Importance of Stereospecific Positioning of the Upstream cis-Acting DNA Element Containing a Curved DNA Structure for the Functioning of the Escherichia coli proV Promoter

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The mechanism by which the Escherichia coli proV promoter is activated more than 100-fold in response to the medium osmolarity, without the help of any known trans-acting activators, is not yet fully understood. In this context, it has recently begun to be realized that structural features, not the primary sequences, of cis-acting DNA elements may be important for transcriptional regulation in prokaryotes. From this point of view, in this study the proV promoter was characterized by constructing a series of spacer-insertion mutants in a proV-lacZ fusion on the chromosome. Here it was found that the upstream cis-acting sequence must be positioned stereospecifically with respect to the principal −35 and −10 regions for the proV promoter to be fully activated. In this regard, it was suggested that an overall DNA structure, particularly DNA curvature, is an important cis-acting parameter for activation of the proV promoter.

In Escherichia coli, the expression of most genes is tightly regulated in response to a wide variety of environmental stimuli. As is generally recognized, trans-acting transcriptional regulators (activators or repressors) are crucially involved in these processes, and the underlying molecular mechanisms have become central to the current extensive study of transcriptional regulation in prokaryotes. However, it is also conceivable that not only such trans-acting protein factors but also cis-acting DNA elements are crucially involved in transcriptional regulation. Needless to say, such cis-acting DNA elements serve as specific target (or binding) sites for trans-acting factors, in which the basal transcriptional machinery, RNA polymerase, is included. However, it has begun to be realized that structural features (not the primary sequence itself) of cis-acting DNA elements may also be important constraints for transcriptional regulation. Such structural features of DNA that should be taken into consideration are superhelicity, flexibility and curvature (or bendability), and unusually adapted DNAs like Z-DNA. Many efforts addressing these relevant issues have been reported recently. Nevertheless, the underlying molecular mechanisms are not yet fully understood even in prokaryotic systems. In this study, we addressed this issue with special reference to the E. coli proV promoter, which is regulated in response to the medium osmolarity.

The proU locus containing three cistrons, proV, proW, and proX, encodes a high-affinity glycine betaine transporter in E. coli. Previous studies involving transcriptional and translational proV-lacZ fusions on the chromosome demonstrated about 100-fold induction of proV on osmotic up-shift with regulation at the level of transcription. An interesting fact about this proV promoter is that extensive genetic searches for trans-acting factors causing such great induction of proV failed to find any trans-acting positive regulators, but found only partially constitutive mutations. The latter types of mutations caused only partial derepression (less than 10-fold) and were still osmotically regulated. These mutations were mapped to either hns or topA, which respectively encode a nucleoid protein (H-NS) and topoisomerase I. Thus, it is tempting to speculate that the 100-fold transcriptional induction of proV is mainly governed by a cis-acting DNA element(s). From these previous observations on the proV promoter, we supposed that characterization of this promoter should provide us with a paradigm of gene regulation, in which the DNA structure of a cis-acting element(s) may be crucial. To analyze the proV promoter according to this experimental rationale, in this study we constructed a series of proV promoter-lacZ transcriptional fusions. The results of intensive analyses of these mutant promoters provided us with an intriguing insight into the function of this promoter in the context of an unusual DNA structure, DNA curvature.

Materials and Methods

Bacterial strains and media. The E. coli strains used in this study were all derivatives of CSH26 (Δlac-pro-thr thia). A set of CSH26 derivatives, each carrying an appropriate promoter-lacZ transcriptional fusion gene was constructed by lysogenization of a set of phages, which was constructed from ApF13 (for details, see below). Cells were mainly cultivated in Luria broth. When β-galactosidase activity was measured, cells were grown in Luria broth without NaCl.

