds of linearized template DNAs, which encompass the *ompF*, *ompC* and *lacUV5* promoters, in an in vitro transcription buffer. The latter promoter, which is presumably an OmpR-independent one, was used as an internal standard for the in vitro transcription. To the reaction mixture, various amounts of EnvZ*, which was radioactively prephosphorylated with ^{32}P, were added, followed by incubation for 1 min to phosphorylate OmpR. After adding RNA polymerase, incubation was continued for 10 min to make

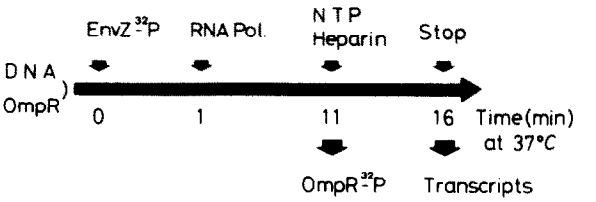

Fig.1. Strategy of the in vitro transcription experiment. Single-round in vitro transcription experiments were carried out by mixing the indicated factors at the times indicated. The amounts of phosphorylated OmpR and RNA transcripts were measured as described in section 2.

transcriptional open complexes. Just before starting the in vitro transcription by adding the substrates (ATP, GTP and CTP plus [α-^{32}P]UTP) and heparin, aliquots of the reaction mixtures were immediately subjected to SDS-polyacrylamide gel electrophoresis, followed by autoradiography. This analysis allowed us to determine the amounts of the phosphorylated form of OmpR in the transcription mixtures. The rest of the reaction mixtures were subjected to single-round in vitro transcription.

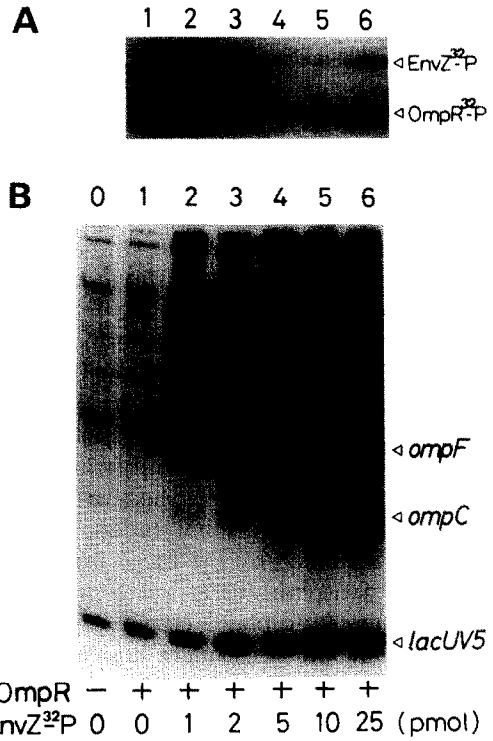

Fig.2. Phosphorylated OmpR-dependent in vitro transcription. (A) Autoradiogram showing the relative amounts of phosphorylated OmpR in the in vitro transcription mixtures. Phosphorylation of OmpR was carried out by incubating with 0 pmol (lane 1), 1 pmol (lane 2), 2 pmol (lane 3), 5 pmol (lane 4), 10 pmol (lane 5) and 25 pmol (lane 6) of phosphorylated EnvZ*, as described in section 2. (B) Autoradiogram showing RNA transcripts of the in vitro transcription. The in vitro transcription was carried out in the absence (lane 0) and presence of the OmpR protein (10 pmol) (lanes 1–6), as described in section 2. The amounts of phosphorylated EnvZ* added were the same as those in (A).

3.2. *Phosphorylation of OmpR results in stimulation of the transcription of both the* ompF *and* ompC *promoter*

As shown in fig.2A, the relative amounts of the phosphorylated form of OmpR in the in vitro transcription mixtures increased in proportion to the amounts of the phosphorylated EnvZ* added. Under these particular conditions, in vitro transcription experiments were carried out and the resultant transcripts were analyzed, as shown in fig.2B. Among several transcripts of different sizes, in nucleotides, we could easily identify each transcript; namely, *ompF*, *ompC* and *lacUV5*, on the basis of their sizes predicted from their nucleotide sequences. At present, the origins of the other transcripts of relatively larger sizes are not known. These transcripts are most likely non-specific products generated under the in vitro conditions used. In any event, the results presented in fig.2 clearly demonstrate that the transcription of both the *ompF* and *ompC* promoters was significantly stimulated under the conditions when the phosphorylated form of OmpR was supplied, while the *lacUV5* promoter was transcribed in an OmpR-independent manner as expected.

