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MINIREVIEW

Mechanism of Transcriptional Activation of the Phosphate Regulon in *Escherichia coli*

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In *Escherichia coli*, at least 31 genes, which are involved in the roles related to the transport and assimilation of phosphate and phosphorus compounds, are induced by phosphate starvation. They constitute a single phosphate (*pho*) regulon, and are under the same physiological and genetic control (30, 36, 46). Proteins PhoB and PhoR, which are regulatory systems for the transcriptional regulation of the *pho* genes, belong to a large family of two-component regulatory systems that respond to a variety of environmental stimuli in bacteria (23, 24, 33, 39). PhoB is the transcriptional activator, which binds to the promoters of the *pho* genes (21, 22). PhoR is a transmembrane protein that modulates the activity of PhoB by promoting specific phosphorylation and dephosphorylation of PhoB in response to the phosphate signal in the medium (19, 21, 37, 50). The phosphorylation of PhoB protein occurs concurrently with the acquisition of the ability to activate transcription from the *pho* promoters (Fig. 1). In the absence of the PhoR functions, PhoB is phosphorylated independently of the phosphate levels by PhoM, a PhoR like protein (2, 3, 26), which was renamed CreC by Wanner (45).

In this article, we describe our recent studies on the mechanism of the transcriptional regulation of the *pho* regulon.

Genes in the *pho* regulon

Fig. 2 shows the positions of the *pho* regulon genes and related genes on the genetic map of *E. coli*. The regulatory genes, *phoB* and *phoR*, are located at about 9 minutes and constitute a single operon (23~25, 27). *phoA* encodes periplasmic alkaline phosphatase (10), and *phoE* encodes an outer membrane protein, porin (31). The *phn* operon that consists of 14 genes encodes proteins related to the transport and utilization of phosphonates, and is active in *E. coli* B but cryptic in *E. coli* K-12 due to a mutation (4, 20, 29, 43, 47). *psiE* was identified by Wanner *et al.* (49) as one of phosphate-starvation-inducible genes and the position of the gene determined by Metcalf *et al* (28). Function of the gene is not known at present, but expression of the gene is positively regulated by

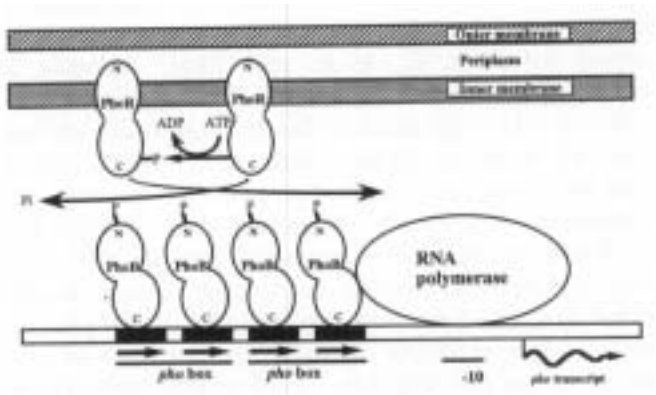

Fig. 1. Schematic diagram of PhoR-mediated phosphorylation and dephosphorylation of PhoB in relation to the transcriptional regulation of the *pstS* promoter. Filled boxes with arrows indicate repeated sequences (5'-TGTCA) in the *pho* boxes (details are described in the text). N and C indicate amino- and carboxy-terminal regions of PhoB and PhoR proteins, respectively. With limited phosphate, PhoR autophosphrylates using ATP and promotes phosphorylation of PhoB protein. Phosphorylation of PhoB enhances its binding activity to the *pho* boxes and activates transcription from the *pstS* promoter in concert with RNA polymerase. The number of the PhoB molecules bound to the *pho* boxes of the *pstS* promoter is putative. With excess phosphate, PhoR promotes dephosphorylation of the phospho-PhoB and dephosphorylation of PhoB decreases its affinity for the *pstS* promoter, which is inactive as the transcriptional activator. Pi indicates the product of dephosphorylation of phospho-PhoB by PhoR.