Plasmid construction. A 832-bp EcoRV-HaeIII fragment encompassing the proV promoter region (see Fig. 1) was first cloned into the Smal site of vector pUC19. The resultant plasmid was designated p445-05. A series of insertion mutants of the proV promoter was then constructed from this particular plasmid. Briefly, the Sph I site, which is at nucleotide position −61, was modified by means of insertion of Bgl II-linkers (8 mer or 10 mer) and/or a Smal-linker (6 mer), and also by treatment either with T4 DNA polymerase or mung bean nuclease. Appropriate combinations of these manipulations yielded a series of insertion-mutants on plasmids (pPRO-series), the sequences of which are shown in Fig. 2. Each EcoRI BamHI fragment carrying the proV promoter region was isolated from the resultant series of plasmids, and then connected with a trpA-lacZ gene on plasmid pMS434HS (plasmids pMS-PRO-series, see Fig. 2). In particular, the plasmid carrying the original proV promoter fused to the trpA-lacZ gene was designated pMS-PRO-0. Plasmid pMK6 carrying the tac promoter-lacZ fusion, schematically shown in Fig. 1, was constructed previously. From these plasmids, pMS-PRO-0 and pMK6, two plasmids carrying chimeric type of promoters of proV and tac
were also constructed by using the respective restriction sites, SspI and HindIII (see Fig. 1).

Construction of a set of λ phage lysogens harboring an appropriate promoter-lacZ transcriptional fusion gene. The construction of a λ phage lysogen harboring a promoter-lacZ fusion was done by the strategy described previously. Each plasmid of the pMS-PRO-series was transferred into strain H2017 (pF13) harboring a lysogenic phage, pF13, and then a λ phage lysate was prepared by UV irradiation. Strain CSH26 was infected with the λ phage lysate. Candidates for lysogens carrying an appropriate promoter-lacZ fusion on the chromosome were selected from among blue plaques on agar plates containing X-gal. The lysogens thus purified were scored for the Lac⁺ phenotype. The structures of the promoters analyzed in this study are schematically shown in Figs. 1 and 2.

Assaying of β-galactosidase activity. β-Galactosidase was assayed essentially as described by Miller. Cells grown to the exponential growth phase were collected and then suspended in a volume of 250 mM sodium phosphate (pH 7.2) for counting the cells. A portion of the cell suspension was assayed for to β-galactosidase. The cells were permeabilized with toluene before the assay.

Mobility analysis of restriction DNA fragments by polyacrylamide gel electrophoresis. DNA fragments were electrophoresed on 6% polyacrylamide gel (30:0.5, monomer : bis) electrophoresis, as described previously. The running buffer comprised 40 mM Tris-acetate (pH 7.4), 5 mM Na-acetate, and 1 mM EDTA. The applied voltage was 6 V/cm. The electrophoresis was done at 4 °C. DNA fragments in the gel were detected by staining with ethidium bromide.

Results and Discussion

Construction and characterization of a set of mutants of the proV promoter

We first constructed an appropriate proV–lacZ transcriptional fusion in a single copy on the E. coli chromosome, as shown in Fig. 1. The principal transcription start site of the proU operon including proV, proW, and proX has been found to be 60-bp upstream of the translation start site of the first cistron, proV. Therefore, the term 'proV promoter' is used hereafter in this text, and the transcription start site was taken as nucleotide position +1 (see Fig. 1). In this promoter-lacZ fusion, a 832-bp DNA fragment encompassing the principal proV promoter (−35 and −10 regions), and its upstream and downstream sequences were placed in front of a functional trpA′–lacZ gene. The proV region covers the sequence extending from −624 to +208, in which the essential sequence for the expression of proV seems to be included. This construct allowed us to monitor the level of proV transcription as β-galactosidase activity. Similarly, a tac promoter-lacZ transcriptional fusion was also constructed as an appropriate reference (Fig. 1). The two other constructs were chimeric types of the proV and tac promoters, which were constructed from the two promoters by exchanging their principal promoter regions and the upstream sequences, as shown in Fig. 1. Strains harboring the respective fusions on the chromosome were named CSH26-P, CSH26-T, CSH26-TP, and CSH26-PT, being derivatives of strain CSH26.

