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Enhancement of RNA polymerase binding to promoters by a transcriptional activator, OmpR, in *Escherichia coli*: Its positive and negative effects on transcription

(enhancer sequence/osmoregulation/porins)

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ABSTRACT The OmpR binding sequence (OBS) in the upstream region of the *ompF* promoter of *Escherichia coli* was fused to 27 synthetic promoters. Transcription from a number of weak promoters, regardless of their sequences, was dramatically activated in the presence of OmpR, a transcriptional activator. *In vivo* DNA footprinting revealed that OmpR enhanced the binding of RNA polymerase to the promoters. This enhancement was essential for transcription of weak promoters, while OmpR binding to the OBS fused to a strong promoter was inhibitory for transcription. These results indicate that OmpR stabilizes the formation of an RNA polymerase–promoter complex, possibly a closed promoter complex, and that a transcription activator can serve not only as a positive but also as a negative regulator for gene expression.

Studies on transcriptional activation in eukaryotic cells have demonstrated that certain eukaryotic transcriptional activators for RNA polymerase II are able to function in heterologous systems as long as the test genes contain DNA sequences recognized by the transcriptional activators (for a review, see ref. 1). In contrast, no transcriptional activators in *Escherichia coli* have been shown to work in heterologous systems, and efforts to construct generalized activators have failed (1). In the present report, we demonstrate that OmpR, a transcriptional activator for *ompC* and *ompF* of *E. coli*, the genes for major outer membrane porins (2), is able to function as a generalized activator for various unrelated promoters.

There are a number of *E. coli* genes whose transcription is activated by a protein factor binding to a specific sequence upstream of the RNA polymerase recognition site (3–5). The RNA polymerase recognition sites for genes requiring transcriptional activators for σ^{70} RNA polymerase are considerably different from the consensus sequence consisting of the −35 region (TTGACA) and the −10 region (TATAAT) relative to +1, the transcription initiation site (6). Because the sequences of these promoters diverge from the consensus sequence, RNA polymerase is either unable to bind to the promoters or to isomerize the closed complex of the promoter and RNA polymerase to the open complex without the aid of transcriptional activators. In the case of *ompC* and *ompF*, RNA polymerase is unable to transcribe these genes without OmpR because the −35 and −10 regions of these genes are quite different from the consensus sequence (7, 8). The OmpR binding sites for both genes have been shown to exist in the regions from −40 to −100 (9, 10). Recently, by *in vivo* DNA footprinting we demonstrated that within the OmpR binding regions there are two different motifs, the F and C boxes, and that OmpR binding to these motifs plays important roles in the regulation of *ompF* and *ompC* expression (11).

We now examine whether OmpR is able to activate transcription from promoters with different sequences and whether OmpR binding to OmpR binding sequences (OBSs) enhances binding of RNA polymerase to a promoter sequence. For this purpose, the OBS from the *ompF* gene was fused to 27 different synthetic promoters and transcription from these promoters *in vivo* was examined in the absence and presence of OmpR with *lacZ* used as a reporter gene.