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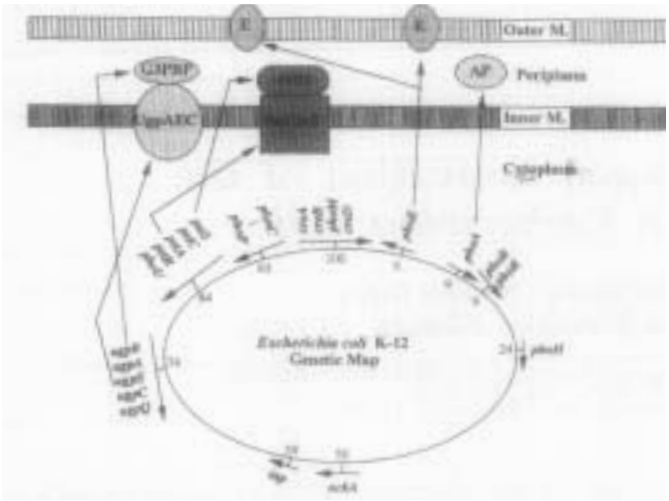

Fig. 2. The positions of the *pho* regulon genes on the genetic map of *E. coli* K-12. The circular map, nomenclature, and map positions are according to Rudd (35). The arrows show the direction of transcription.

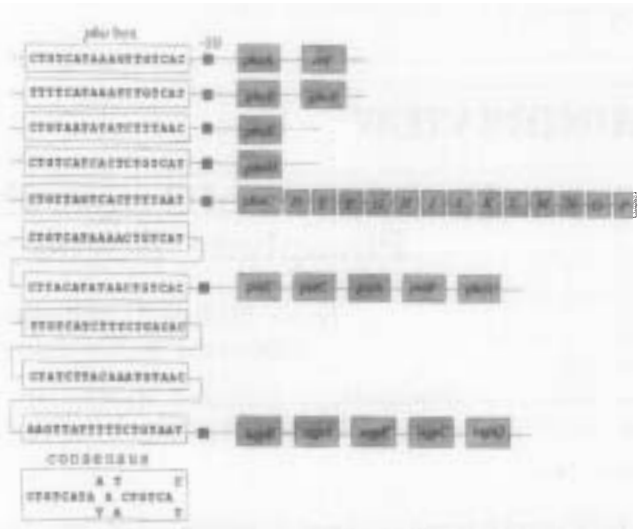

Fig. 3. Schematic presentation of the *pho* regulon genes. The homologous sequences shared by the genes belonging to the *pho* regulon (the *pho* boxes) are shown. The *pho* boxes, -10 sequences and coding regions are illustrated as indicated. The *pst* and *ugp* promoters contain 2 and 3 *pho* boxes, respectively.

the product of *phoB* and negatively regulated by the product of *crp* (44). Footprinting experiments using phospho-PhoB and cAMP-CRP indicated that the binding sites of these proteins on the promoter region partially overlap (unpublished data). The *pst-phoU* operon encodes proteins involved in the phosphate specific transport system and signal transduction in the *pho* regulon. An external phosphate signal received by PstS, C, A, B, and PhoU proteins is transmitted to PhoR (1, 16, 41). The *ugp* operon encodes proteins involved in the transport and utilization of *sn*-glycerol-3-phosphate (32). *phoH*, which had been originally named as *psiH* by Wanner and McSharry (48), encodes a protein that contains the ATP-binding motif and actually binds to ATP (12), but its function *in vivo* is not known. Though *phoM*, *ackA* and *iap* genes are not involved in the *pho* regulon, their products regulate the functions of proteins in the *pho* regulon. PhoM autophosphorylates in the presence of ATP and catalyzes the phosphorylation of PhoB (3). The *ackA* encodes acetate kinase which catalyzes the formation of acetyl phosphate and ADP in the presence ATP and acetate. The acetyl phosphate directly phosphorylates PhoB in the absence of the cognate kinases and activates transcription from the *pho* genes in concert with RNA polymerase in vitro (6). The *iap* encodes a protein which works as a protease to make isozymes of alkaline phosphatase.