These strains were grown in Luria broth to the exponential phase in the absence and presence of 0.5 M NaCl, and then β-galactosidase expressed by the cells was measured (Fig. 1). Strain CSH26-P carrying the proV–lacZ fusion had a certain level of α-galactosidase activity in response to the medium osmolarity (more than 100-fold induction in medium of high osmolarity), whereas strain CSH26-T carrying the tac-lacZ fusion had a constitutive level of β-galactosidase activity irrespective of the medium osmolarity. It was thus found that the cis-acting DNA element essential for the osmotic induction of proV resides within the 832-bp region. In strain CSH26-TP (i.e., when the upstream sequence of proV was replaced by that from tac), the expressed β-galactosidase activity still significantly fluctuated in response to the medium osmolarity. However, the basal level of expression in the medium of low osmolarity increased, and the induced level of the expression in the medium of high osmolarity decreased, resulting in only an overall 8.5-fold induction of β-galactosidase activity (Fig. 1). These results suggested two things. First, the −35 and −10 regions of proV, and the downstream sequence (nucleotide positions −60 to +208) were essential for the osmotic regulation, but were not sufficient by themselves. This view is consistent with the results reported previously by other investigators. Second, the upstream DNA sequence of proV may be involved in the full regulation of proV by assisting the function of the principal proV promoter. Interestingly, however, the upstream sequence itself had no effect on the expression of β-galactosidase activity, when this region was combined with the −35 and −10 regions of tac in CSH-PT. Several interpretations can be envisioned to account for these apparently contradictory phenomena. One plausible explanation is that the overall (or entire) DNA structure surrounding the proV promoter regions is crucial for the full regulation of proV in response to the medium osmolarity. In other words, the upstream DNA element can play a role particularly in coordination with the −35 and −10 regions (and/or the downstream DNA element) of proV. This preliminary view was tested experimentally, as described below.

![Fig. 1. Schematic Representation of the Structures of a Set of Promoter-lacZ Fusions on the E. coli Chromosome, and β-Galactosidase Activities Expressed by the Fusions.](image-url)
Characterization of a series of spacer-insertion mutants of the proV promoter

A simple and well established method for analyzing the functional importance of an overall DNA structure of a given promoter region is short spacer-insertion mutagenesis. To ensure appropriate results are obtained, however, varied numbers of nucleotides must be introduced as spacers (see Fig. 2). Considering this, the proV-lacZ fusion gene was treated by such spacer-insertion mutagenesis. The resultant series of spacer-insertion mutants of the proV-lacZ fusion are schematically shown in Fig. 2. It should be noted that these mutants were also placed in a single copy on the E. coli chromosome. The nucleotide numbers of spacers thus inserted were +4 to +22, which corresponded, at the most, to two helical turns of the DNA helix. The β-galactosidase activities measured for these mutant promoters produced a striking profile in terms of the promoter activity, as shown in Fig. 3. The level of β-galactosidase activity for cells grown in the medium of high osmolality varied dramatically and periodically, depending on the nucleotide number of spacers inserted, while the basal levels for the medium of low osmolality were very low. That is, the insertion of certain nucleotide numbers of spacers (e.g., +4, +6, +14, +16, and +18) dramatically decreased β-galactosidase activity, but the β-galactosidase activity expressed by the spacing-mutants with +10 and +20 insertions was essentially the same as that of the wild-type promoter. In other words, only when short spacers with lengths corresponding to approximately integral numbers of one turn of the DNA helix (e.g., +10 and +20) were introduced was the proV promoter fully active. These results are compatible with the idea that the overall structure (or stereospecific configuration) of both the −35 and −10 regions of proV, and its upstream DNA element is an important parameter for the proV promoter to be fully activated in response to the medium osmolality, as discussed below.

A model implicating DNA curvature with respect to the function of the upstream sequence of the proV promoter

i) Theoretical consideration. How can these striking observations as to the series of spacer-insertion mutants be explained according our current knowledge of DNA structure? Among several features with regard to DNA structure, we supposed that DNA curvatures is most likely such a parameter that may be implicated in the phenomenon described above, because DNA curvature in a given DNA sequence is most severely affected by the periodicity along the DNA helix (see below), while DNA superhelicity and flexibility are essentially not. In fact, the proV promoter region, contains a significantly curved DNA structure, as reported by us and other investigators.12,23,24 It is worth mentioning that the upstream sequence of the lac promoter, used as a reference in this study (see Fig. 1), does not contain any significant DNA curvature (data not shown). Considering these facts, we can envision that the proV promoter region displays an overall curvature, as schematically shown in Fig. 4. In this context, it is known that curved DNA sequences are characterized by the periodical arrangement of stretches of certain nucleotides (e.g., short [dA], and [dT], stretches) in a given DNA sequence along one face of the DNA helix.25 Such periodicity along the DNA helix is crucial for a pronounced DNA curvature.25 Therefore, when short spacer-insertions with lengths corresponding to non-integral numbers of one turn of the DNA helix are introduced into a certain position, the relative direction of the curvature of each segment (in this case, the −35 and −10 regions, and its upstream sequence) should change, as shown in Fig. 4. The spacer-insertions would thus change the stereospecific positioning of these two DNA elements, and also reduce the magnitude of overall curvature. On the contrary, even when short spacer-insertions with lengths corresponding to integral numbers of one turn of the DNA helix were introduced, the relative direction and the magnitude of overall curvature should essentially be maintained. Therefore, the magni-
tude of overall DNA curvature in a given DNA region would change periodically with such a series of spacer-insertions.