MATERIALS AND METHODS

Construction of Synthetic Promoters. Plasmid pTB0533 (pBR322 derivative) contains the *Xba* I/*Bam*HI DNA fragment of the *ompF* promoter from −195 to +118, which encompasses a part of the coding sequence from the initiation codon to the fourth codon. This fragment also contains four OBSs (Fa, Fb, Fc, and Cd boxes; see Fig. 1*A*) as well as the −35 and −10 promoter sequence. The *lacZ* gene coding sequence starting from the eighth codon is fused in-frame to the above *ompF* sequence. By site-specific mutagenesis the 7-base-pair (bp) DNA sequence immediately after the Cd box (Fig. 1*A*) of *ompF*, TCACGGT* (asterisk indicates the first base in the −35 region of *ompF*), was replaced with the sequence AAGATCT to generate a unique *Bgl* II site (underlined). The DNA sequence between this *Bgl* II site and an original *Pst* I site at the +1 position (transcription initiation site) of *ompF* was then replaced with synthetic oligonucleotides. For example, the sequence for promoter 1 consists of the consensus −35 sequence (TTGACA), an 18-bp spacer sequence (CTTTAA*GCTTCCGGCTCG), the −10 sequence of the *ompC* promoter (GAGAAT), and a 9-bp sequence (GTCGACA*AT) after the −10 sequence to generate a unique *Sal* I site (underlined). The spacer sequence is identical to that of the *lac* promoter (6) except that the sixth T residue (indicated by an asterisk) was changed to A to create a unique *Hin*dIII site (underlined). The 9-bp sequence after the −10 sequence is from the *lacZ* promoter sequence, where the A residue with an asterisk indicates the transcription initiation site. Other synthetic promoters (from no. 2 to no. 27) were constructed by replacing DNA sequences either between the *Bgl* II and *Hin*dIII sites (for the −35 region) or between the *Hin*dIII and *Sal* I sites (for the −10 region) with their respective oligonucleotides. In all promoters except for promoter 10, the DNA sequences beside the −35 and −10 sequences are identical to those for promoter 1 described above. In promoter 10, the 2-bp sequence (CG) at the 3′ end of the 18-bp spacer sequence was removed to reduce the promoter activity, since the promoter with the 18-bp sequence was lethal to cells. In all promoters, the −35 regions

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Abbreviation: OBS, OmpR binding sequence.

are 5 bp downstream of the Cd box (see Fig. 1). Because of the difference of the first base in the −35 regions in different promoters, only those promoters whose −35 sequences start with a T residue retain the *Bgl* II site. An *ompR*$^+$ *E. coli* strain (MC4100; ref. 2) and its isogenic *ompR*$^-$ strain (MH1160; ref. 2) were then transformed with these plasmids constructed as described above.

***In Vivo* Dimethyl Sulfate DNA Footprinting.** Cells carrying the synthetic promoter plasmids were grown in L-broth medium (20 ml) supplemented with ampicillin (50 μg/ml) to midlogarithmic phase. Dimethyl sulfate [final concentration, 0.1% (vol/vol)] was directly added to the cells under vigorous shaking at 37°C. The treatment was continued for 20 sec before 2 ml of 0.5 M EDTA (pH 8.0) was added to the culture. After 30 sec, 15 g of ice was added directly to the culture. The plasmid DNA was isolated and digested with *Bam*HI, which cuts the plasmid DNA at ≈120 bp downstream from the +1 position (transcription initiation site). The linear DNA fragment was then gel isolated and end labeled with [γ-^{32}P]ATP. The labeled DNA fragment was then digested with *Xba* I, which cuts at the −195 position. The resultant *Xba* I/*Bam*HI fragment was again gel purified and cleaved with 1 M piperidine (G>A cleavage) and analyzed on a 6% sequencing gel. DNA manipulation is according to Maniatis *et al.* (12).

β-Galactosidase Activity Assay. β-Galactosidase assay was performed as described and expressed in Miller units (13). Since the promoter 10 plasmid was unstable, Lac$^-$ cells were constantly generated. Therefore, the β-galactosidase activity was corrected by using the ratio of Lac$^+$ to Lac$^-$ cells in a given culture, which was obtained by plating the culture on Lac indicator plates.

