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Evidence for Multiple OmpR-Binding Sites in the Upstream Activation Sequence of the *ompC* Promoter in *Escherichia coli*: a Single OmpR-Binding Site Is Capable of Activating the Promoter

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We present experimental evidence for the existence of multiple activator-binding sites in the upstream sequence of the *ompC* promoter, the expression of which is activated by the positive regulator OmpR in response to the osmolarity of the medium. We also found that a single OmpR-binding site can activate the *ompC* promoter, providing that the binding site is close and placed stereospecifically with respect to the canonical −35 and −10 regions.

In *Escherichia coli*, the expression of many genes is activated by a specific protein factor that is known to interact with a DNA domain some distance from a canonical promoter (14). The definition of such transcriptional regulatory signals and their functions is essential for our understanding of the molecular mechanisms underlying gene expression. Recent extensive studies on the *ompC* gene, which codes for an outer membrane protein of *E. coli*, provided us with a paradigm of gene activation in procaryotes (4, 6, 8, 9). Expression of the *ompC* gene is activated by a *trans*-acting factor, OmpR, as the osmolarity of the culture medium increases (4, 5, 7, 12, 13). The OmpR-binding region in the *ompC* promoter, the sequence of which was previously determined by DNase I footprint analysis, is relatively long, i.e., the OmpR-binding region comprises a stretch of more than 60 nucleotides upstream of the −35 and −10 regions (8, 9). It was thus suspected that the region protected by the OmpR protein in the *ompC* promoter may comprise two or more OmpR-binding sites. In fact, from the results of footprint analyses in which the OmpR concentration was varied, it was suggested that the upstream region contains at least two binding sites with different affinities to the OmpR protein (9). In this study, to further elucidate the molecular mechanism underlying the activation of the *ompC* promoter by the OmpR protein, we addressed the question of how many OmpR-binding sites are present in the upstream sequence of the *ompC* promoter.

In the upstream sequence of the *ompC* promoter, there are three repetitive 10-base-pair sequences with 11- and 10-base-pair intervals (Fig. 1). These repetitive sequences were suggested to play roles in the recognition of the upstream sequence by the OmpR protein (9, 11). In the present study, we constructed a set of deletion mutants with respect to the upstream sequence which were connected with the canonical −35 and −10 regions of the *ompC* promoter followed by the *lacZ* coding sequence on a low-copy-number plasmid (9) (Fig. 1). In addition to the wild-type plasmid (pCI-0), these deletion mutant DNAs encompassing the *ompC* promoter regions were subjected to DNase I footprint analysis (3, 9) with the purified OmpR protein (6). The top DNA strand was used for the analysis, and the results are shown in Fig. 2. The footprint profiles revealed that the upstream sequence of the deletion mutant pCD-94, in which the deletion proceeded from upstream to nucleotide −95, was well protected against DNase I digestion by the OmpR protein (Fig. 2, lanes 3 and 4). Although a slight difference was observed, the protection profile was essentially the same as that observed in the wild-type (pCI-0) (Fig. 2, lanes 1 and 2), namely, the protected region comprised nucleotides −94 to −37. It should be noted that, upon OmpR binding to the region, remarkable enhancement of DNase I digestion was observed at nucleotides −74 and −73. Even in the case of the deletion mutant pCD-78, in which the deletion extended to nucleotide −79, significant protection was observed in the region between nucleotides −70 and −37 (Fig. 2, lanes 5 and 6), although the protection seems to be less than in the case of pCD-94. The upstream region of the deletion mutant pCD-35, in which nucleotides from −58 to −35 were replaced by a nonspecific linker sequence (9), was also found to be protected by the OmpR protein, and the protected region comprised nucleotides −101 to −55 (Fig. 2, lanes 7 and 8). Interestingly, it was further revealed that when the upstream sequence of pCD-7135 (in which nucleotides from −71 to −35 were deleted) was examined, the region between nucleotides −101 and −78 was clearly protected (Fig. 2, lanes 9 and 10). These results are summarized in Fig. 1. The simplest and most reasonable explanation for these observations is that the upstream sequence of the *ompC* promoter comprises at least three distinct OmpR-binding sites. At present, we cannot determine the precise boundaries of each OmpR-binding site in the nucleotide sequence. On the basis of results presented in this study, however, it was assumed that one site is located between nucleotides −94 and −75, another site is located between nucleotides −74 and −55, and the third site is located between nucleotides −54 and −35, although several nucleotides upstream from −95 may also help the OmpR binding. It should be noted that the OmpR binding to the third site is somewhat dependent on the OmpR binding to the most upstream site (Fig. 2, lanes 5 and 6). It is also noteworthy that each of the proposed OmpR-binding sites comprising 20 nucleotides contains each one of the repetitive 10-base-pair sequences.