ii) Experimental support. The crucial question then arose as to whether the phenomena considered above would indeed occur in the proV promoter. The magnitude of overall curvature of a DNA segment can be estimated conventionally by means of simple polyacrylamide gel electrophoresis. More specifically, a curved DNA segment has an anomalously slow electrophoretic mobility, depending on the magnitude of its overall curvature, when analyzed by polyacrylamide gel electrophoresis at a low temperature (4°C).25–27 In such a gel, a curved DNA fragment migrates to a position higher (or slower) than that expected from its nucleotide length. Such analyses were done for the series of spacer-insertion mutants of the proV promoter. Each DNA fragment encompassing the region corresponding to the EcoRV–HaeIII fragments was isolated from the series of mutants on plasmids, by digestion with EcoRI and HindIII (see Fig. 2), and then analyzed by polyacrylamide gel electrophoresis (Fig. 3A). Indeed, the 883-bp EcoRI–HindIII fragment with no insertion had an extremely slow electrophoretic mobility, more than anticipated; it migrated to the position corresponding to that of a 1.29-kb fragment. It was thus suggested that this particular fragment contains a significantly curved DNA structure. Furthermore, the relative mobilities of these respective fragments varied significantly, as shown in Fig. 3A. For example, it was found that the fragment with a 10-bp insertion migrated much more slowly than that with a 12-bp insertion on the gel. Then, the relative mobilities on the gel measured for these fragments were plotted as a function of the number of nucleotides, and the profile was superimposed on that of β-galactosidase activity in Fig. 3. It was seen that the profile of the relative magnitude of DNA curvature almost perfectly matched that observed for the relative strength of these promoters. Namely, the variation of the proV promoter strength (i.e., more active or less active) and that of the magnitude of DNA curvature (i.e., more curved and less curved), observed for the series of spacer-insertion mutants, were well correlated to each other. These findings appear to be indicative of the functional importance of DNA curvature for the full-activation of proV. Based on the consideration and experimentation, it was suggested that the upstream sequence of the −35 region of proV must be positioned stereospecifically with respect to the principal −35 and −10 regions, so that this upstream region functions as a cis-acting DNA element. Considering the fact that no trans-acting activator has been identified for proV, such a stereospecific DNA curvature itself appears to be an important parameter for activation of the proV promoter, as schematically shown in Fig. 4. It is worth mentioning, however, that the basal level of proV expression in the low osmolarity medium was not affected significantly by the spacer-insertion mutation, although it was fluctuated in a parallel fashion with that in the high osmolarity (Fig. 3). Therefore, the overall DNA curvature may not be implicated in the osmotic regulation of proV per se. Interestingly, if this cis-acting DNA element was artificially placed in an inadequate configuration, the proV promoter activity was rather weakened.

Conclusion

The molecular mechanism by which the proV promoter is activated more than 100-fold in response to the medium osmolarity, without the help of any known trans-acting activators, is not yet understood. In this particular case of proV, we suggest that DNA curvature must be taken into consideration to fully understand the underlying mechanism. The model proposed here is the one we favor most so far. Although other explanations can be envisioned, this model is rather simple and consistent with the experimental data we and others have obtained. In any case, the results of studies on the proV promoter provided us with clear evidence that the overall DNA structure including DNA curvature is an important parameter for a given promoter-function in prokaryotes. It should be mentioned here that the similar instances have been reported previously for other prokaryotic promoters.4,5,28,29 Thus, our results further support the view, emerging from these recent studies, that DNA curvature is crucially involved in the function and regulation of a subset of prokaryotic promoters.30

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References