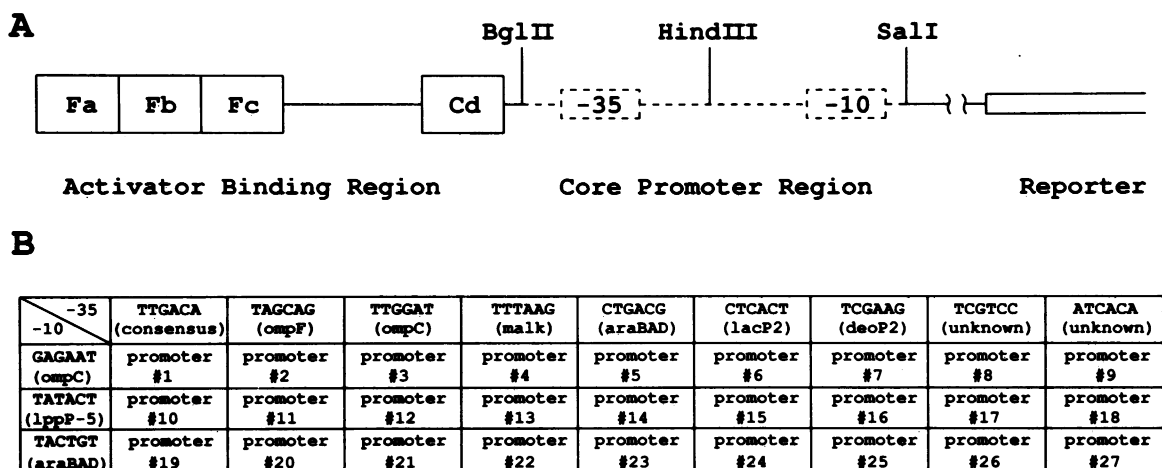
RESULTS

Construction of Synthetic Promoters. The fragment containing the OBS used in the present experiments (Fig. 1*A*) was taken from the *ompF* gene and encompasses −42 to −195. By *in vivo* DNA footprinting, we demonstrated that the *ompF* OBS contains three tandemly repeated F boxes—Fa, Fb, and Fc (Fa, $^{-100}$TTTACTTTTG^{-91}; Fb, $^{-90}$GTTACA-TATT^{-81}; Fc, $^{-80}$TTTTCTTTTT^{-71})—and a C box (Cd, $^{-51}$TGTAGCACTT^{-42}) (11). This DNA fragment was fused to a small DNA fragment (the core promoter region) containing DNA sequence features recognized by the *E. coli* σ^{70} RNA polymerase. The core promoter region was engineered in such a way that the critical −35 and −10 motifs were bound by unique restriction sites as diagrammed in Fig. 1*A* (see also *Materials and Methods* for construction details). The variation of promoter sequences was created by replacing either the −35 or the −10 regions with oligonucleotides containing different sequences. To monitor the activities of the synthetic promoters thus constructed, the *lacZ* gene was fused downstream to the promoters as a reporter gene *in vivo*.

Nine *Bgl* II/*Hin*dIII synthetic fragments containing different −35 sequences from various promoters (see Fig. 1*B*) and three *Hin*dIII/*Sal* I synthetic fragments containing different −10 sequences (see Fig. 1*B*) were used. Of the nine −35 sequences, the following were used: TTGACA for the consensus sequence (6), TAGCAG from *ompF*, TTGGAT from *ompC* (7–9), TTTAAG from the MalT-dependent *malK* promoter (4, 6), CTGACG from the AraC-dependent *araBAD* promoter (6, 14), and CTCACT from cAMP receptor protein-dependent *lac*P2 promoters (6, 15) TCGAAG, TCGTCC, and ATCACA. The last three sequences were designed to generate three mismatches at different positions within the consensus sequences. The sequence TCGAAG happened to be identical to the −35 sequence of *deo*P2 promoter (6). The three −10 sequences include GAGAAT from the OmpR-dependent *ompC* promoter (8, 9), TACTGT from the AraC-dependent *araBAD* promoter (6, 14), and TATACT from the constitutive *lpp* P-5 promoter (16). The last −10 sequence has been shown to exert stronger transcriptional activities than the consensus −10 sequence (TATAAT; ref. 6) when it was combined with the consensus −35 sequence (16).