We next examined the promoter activities of these deletion mutants of the *ompC* promoter fused to the *lacZ* gene. The plasmids listed in Fig. 1 were transferred into strains MC4100 (Δ*lacZ*, wild type with respect to *ompR*) and MH1160 (Δ*lacZ*, an *ompR1* mutant) (see references 2 and 5), and the β-galactosidase activity expressed by the cells

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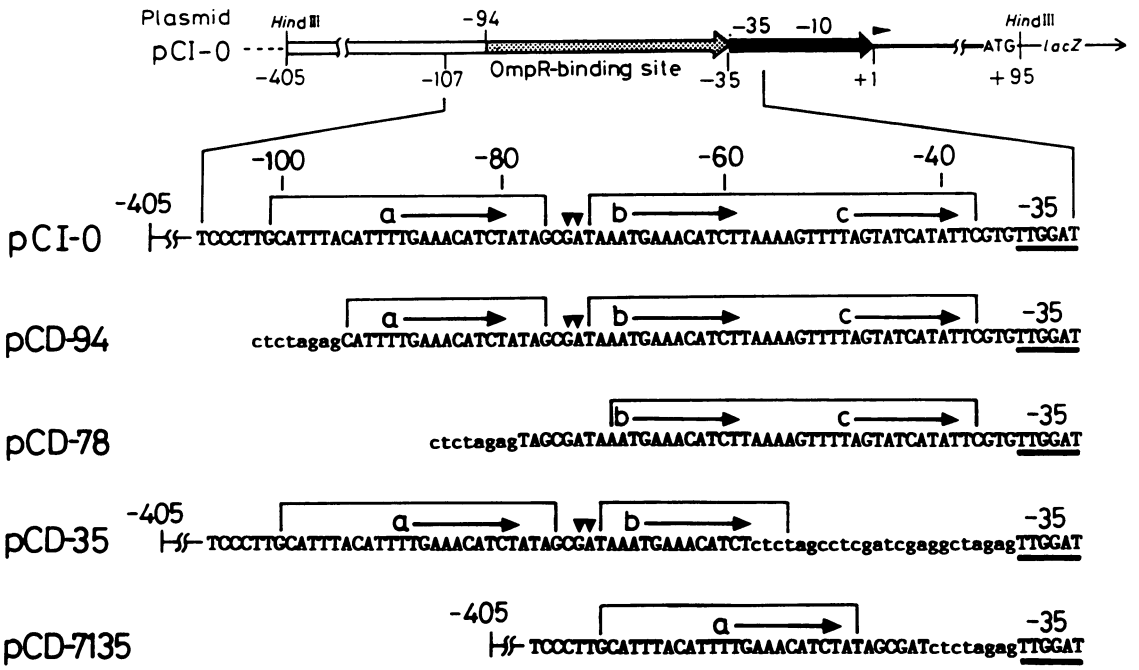

FIG. 1. Schematic representation of the structures of the wild-type and deletion mutants of the *ompC* promoter region. The structure of the wild-type *ompC* promoter fused to the *lacZ* gene, which is carried on plasmid pCI-0, is schematically presented. The *ompC* transcription starting site (▶), the canonical −35 and −10 regions (▬▶), and the essential upstream sequence (⟹) are shown. The numbers denote the positions of the nucleotides (the *ompC* transcription start site was taken as +1). The nucleotide sequences of a series of deletion mutants are presented. The three repetitive sequences are indicated by thin arrows preceded by the large lowercase letters a, b, and c. Nucleotides derived from linkers are indicated by small lowercase letters. Note that the same nucleotide numbers as those shown in the wild-type sequence are adopted for the deletion mutants in the text. The regions protected by the OmpR protein against DNase I digestion are indicated by brackets above the sequences (Fig. 2). The nucleotides at which enhancement of DNase I digestion was observed (▼) are also indicated.

harboring one of the plasmids was then measured (10) after the cells were grown in medium A containing 15% sucrose (7). Cells harboring plasmid pCD-94 exhibited OmpR-dependent expression of β-galactosidase activity, while β-galactosidase activities expressed in the cells harboring either plasmid pCD-78 or plasmid pCD-35 were significantly reduced (Table 1). More importantly, it was revealed that the deletion mutant (pCD-7135) in which only one OmpR-binding site (the most upstream one) is just in front of the −35 and −10 regions of the *ompC* promoter displayed significant β-galactosidase activity, which was expressed in an OmpR-dependent manner. The critical question of whether the transcription start site of this particular deletion mutant (pCD-7135) is the same as that of the wild-type *ompC* promoter then arose. The transcription start site of the mutant promoter was determined by reverse transcriptase mapping (1, 9). The transcription start site of the deletion mutant was the same as that of the wild-type promoter (Fig. 3, lanes 1 and 2). Furthermore, the transcription in the case of pCD-7135 appears to be dependent on the OmpR function (Fig. 3, lane 3). Considering the fact that the deletion mutant in plasmid pCD-78 did not exhibit significant β-galactosidase activity, the following was suggested. First, the most up-