The *pho* box as a consensus sequence for the *pho* promoters

The genes in the *pho* regulon are transcriptionally activated by PhoB protein. Therefore, they are likely to share a common regulatory element in the promoter regions which are recognized by PhoB. In agreement with this idea, the well-conserved 18-nucleotide sequence, 5'-CTGTCATAA(/T)AT(/A)CTGTCAC(/T), which we named the *pho* box, is found 10 nucleotides upstream from the putative -10 regions in all cases as shown in Fig. 3 (9, 12, 20, 23, 31). However, the sequences homologous to the -35 region are not found. DNase I footprinting using phospho-PhoB revealed that these *pho* boxes are the target sequences for the PhoB protein. The *pho* box is tandemly repeated with twice and more in the regulatory regions of *pstS* and *ugpB*, respectively. Mutational analysis of the *pstS* promoter region was performed (13, 17). Deletions extending into the upstream *pho* box but retaining the downstream *pho* box greatly reduced the promoter activity, but the remaining activity was still regulated by phosphate levels in the medium and by the PhoB protein, indicating that each *pho* box is functional and cooperative binding of PhoB to the *pho* boxes is required for maximum expression. No activity was observed in deletion mutants that lacked the remaining *pho* box or the -10 region. The binding region of PhoB in the *pstS* regulatory region was studied by a gel-mobility retardation assay with the truncated promoter fragments (13) and by DNase I footprinting (21, 22). The results of these in vitro experiments also indicate that PhoB binds to each *pho* box of the *pstS* promoter. The *pho* box is likely to consist of two direct repeats of 5'-TGTCA that flank the AT-rich 6-bp spacer. Single-point mu-

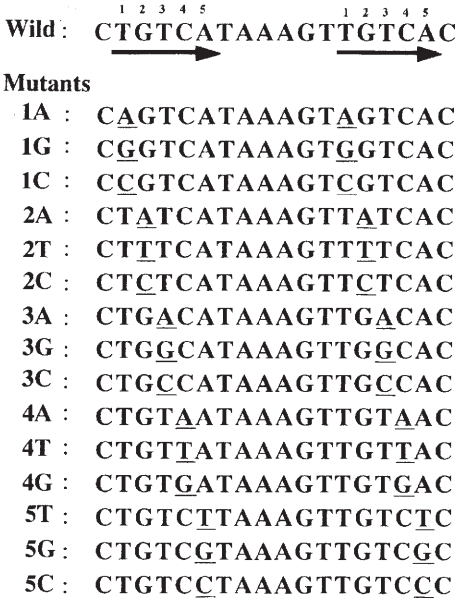

Fig. 4. Mutant *pho* boxes. Mutations were introduced at the same positions of the direct repeats. For example, mutant 1A has two T to A mutations at position 1 in each direct repeat. The wild-type *pho* promoter was fused to a repoter gene, *lacZ*. The wild-type *pho* box was replaced by these mutant *pho* boxes and the PhoB-dependent promoter activityies were measured. Positions in direct repeats are shown at the top of the wild-type *pho* box.

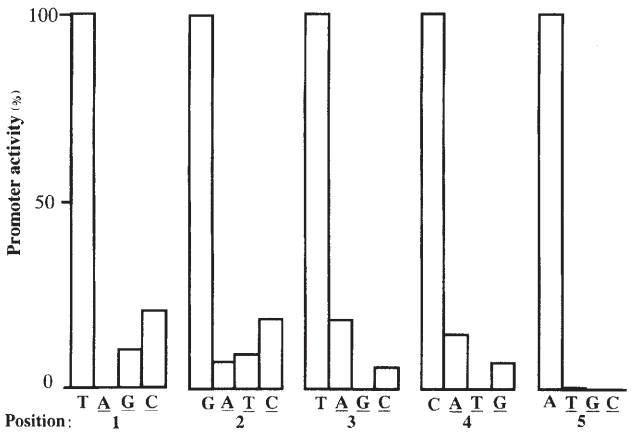

Fig. 5. Relative promoter activities of the mutant *pho* promoters. Mutant *pho* boxes used are shown in Fig. 4. Mutated nucleotides and positions were indicated by underlines and numbers, respectively. Promoter activity of the wild-type promoter is shown as 100%.