All possible combinations of these −35 and −10 synthetic fragments were fused with the OBS sequence and the reporter sequence in a pBR322 derivative yielding 27 different promoters (numbered 1–27) as listed in Fig. 1*B*. In all cases, the promoter sequences were extended by 1 base (because of the distance between the −35 and the −10 regions) from the *ompF* promoter, which has a 17-bp spacer (7, 9) and transcription is considered to initiate from the A residue immediately after the *Sal* I site (see *Materials and Methods*). Note that for the no. 10 promoter a 16-bp sequence was used instead of the 18-bp sequence between the −35 and the −10 region (see *Materials and Methods*) to reduce the promoter activity. Construction of the promoter with the 18-bp spacer was unsuccessful, and even the cells harboring the no. 10

Assembly of Synthetic Promoters



-35 / -10	TTGACA (consensus)	TAGCAG (ompF)	TTGGAT (ompC)	TTTAAG (malk)	CTGACG (araBAD)	CTCACT (lacP2)	TCGAAG (deoP2)	TCGTCC (unknown)	ATCACA (unknown)
GAGAAT (ompC)	promoter #1	promoter #2	promoter #3	promoter #4	promoter #5	promoter #6	promoter #7	promoter #8	promoter #9
TATACT (lppP-5)	promoter #10	promoter #11	promoter #12	promoter #13	promoter #14	promoter #15	promoter #16	promoter #17	promoter #18
TACTGT (araBAD)	promoter #19	promoter #20	promoter #21	promoter #22	promoter #23	promoter #24	promoter #25	promoter #26	promoter #27

FIG. 1. Construction of synthetic promoters. (*A*) The activator binding region is taken from the *ompF* promoter, which contains OmpR binding sites Fa, Fb, and Fc (−100 to −71) and Cd (−51 to −42). The core promoter region contains variable −35 and −10 motifs and a constant 18-bp (16 bp in promoter 10) spacer sequence between these two motifs. Promoter sequence variations can be achieved through replacing the *Bgl* II/*Hin*dIII or the *Hin*dIII/*Sal* I fragments with synthetic oligonucleotides. The *lacZ* coding sequence is used as the reporter gene in this study. (*B*) The −35 and −10 sequences of the synthetic promoters used in this study.

promoter fused with *lacZ* grew very slowly because of high production of β-galactosidase.

Promoter Activities in the Presence and Absence of OmpR. *E. coli* MC4100 ($ompR^+$) and its isogenetic strain MH1160 ($ompR^-$) (2, 11) were transformed with the plasmids constructed as described above. β-Galactosidase activities of the transformed cells were measured at midlogarithmic phase and are shown in Fig. 2. The results can be summarized as follows: (*i*) Transcription of 19 of 27 promoters was enhanced by OmpR. These promoters (nos. 1–4, 11–23, 25, and 26) do not share specific sequences, indicating that OmpR-mediated transcriptional activation is not limited to specific −35 and −10 sequences. Furthermore, promoter-nonspecific activation by OmpR suggests that OmpR enhances the binding of RNA polymerase to the promoters by its interaction with RNA polymerase containing σ^{70}. σ^{70} has been shown to be required for OmpR-dependent activation of *ompF* transcription *in vitro* (S. Norioka and M.I., unpublished result). This OmpR enhancement of RNA polymerase binding to the promoters will be demonstrated by *in vivo* footprinting as described later. (*ii*) Transcription from a strong consensus promoter (no. 10; see Fig. 2*B*) was inhibited more than 5-fold by OmpR. Since promoter 10 is arranged in the same way as the other promoters described in *i*, OmpR bound to OBS of promoter 10 is most likely to interact with RNA polymerase in the same manner as in the case of other promoters described in *i*. Therefore, this same interaction that stimulates transcription of the weak promoters is considered to inhibit transcription of promoter 10. This will also be demonstrated by *in vivo* footprinting as described later. (*iii*) Seven promoters (nos. 5–9, 24, and 27) were not affected by OmpR. These promoters, using either the *ompC* or *araBAD* −10 sequence, are inactive in the absence of OmpR and are probably very weak promoters so that even OmpR binding to OBS is unable to assist RNA polymerase to bind the promoters.

