Identification of the Promoter Region and the Transcriptional Regulatory Sequence of the evgAS Operon of Escherichia coli

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The evgAS operon of Escherichia coli encodes the EvgA response regulator and the EvgS sensory kinase, which are members of one of the two-component signal transduction systems of Escherichia coli. In this study, we identified the evg promoter and the EvgA-responsive element. Primer extension analysis found two evg transcriptional initiation sites, designated P1 (+1) and P2 (−10), and placed them 114 bp and 124 bp upstream of evgA, respectively. A gel retardation assay demonstrated that EvgA specifically bound to an inverted repeat located between −102 and −128 counting from P1. We also did a β-galactosidase induction experiment using a promoter-probing vector and found that the EvgA-binding sequence was important to stimulate the evg promoter. These results suggest that the expression of evgAS is positively regulated by its own product, EvgA.

Key words: EvgA; evgAS; transcription factor; two-component system; signal transduction

Prokaryotic organisms use two-component signal transduction systems to control cellular functions in response to environmental conditions. In the two-component system, environmental signals sensed by a membrane receptor (sensor kinase) are passed to a cytoplasmic translocation factor (response regulator) via phosphorylation, which activates the latter protein, resulting in an alteration of gene expression.1) We have isolated an operon named evgAS from E. coli.2) The sequence similarity between the evgAS products and the proteins of several two-component systems predicted that EvgS is a sensor kinase that activates its cognate response regulator, EvgA, when proper environmental stimuli are present.2) Actually, in vitro experiments showed that EvgA and EvgS communicated through phosphorylation/phosphotransfer.3) Although in vivo studies suggested that the sensory transduction and subsequent transcriptional activation were also tightly coupled in the evg system,2) little is known about the mechanism of evg-regulated gene expression.

EvgA and EvgS are very similar to BvgA and BvgS, respectively.2) BvgA/BvgS produced by Bordetella pertussis, the human pathogen that causes whooping cough, also fall into the two-component family and the bvgAS operon encoding these proteins is a central regulatory locus necessary for expression of the virulence of this pathogen.5) The bvgAS locus is linked to the fhaB locus, a virulence gene encoding filamentous haemagglutinin, and is transcribed in the opposite direction. The intergenic area between the bvg and fhaB genes contains the respective promoters. BvgA is a transcription factor and transactivates the two genes through interaction with cis-active elements situated 5′ to each promoter.6) The close proximity of these promoters may facilitate regulatory interactions.

We recently found an operon composed of emrK and emrY, which are similar to the E. coli multidrug resistant genes emrA and emrB,10) in the upstream domain of evgA.4) Since the evgAS and emrKY operons point in opposite directions as with bvgAS and fhaB, there should be at least two divergent promoters. The similarity between evg/emr and bvg/fha allows us to hypothesize that there exist cis-active sequences for expression of evg and emr near these genes and EvgA facilitates transcription of both genes after working on the sequences. In this study, we investigated the mechanism by which expression of the evgAS operon is regulated.

Materials and Methods

Analysis of transcription products. Total cellular RNA was isolated from midlogarithmic phase cultures of E. coli MC4100 grown in 1-broth at 37°C by extraction with hot phenol as mentioned.11,12) Primer extension analysis was done by the previously described method.13) The primer used was a 20-mer oligonucleotide (5′-TCCCTTTGTGCAATGAAGCATC-3′) complementary to 8 to 27 nucleotides upstream from the evgA initiation codon. The protocol for the S1 nuclease protection experiment is described elsewhere.12) A DNA fragment containing the evg transcription start sites was used for the primer.

Gel retardation assay. The binding reaction was done in a total volume of 10 μl of a binding buffer of 10 mm HEPES-NaOH, pH 7.5, 50 mm KCl, 10% glycerol, and 0.5 mm dithiothreitol. Each reaction mixture contained 1 μg of purified EvgA9) or bovine serum albumin as a negative control as well as 1 μg of probe DNA. The probe DNA was prepared by the polymerase chain reaction (PCR) and, when necessary, digested with specified

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Abbreviations: lacZY, attached to the promoterless lactose operon; β-gal, β-galactosidase; PCR, polymerase chain reaction
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Restriction enzymes before use. After they were incubated at 37°C for 30 min, the mixtures were put onto a 4% NuSieve 3:1 (FMC BioProducts) agarose gel containing 1× TAE buffer (40 mM Tris, 5 mM sodium acetate, 2 mM EDTA, pH 8.0) and run at 8.5 volts/cm in 0.5× TAE at room temperature. When bromophenol blue reached the opposite end of the gel, the electrophoresis was stopped. The gel was stained with 1 μg/ml of ethidium bromide and photographed under ultraviolet illumination.