tations in any of these sequences in a truncated *pstS* promoter that contains only the distal *pho* box caused reduction in transcriptional activity, but other mutations in the *pho* box affected only marginally at most, indicating that these direct repeats play an important role in the interaction with PhoB (17). More systematic mutation analysis of the *phoA* promoter was performed. In this study, same two positions in each direct repeat of the *pho* box were mutagenized as shown in Fig. 4 and fused to a reporter gene, *lacZ*. Relative promoter activities with each mutant *pho* box are shown in Fig. 5. As compared with single point mutants (17), promoter activities in each mutant were more severely defective, especially in position 5 mutants (Fig. 5). We also examined the binding of PhoB to the regulatory regions of the *pho* genes by methylation protection experiments (9, 12, 17, 22). In most cases, methylation of Gs at nucleotides 5'-TGTCA in the upper strands and 5'-TGACA in the lower strands was most reduced by PhoB. Therefore, these Gs are very important for the interaction with PhoB, probably by directly making hydrogen bonds with PhoB. Radical footprinting experiment to the *pstS* promoter also showed that the direct repeats are the target sites for PhoB. The observed protection pattern suggests that PhoB molecules (probably 4 molecules) tandemly bind on the *pstS* promoter in the same manner (Fig. 6).

Structure of PhoB protein

The transcriptional activator PhoB composed of 229 amino acids is postulated to contain at least three functional domains (21): (I) a domain for phosphorylation, (II) a domain for DNA (the *pho* box)-binding, and (III) a domain for interaction with RNA polymerase holoenzyme. To identify these domains in PhoB, we constructed a series of *phoB* mutants, including those that encode amino (N)-terminal or carboxy (C)-terminal truncated PhoB and point mutants, and analyzed transcription-enhancing, phosphate-accepting, and

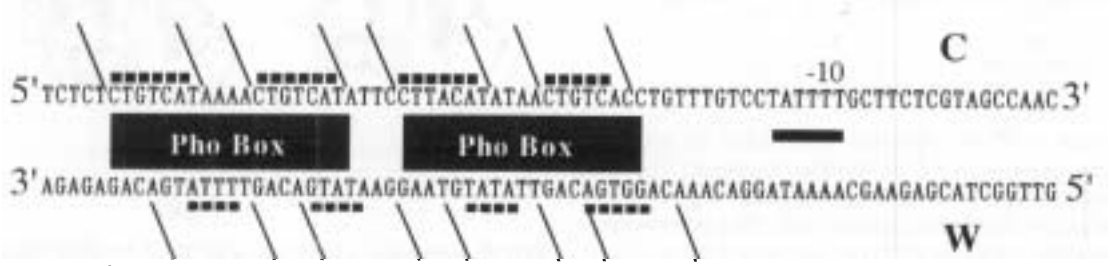

Fig. 6. Summary of the hydroxyl radical footprinting experiment. The positions that were protected are indicated by filled squares. Four binding sites are identified. C and W indicate the Watson and Crick strands.

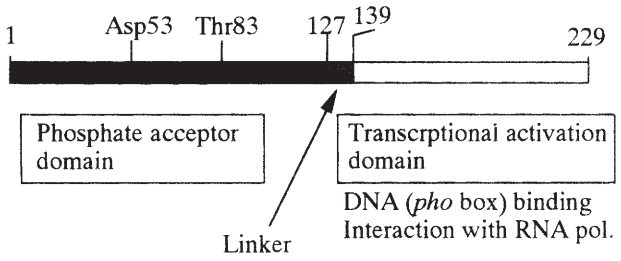

Fig. 7. Schematic presentation of domains of PhoB.