***In Vivo* DNA Footprinting.** The results described above suggest that OmpR binding to the OBS of most of the promoters is responsible for transcriptional activation in the presence of OmpR. Similarly OmpR binding to the OBS of promoter 10 is considered to inhibit RNA polymerase to initiate transcription. To elucidate how OmpR interacts with the various promoters used in the present study, *in vivo* DNA footprinting (11) was carried out. Fig. 3 shows DNA footprinting on four synthetic promoters (nos. 1, 5, 10, and 14). Both promoters 5 and 14 contain the *araBAD* −35 region. However, promoter 5 is transcriptionally inactive with or without OmpR (Fig. 2*A*), while promoter 14 is active and its transcription is significantly enhanced in the presence of OmpR (Fig. 2*B*). This difference is most likely to be attributed to the −10 regions; promoter 14 has a more consensus-like sequence than promoter 5 (see Fig. 1*B*). It is important to notice that even if transcriptionally inactive, the F and C boxes of promoter 5 were protected in the presence of OmpR (Fig. 3*A*, lane 1) to the same extent as those of promoter 14 (lane 3). This indicates that OmpR can bind to OBS independently from the −35 and −10 sequences, and that OmpR binding to OBS is essential but not sufficient for transcriptional activation. The difference between promoter 5 and promoter 14 is probably that promoter 5 cannot be recognized by RNA polymerase, while promoter 14 can be recognized by RNA polymerase, although the enzyme is unable to form a stable promoter complex without the aid of OmpR.

For DNA footprinting of promoter 14, as in the case with the *ompF* and *ompC* promoters (11), we were unable to demonstrate the binding of RNA polymerase to the promoter.