We then roughly calculated the stoichiometric amounts of the phosphorylated form of OmpR in the reaction mixtures, on the basis of the amounts of the radioisotope incorporated into OmpR. The maximum value was calculated to be 0.11 mol phosphate/mol OmpR (fig.2A, lane 6). Assuming that the OmpR molecule contains a single phosphorylation site, only 10% of the OmpR molecules in the reaction mixtures appeared to be phosphorylated at the maximum level. When a large excess of EnvZ* was added to the in vitro transcription mixture, a non-specific inhibitory effect on the in vitro transcription itself appeared to occur. In order to achieve better in vitro transcription conditions under which OmpR molecules are phosphorylated more efficiently, we used a mutant EnvZ*, named EnvZ11*, as an alternative phosphate donor for OmpR phosphorylation, since it is known that EnvZ11* is capable of phosphorylating OmpR efficiently in vitro, but cannot mediate OmpR dephosphorylation [8]. Here we assume that as far as in vitro OmpR phosphorylation is concerned, EnvZ* and EnvZ11* are the same in nature [9]. Using the phosphorylated EnvZ11*, essentially the same in vitro transcription experiments as in fig.2 were carried out (fig.3). Similar results as in fig.2 were obtained. However, under these conditions, the maximum level of OmpR phosphorylation was calculated to be 0.47 mol phosphate/mol OmpR (fig.3A, lane 6). It was

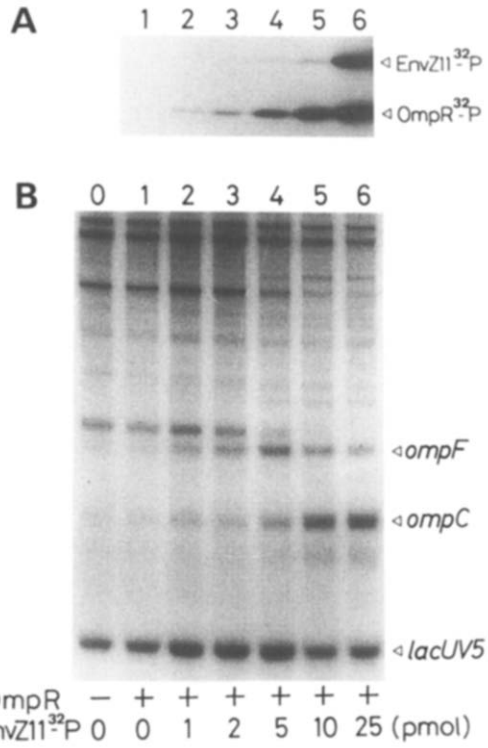

Fig.3. Phosphorylated OmpR-dependent in vitro transcription. To phosphorylate the OmpR protein in the in vitro transcription mixtures, phosphorylated EnvZ11* was used. Other details were the same as those described in the legend to fig.2.