DNA-binding activities. The truncated proteins containing at least N-terminal 127 amino acids (domain I) were fully competent for accepting phosphate from phospho-PhoR (Fig. 7). Biochemical and mutational analysis revealed that the Asp53 of PhoB is the phosphate-accepting residue from phospho-PhoR and the Thr83 plays an important role for the phosphate transfer reaction. Domains (II) and (III) were contained in the C-terminal 90-amino acids of PhoB protein. This 90-amino acid PhoB protein, which was expressed by a plasmid pBC107, constitutively activates transcription from the *pho* genes (Fig. 7). Therefore, we propose that the N-terminal domain physically blocks the C-terminal activator domain, and the phosphorylation of Asp53 releases this block by bringing about the conformational change in the domain.

With the hope of identifing the DNA-binding domain (DBD) of PhoB protein, the *phoB* gene was randomly mutated (17). Ninety mutants that failed to express the *phoA* gene were obtained and 53 of these were independent mutants, among which five were nonsense mutants, five were double mutants, and the remaining 43 were single mutants (Fig. 8). Binding of the mutant protein to the *pstS* promoter

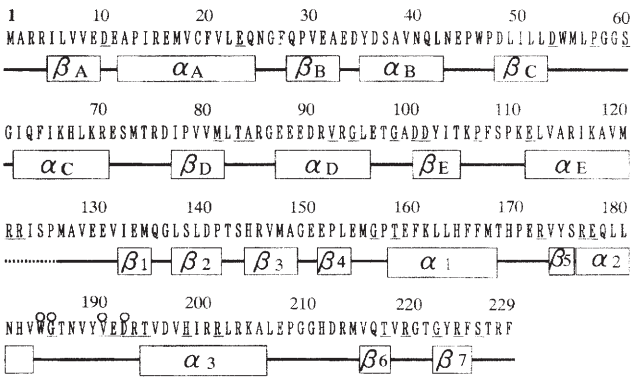

Fig. 8. Structure of PhoB. Mutants that failed to activate *phoA* expression were selected by random mutation. The positions of mutations are indicated by underlines. Open circles show the positions of PhoB that interact with RNA polymerase. Proposed secondary structure of PhoB are shown by α(α-helices, A to E and 1 to 3), β(β-strands, A to E and 1 to 7) and turn (lines) structures. Dotted line shows unknown structure.

fragment was measured by a filter binding assay. Of the 43 single mutants, 18 had mutations at the C terminus, while the others were at the N terminus. Most of the C-terminal mutants showed poor binding to the target DNA. In contrast, among the 25 N-terminal mutants, all but one, M81I, showed more than 50% normal DNA-binding ability. These data also indicate that the DBD is located at the C-terminal region of PhoB and the mutated amino acid residues should play important roles in the DNA-binding or/and conformational stability of PhoB.

To try to identify the positions of PhoB that are used for interaction with RNA polymerase, another type of random mutagenesis experiment was carried out on pBC107 to obtain mutant proteins that were able to compete with the genomic *phoB* product and thereby suppress expression of *phoA* activity (17). Twelve such clones were obtained. All of them were single point mutants and four independent types, W184R, G185R, V190N, and D192G, were identified. These mutant PhoB proteins were purified and used for footprinting experiment. The result indicated that these mutant PhoB proteins efficiently bound to the Pho promoter as compared with wild-type PhoB protein. In the case of wild-type PhoB protein, when RNA polymerase was added, more protection extending to the -10 region was occurred. However such protection was not observed with the mutant PhoB proteins, indicating these 4 positions of PhoB play an important role to interact with RNA polymerase on the *pho* promoter.