Construction of evg::lacZ fusion plasmid. Sequential deletion fragments of 5′-flanking DNA of evgA were generated by PCR to bear an EcoRI and a BamHI site at the 5′- and 3′-ends, respectively. These DNA fragments were restricted and inserted in frame upstream of the promoterless lacZ gene on the multicopy plasmid pMC1403 which had been cut with EcoRI and BamHI. The resulting plasmids with the evg::lacZ fusion code for a hybrid β-galactosidase (β-gal) in which the amino terminal five amino acids of EvgA were joined with the eighth amino acid of β-gal via three linker amino acids corresponding to the BamHI site and direct synthesis of the protein under control of the evg promoter.

β-Galactosidase assay. E. coli strains transformed with an evg::lacZ fusion plasmid were grown overnight in LB-broth at 37°C. The next day the bacteria were diluted 1/100 in LB-broth and grown at 37°C to an optical density at 600 nm of approximately 0.4. Nicotinic acid (10 mM, final concentration) was added when necessary. β-Gal activity was measured by Miller’s method.

Results and Discussion

Location of the evg transcription start

To pinpoint the start position of the evg mRNA, we did a primer extension experiment on total RNA isolated from E. coli MC4100 (Fig. 1). Two different sites, P1 and P2, were present upstream of evgA; they were located 114 bp and 124 bp ahead of the ATG start codon of evgA, respectively. A S1 nuclease protection assay on the total RNA displayed two protected bands of almost the same length, confirming the position of P1 and P2 (data not shown).

Transcription at the bvg locus of B. pertussis is directed by three autoregulated promoters and one bvg-independent promoter. Similar results have been obtained from B. parapertussis, in which one of the bvg-dependent promoters is inactive. All the autoregulated promoters are negatively controlled by modulating signals, such as nicotinic acid, magnesium sulfate, and low temperature, from the surrounding environment. The function of the evgAS operon is also sensitive to these signals, though the degree of repression is not as serious as in the case of bvg. In the protection assay, we observed a coordinate decrease in the amount of these two evg transcripts when bacteria were grown in the presence of 10 mM nicotinic acid (data not shown), which implies that both promoters are controlled by the evg products. In these transcription analyses, however, we may miss (an) evg-independent promoter(s). Further biochemical and genetic experiments on the evg promoters are required to clarify the role of each promoter in evg expression.

Sequence-specific EvgA-DNA interaction

Structural analysis of evgA predicted that EvgA is a typical DNA binding protein with a carboxy terminal helix-turn-helix motif. On the basis of our above mentioned hypothesis about the function of EvgA, we searched for EvgA-recognition loci on the 525-bp evgA/emrK intergenic region (see Fig. 1 in ref. 2 for the nucleotide sequence) by the gel retardation method using DNA fragments covering this region as probes (Fig. 2). First, a 550-bp fragment encompassing the entire intergenic region was examined for the interaction with EvgA after digestion with HaeIII. Out of the two resulting fragments, the longer one (the 3′ moiety) caused an upward band shift in the binding assay, while the mobility of the shorter fragment was not affected by EvgA (data not shown). We next prepared a DNA fragment of 380 bp spanning −250 to +130 relative to the P1 start of transcription and cut it into two parts with MboII or DraI. After incubated with these fragments, EvgA preferentially retarded the electrophoretic mobility of the 5′-part of the DNA restricted with MboII and the 3′-part with DraI (not shown). Thus, the EvgA target was narrowed down to the 91 bp flanked on one side by the DraI site and on the other by the MboII site.