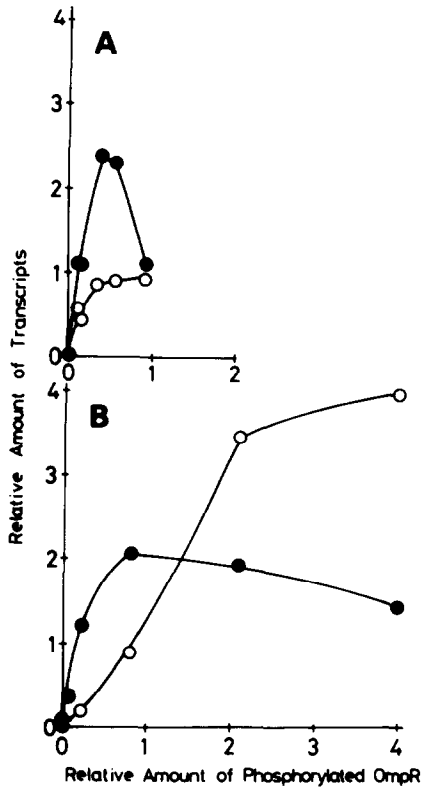

Fig.4. Quantitative representation of RNA transcripts of phosphorylated OmpR-dependent in vitro transcription. The amounts of phosphorylated OmpR and RNA transcripts were determined by scanning the autoradiograms shown in fig.2 (A) and fig.3 (B). The relative amounts of *ompF* (●) and *ompC* (○) transcripts were normalized and plotted as a function of the relative amounts of phosphorylated OmpR, the *lacUV5* transcripts being taken as an internal standard.

thus revealed that as OmpR was phosphorylated more extensively, the *ompC* transcription was more preferentially stimulated. The relative amounts of the respective transcripts of *ompF* and *ompC* in figs.2 and 3 were determined by scanning the autoradiograms densitometrically, the *lacUV5* transcript being taken as an internal standard. As shown in fig.4, the relative amounts of the *ompF* and *ompC* transcripts were plotted as a function of the relative amounts of the phosphorylated form of OmpR in the in vitro transcription mixtures. These results will be discussed in relation to the mechanism underlying the osmoregulatory expression of the *ompF* and *ompC* genes.

4. DISCUSSION

The EnvZ-OmpR phosphotransfer reaction was suggested to play a crucial role in the signal transduction and the consequent osmoregulatory expression of the *ompF* and *ompC* genes [5–9]. In the present study, we indeed demonstrated in vitro that the phosphorylation of OmpR results in stimulation of the transcription of both the *ompF* and *ompC* genes. This is consistent with similar observations by Igo et al. [7], although they did not observe such activation of the *ompC* gene. In vivo, as the medium osmolarity increases, the *ompC* gene is preferentially activated, whereas as the osmolarity decreases, the *ompF* gene is preferentially activated. How might the in vitro findings as to the activation of the *ompF* and *ompC* genes by the phosphorylated form of OmpR account for the differential expression of these genes in vivo? Based on the results of genetic studies, we previously suggested that the accumulation of the phosphorylated form of OmpR in cells results in preferential activation of the *ompC* gene [8]. In this respect, the results presented in this study provide us with a further clue for understanding the complex mechanism underlying the osmoregulatory expression of the *ompF* and *ompC* genes. As shown in fig.4, provided that the amounts of the phosphorylated form of OmpR were relatively low in the in vitro transcription mixtures, the transcription of the *ompF* gene was triggered more efficiently than that of the *ompC* gene. On the contrary, the transcription of the *ompC* gene was triggered more efficiently under the conditions when the amounts of the phosphorylated form of the OmpR were relatively high. Based on these lines of experimental evidence, together with the results reported previously [5,8,9,15], we propose the following model for the osmoregulatory expression of the *ompF* and *ompC* genes in vivo. The EnvZ protein somehow senses an environmental osmotic stimulus and its protein kinase activity and/or its putative phosphoprotein phosphatase activity change. The EnvZ protein thus signals the OmpR protein through the phosphorylation and dephosphorylation reactions. Consequently, the relative amount of the phosphorylated form of OmpR in cells varies in response to the medium osmolarity, the total amount of the OmpR protein being constant, irrespective of the medium osmolarity (data not shown). When the medium osmolarity is relatively low, the relative amount of the phosphorylated form of OmpR is regulated so as to be small. Under this particular condition, the *ompF* gene is preferentially activated. As the medium osmolarity increases, the relative amount of the phosphorylated form of OmpR increases, which in turn results in preferential activation of the *ompC* gene. The transcription of the *ompF* gene appears to be inhibited under the conditions when the relative amount of the phosphorylated form of OmpR is high. To substantiate this tentative model and to understand the molecular basis of the complex mechanism described above, more extensive studies on the in vitro transcription system developed in this study are needed.

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