The N-terminal 127 residues are homologous to the phosphorylation domain of another protein, CheY, the structure of which has been determined by crystallography (40). Based on the structure, we assigned N-terminal 127 region of PhoB, which is composed of five β-strands and five α-helices (Fig. 8). In

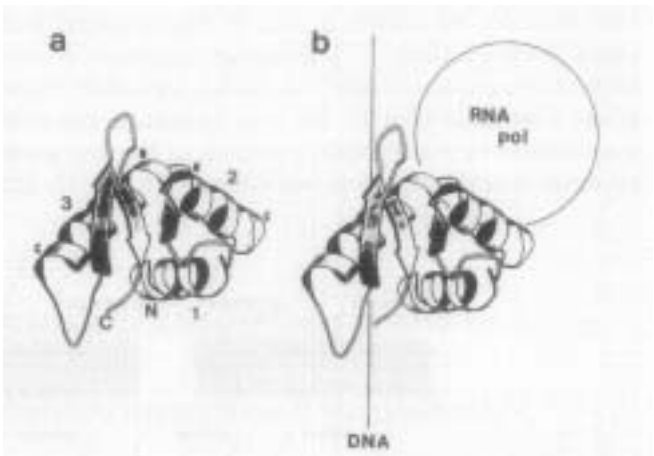

Fig. 9. Proposed structure and DNA-binding mode of the PhoB DBD. (a) The N to C direction is shown. (b) The DNA and the proposed positioning of RNA polymerase are shown.

our earlier paper (42), we have stated that C-terminal part of PhoB (about 80 residues) resembles the sequence of the DBD of histone H5, and thus we expect the structure of PhoB DBD to be similar to that of the H5 DBD, which has been detremined by X-ray crystallography (34). The structure was confirmed by NMR study (unpublished results). The C-terminal half of PhoB (residues 130-229) contains 7 β-strands and 3 α-helices (Fig. 8). We have proposed that the motif of PhoB DBD is a helix-turn-helix motif that contains longer turn than other known helix-turn-helix motifs and the helix 3 is the direct recognition helix. The four amino acid positions that are likely to contact with RNA polymerase fall around the long turn connecting the two helices 2 and 3 (Fig. 8). Proposed structure and DNA-binding mode of PhoB DBD are shown in Fig. 9.

Role of the σ^{70} subunit of RNA polymerase in transcriptional activation by PhoB

Using a series of C-terminally truncated α subunits of RNA polymerase, Ishihama and co-workers have demonstrated that some activators such as CRP, which binds to the upstream region from the promoter, make direct contact with C-terminal region of the α subunit in transcriptional activation (7, 8). Genetical studies also supported that activators such as CRP, Fnr, and OmpR make direct contact with C-terminal region of the α subunit in transcriptional activation (15, 38, 51). However, in vitro transcription analysis using PhoB indicated that the C-terminal region of the α subunit of RNA polymerase is dispensable for the activation of transcription from the PhoB-dependent promoters (7). From these results, we postulated that PhoB specifically interacts with other subunit of RNA polymerase in transcriptional activation. To identify the subunit of RNA polymerase involved in the specific interaction with PhoB, we attempted by localized mutagenesis on the chromosome to isolate *rpo* mutants which are specifically defective in the expression of the *pho* genes. We isolated two mutants with such properties, and they had mutations in the *rpoD* gene encoding the σ^{70} subunit of RNA polymerase (18). The *rpoD* mutations altered amino acids within and near the first helix of the putative helix-turn-helix (HTH) motif in the C-terminal region (region 4.2) of σ^{70}. Transcription from the *pho* promoters in vivo was greatly reduced in these mutants while transcription from the PhoB-independent promoters was affected only marginally at most. The reconstituted RNA polymerase holoenzymes containing the mutant σ^{70}s were very defective in the transcription from the *pstS* promoter *in vitro*, while they were proficient in transcribing the PhoB-independent promoters. Phosphorylated PhoB mediated the specific binding of the wild-type holoenzyme to the *pstS* promoter, while it did not mediate the binding of the mutant holoenzymes. These results suggest that PhoB promotes specific interaction between RNA polymerase and the *pho* promoters for the transcriptional activation, and the first helix of the putative HTH motif of σ^{70} plays an essential role in the interaction.