In B. pertussis, the regulatory region found 5′ to fhaB and bvgA, which are involved in BvgA binding and subsequent transcriptional promotion, both contain the consensus sequence 5′-TTTCCTTA-3′, which is one half of a 14-bp inverted repeat upstream of fhaB and a 14-bp direct repeat upstream of bvgA. Upon close examination of the sequence of the 91-bp DraI/MboII region, we discovered two sites that share some similarity to the consensus sequence and form an inverted repeat (Fig. 2). The hexameric sequence 5′-TTCTTA-3′ is identical with six out of the seven nucleotides TTCTTA that is the other half of the 14-bp inverted repeat above the fhaB promoter. The fha heptameric sequences are joined directly, while the evg inverted repeat is separated by a 15-bp spacer sequence. Similar hyphenated half-sites are present upstream of the pts and cya genes coding for B. pertussis pertussis toxin and haemolysin-adenylate cyclase toxin, respectively, and recognized specifically by phosphorylated BvgA. These findings suggest that EvgA may interact with the hyphenated inverted repeat within the 91-bp region.

For fine mapping of the EvgA-responsive sequence, we generated a set of deletions in the 91-bp DNA fragment and then used them as probes for a DNA-binding assay (the lower part of Fig. 2). Probes 2 and 3 are 3′-terminally deleted derivatives of a 90-bp PCR product (Probes 1 in which one base of the 3′-extruding terminal of the 91-bp DraI/MboII fragment is missing) and Probes 4 and 5 are 5′-terminal correspondents. Probes 2 and 4 have both of the half-sites of the inverted repeat, but Probes 3 and 5 lack the 3′- and 5′- half-sites, respectively. The result is shown in Fig. 3. When tested for
Fig. 1. Transcription of the *evgAS* Operon.

Radiolabeled oligonucleotide primer was annealed to 80 μg of total RNA from *E. coli* MC4100 and extended with reverse transcriptase (lane 1). The same primer was used to determine the DNA sequence shown to the left of the primer extension products. The DNA sequence (coding strand) around the *evg* promoters is presented below the primer extension reactions. The locations of P1 and P2 are marked. The putative −10 and −35 regions of each promoter are also indicated.

![Diagram of transcription](image)

Fig. 2. Schematic Representation of Strategy for Location of EvgA-binding Domain.

The partial genetic and restriction map between *emrK* and *evgA* is displayed at the top. Nucleotide positions are given relative to the P1 transcriptional initiation point (+1). The direction and starts of transcription are arrowed. The inverted repeat within the *DraI/MboII* region is indicated by arrows facing each other. The hatched portions represent DNA fragments to which EvgA specifically bound. Restriction sites: D, *DraI*; H, *HaeIII*; M, *MboII*.
affinity with EvgA, Probe 1 presented a retarded migration pattern. Instead, this probe DNA did not react with bovine serum albumin. This observation confirms this result using restricted probes and indicates the interaction to be sequence-specific. Although Probe 2 remained positive in the reaction with EvgA, removal of the TTCTTA sequence from the 3′-end of Probe 2 made the resultant DNA, Probe 3, insensitive to EvgA. The same was evident for the 5′-deletion of Probe 1: EvgA associated with Probe 4, but was inactive to Probe 5. Taken together, we concluded that EvgA exclusively targets the 12-bp inverted repeat with intervening 15 nucleotides.

The BvgA molecule can be separated roughly into two domains from the functional point of view. DNA binding activity is expressed by the carboxy-terminal half of the molecule and does not require the receiver domain, while phosphorylation of the receiver is indispensable for positive autoregulation of transcription. This indicates that DNA binding ability of BvgA does not depend on the state of its phosphorylation, though the phosphorylation raises the affinity of the transcription factor for its DNA target. We presume that the EvgA preparation used in this assay is predominantly composed of unphosphorylated molecules. Therefore, the EvgA-responsive sequence specified here is likely to be one recognized by the unphosphorylated form of the protein. A recent study on the DNA-binding ability of BvgA discovered that in vitro phosphorylated BvgA binds to a large sequence on the fha upstream region, which extends far downstream from the target sequence indicated by unphosphorylated BvgA. Such an extensive target region of phosphorylated BvgA has been reported for the ptx and cya promoters. Fully phosphorylated EvgA may display a different DNA binding profile from that obtained in this study.

Many transcription factors have been grouped into two classes on the basis of the nature of their interaction with RNA polymerase: those that contact the DNA-binding, carboxy-terminal domain of the α-subunit of RNA polymerase (class I factors) and those that contact the σ-subunit (class II). Class I factor binding sites are generally located upstream of −35, while class II factors bind to sites that overlap the −35 element. Based on current data, EvgA appears to act as a class I factor at the evg promoter.