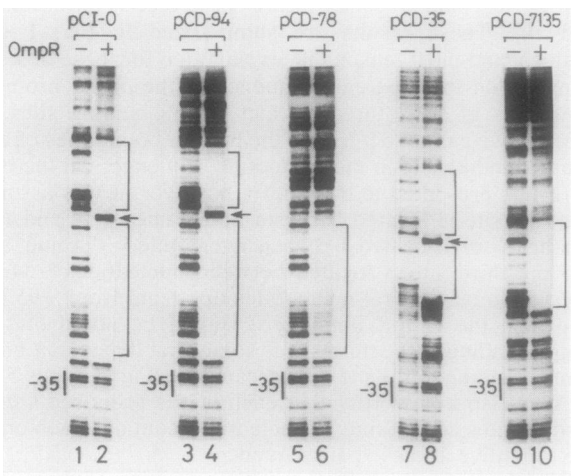

FIG. 2. DNase I footprint of the deletion mutants of the *ompC* promoter with the OmpR protein. The regions protected by the OmpR protein (700 pmol) are indicated by brackets. The enhanced regions are indicated by arrows. The precise positions of the protected nucleotides, which are summarized in Fig. 1, were determined by means of the Maxam-Gilbert G cleavage reaction (data not shown).

TABLE 1. β-Galactosidase activities expressed by the deletion mutants of the *ompC* promoter fused to the *lacZ* gene

Plasmid	β-Galactosidase activity (U) of:	
	MC4100 *recA* (*ompR*$^+$)	MH1160 *recA* (*ompR*)
pCI-0 (wild type)	826	32
pCD-94	515	108
pCD-78	90	128
pCD-35	137	31
pCD-7135	589	39

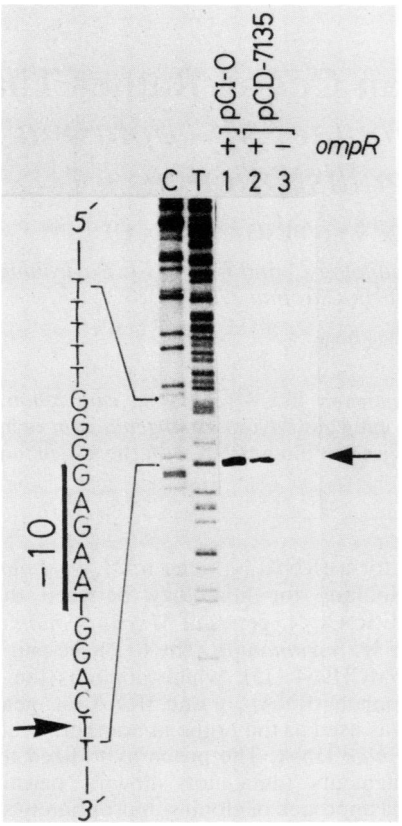

FIG. 3. Reverse transcriptase mapping of the transcription start site in the deletion mutant of the *ompC* promoter. RNA fractions were prepared from MC4100 *recA* (*ompR*$^+$; lanes denoted by +) and MH1160 *recA* (*ompR*; lane denoted by −) harboring either plasmid pCI-0 or pCD-7135. A synthetic oligonucleotide (dGTTTTCCC AGTCACGAC), which hybridized with the 5′-portion of the *lacZ* mRNA, was used as a primer for reverse transcriptase mapping. By using the same primer, nucleotide sequencing of the plasmid DNA (pCI-0) was also carried out by the dideoxy-chain termination method. Samples of the sequencing were electrophoresed adjacent to lane 1. C, C reaction; T, T reaction.

stream OmpR-binding site is essential for activation of the *ompC* promoter. Second, this single OmpR-binding site can function without the other two sites, providing that it is close to the −35 and −10 regions of the *ompC* promoter.

The results presented in this study provided us with several new clues about the mechanism underlying the activation of the *ompC* promoter by the OmpR protein. First of all, the upstream sequence of the *ompC* promoter most probably comprises three OmpR-binding sites. At present, it is not known whether the OmpR proteins that bind to the OmpR-binding sites are monomers or multimers. In any case, the three repetitive sequences located in the OmpR-binding sites are intriguing because if each one confers a contact site for the OmpR molecule, the OmpR molecules that bind to the repetitive sequences are presumably all on one side of the DNA helix. When the most upstream OmpR-binding site was absent, the efficiency of OmpR binding to the most downstream site was significantly reduced (Fig. 2, lanes 5 and 6). This suggests that the OmpR proteins bind cooperatively to the multiple binding sites. Secondly, a single OmpR-binding site, which was located originally in the most upstream region, can activate the *ompC* promoter under certain conditions, i.e., when the binding site is close to the −35 and −10 regions. We previously demonstrated that the stereospecific positioning on the DNA helix of the OmpR-binding sites with respect to the −35 and −10 regions is crucial for activation of the *ompC* promoter (8, 9). In this respect, it should be emphasized that the single OmpR-binding site in pCD-7135 is positioned in keeping with such stereospecificity on the DNA helix (Fig. 1). In conclusion, the results obtained in this study are most easily explained by the fact that the direct interaction between the OmpR protein and RNA polymerase is important in the mechanism underlying the activation of the *ompC* promoter by the OmpR protein. The multiple OmpR binding observed in the upstream region of the *ompC* promoter may play a role in the complex mechanism underlying the *ompC* expression in response to the osmolarity of the medium.

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