To further study the role of the first helix of region 4.2 of σ^{70} in transcriptional activation by PhoB, we made a series of site-directed mutations to alter amino acids in the motif and purified the mutant σ^{70} proteins (11). Most of the reconstituted RNA polymerases containing the mutant σ^{70}s reduced the in vitro transcription from the *pstS* promoter, while they transcribed normally the *tac* promoter. Kumar *et al.* (14) also have demonstrated that RNA polymerase containing a C-terminally truncated σ^{70} with a deletion up to the second helix of the putative HTH, which is proposed to be the direct recognition helix for the -35 sequence (5) could not transcribe most promoters but it could transcribe the *pstS* promoter in the presence of PhoB protein in vitro. However, RNA polymerases with further

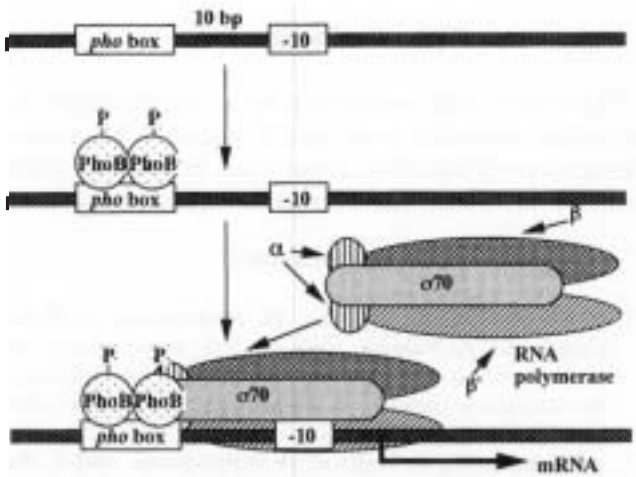

Fig. 10. Schematic presentation of transcription activation in the *pho* genes. The -10 signal and PhoB binding site (*pho* box) in the *pho* promoter are drawn. The heavy arrow means transcription into mRNA when the system is activated. The subunits of RNA polymerase and PhoB are illustrated as indicated: empty (upper), bound by PhoB (middle), or bound by PhoB and RNA polymerase (lower). PhoB bound to the pho box promotes interaction of RNA polymerase with the promoter via direct contact with the σ^{70} subunit. Although σ^{70} interaction with the -10 site of the *pho* promoter is required, it alone is not enough for the formation of stable complex with the promoter. The process requires the function of PhoB bound to the *pho* box.

deletion in σ^{70} could not transcribe *pstS* which does not possess the -35 sequence. These results are consistent with the hypothesis that PhoB directly interacts with the first helix of region 4.2 of σ^{70} to introduce the RNA polymerase into the *pho* promoters for transcriptional activation (Fig. 10).

Conclusions

The pathway of signal transduction in the *pho* regulon involves PstS, C, A, and B (phosphate transporter), PhoU, PhoR (sensor), PhoB (activator), and RNA polymerase. An external phosphate signal received by PstS, C, A, B, and PhoU proteins is transmitted to PhoR. With limited phosphate, PhoR autophosphorylates using ATP and phosphorylates PhoB. The activated PhoB (phospho-PhoB) binds to the *pho* boxes of the *pho* promoters, interacts with RNA polymerase, by making direct contact with the first helix of the HTH motif of σ^{70}, and to facilitate the RNA polymerase to enter the promoters for the initiation of RNA synthesis. The turn structure of the helix-turn-helix of PhoB plays an important role in this interaction. With excess phosphate, PhoR facilitates the dephosphorylation of phospho-PhoB and the transcription from the *pho* promoters is abolished.

Acknowledgments

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