**Transcriptional regulatory element for evg expression**

To analyze the DNA sequences required for regulation of the evg promoters, we constructed pMC1403-derived plasmids carrying lacZY transcriptional fusions to a series of in frame deletions in the 5′-flanking region of evgA. The fusion plasmids were introduced into E. coli MC4100, which has chromosomally located evgA. The resulting transformants were grown at 37°C to mid-logarithmic phase and assayed for β-gal production (Fig. 4). pMK4A retains both evg transcription starts but lacks further upstream sequences. Transformants harboring this plasmid were defective in β-gal expression with or without nicotinic acid like a negative-control transformant with pMC1403. In contrast, when transformed with pMK4A1, which accommodates the entire evg upstream region, and grown in the medium free of nicotinic acid, the bacteria produced a high level of β-gal. Upon expression of pMK4A2, in which the evg upstream region was deleted to −130, a comparable level of β-gal was yielded in the absence of nicotinic acid.

![Fig. 4. Schematic Representation of evg::lacZY Fusions Used to Delineate the Sequences for evg Transcriptional Activation.](image-url)

Each transforming plasmid contains a fragment of the 5′-flanking region of evgA fused to a promoterless lacZY cartridge as illustrated here. E. coli MC4100 was transformed with the fusion plasmid and grown in the presence (+NA) or absence (−NA) of 10 mM nicotinic acid. β-Gal units are expressed as the average of four independent assays which did not differ by more than 15%.
When β-gal activity was measured for pMKA3, which is 32-bp shorter at the 5’-end of the evg insert than pMKA2, however, a drastic decrease in β-gal productivity was observed compared to pMKA2. These results demonstrate that the −130 to −101 region specifies a cis regulatory signal essential for transcriptional enhancement of the evg promoters. Since this region is the target locus of EvgA and host bacteria harbor a single copy of the evgAS operon in trans, this finding suggests that EvgA serves as a trans-acting factor for activation of the evg promoters following contact with its target.

To further examine the relationship between EvgA and evg transcription, β-gal induction was done in the growth medium containing nicotinic acid. In bacterial cells transformed with either pMKA1 or pMKA2, the β-gal productivity was observed in the presence of nicotinic acid to the level as expected from our previous study. On the contrary, no measurable repression of β-gal expression by this modulating agent was seen for pMKA3-transformed bacteria. We do not know whether the pMKA3-borne β-gal reflects either transcription from some unknown evg-independent promoter(s), mere amplification of a background level of transcription from evg-regulated promoters due to the multiplicity of pMKA3, or both. The data obtained here strongly suggest an autogenous regulation of expression of the evgAS operon, but the final conclusion awaits construction of bacterial mutants of EvgA production.

As mentioned above, the fhaB-bvgA intergenic region of B. pertussis contains two BvgA targets, but this study identified only one site that is involved in EvgA binding between emrK and evgA, which serves as a cis-active element for evg transcriptional increases. Two alternative hypotheses to simply explain this difference are the following: (i) The single EvgA target functions also for emr expression; and (ii) we failed to detect another EvgA-binding site that is specific for regulation of emr expression. On the other hand, our experiment on the course of evg expression showed that evg is expressed at early logarithmic phase, but emr expression does not occur until stationary phase. This result is reminiscent of the differential regulation of expression of the B. pertussis virulence factors: fha and bvg transcripts are detected minutes after an inducing signal, but ptx and cya transcripts appear only after several hours. Many workers have proposed that the delay of the expression of ptx and cya following bvgAS induction is due to the additional time needed to reach a threshold concentration of phosphorylated BvgA required to activate the expression of these genes which is higher than that required for fha and bvg activation. Consistent with this proposal, recent DNA binding studies demonstrated that phosphorylation of BvgA is an absolute requirement to detect specific binding by the transcription factor at the ptx and cya promoters. Similarly, EvgA may be able to interact with an unknown low-affinity site at the emrK upstream region only when highly phosphorylated, and thereby activate the emr promoter. We are initiating studies on the regulatory mechanism of the EvgA-mediated emr expression.

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References