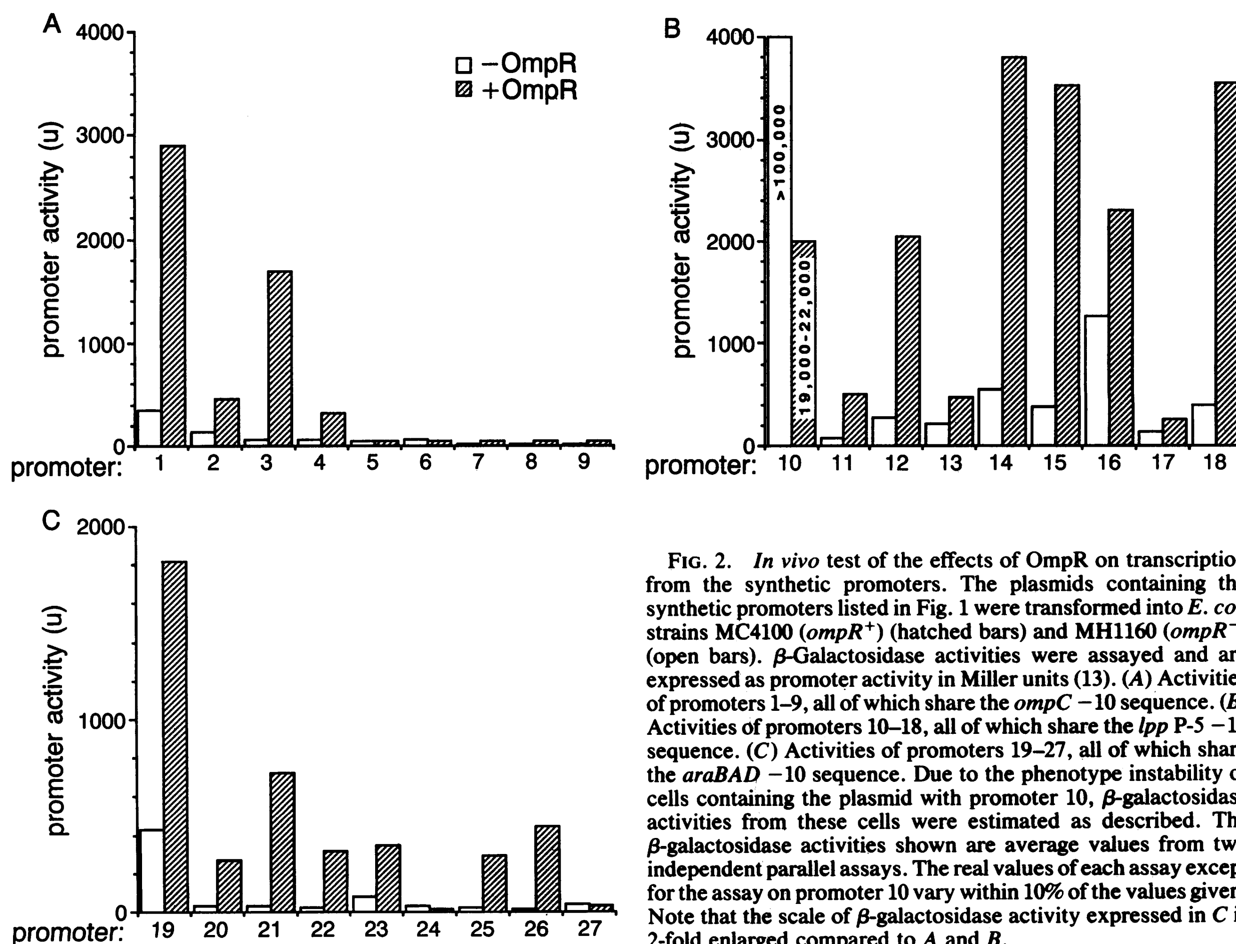


FIG. 2. *In vivo* test of the effects of OmpR on transcription from the synthetic promoters. The plasmids containing the synthetic promoters listed in Fig. 1 were transformed into *E. coli* strains MC4100 ($ompR^+$) (hatched bars) and MH1160 ($ompR^-$) (open bars). β-Galactosidase activities were assayed and are expressed as promoter activity in Miller units (13). (*A*) Activities of promoters 1–9, all of which share the *ompC* −10 sequence. (*B*) Activities of promoters 10–18, all of which share the *lpp* P-5 −10 sequence. (*C*) Activities of promoters 19–27, all of which share the *araBAD* −10 sequence. Due to the phenotype instability of cells containing the plasmid with promoter 10, β-galactosidase activities from these cells were estimated as described. The β-galactosidase activities shown are average values from two independent parallel assays. The real values of each assay except for the assay on promoter 10 vary within 10% of the values given. Note that the scale of β-galactosidase activity expressed in *C* is 2-fold enlarged compared to *A* and *B*.

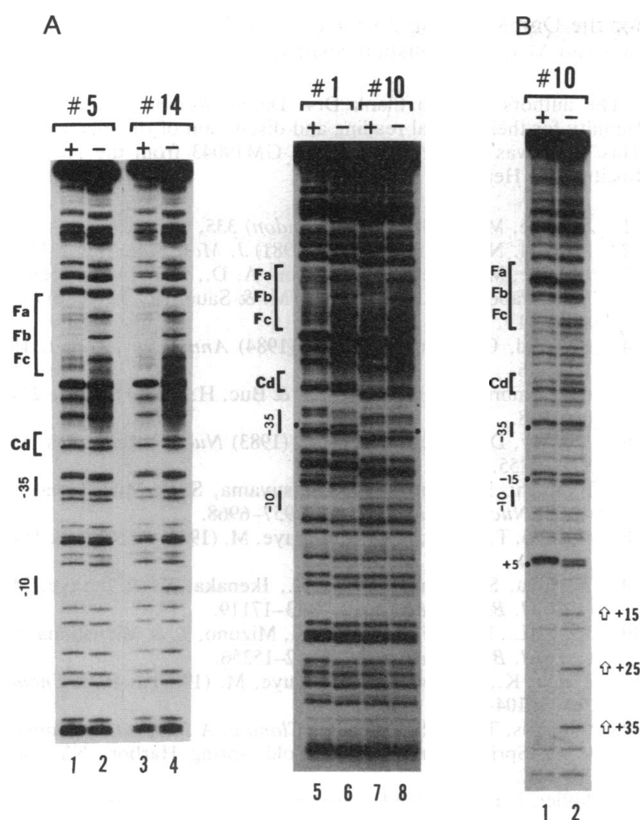


FIG. 3. *In vivo* DNA footprinting assay of various synthetic promoters. *E. coli* MC4100 (*ompR*$^+$) and MH1160 (*ompR*$^-$) cells carrying the synthetic promoter plasmids were grown in 20 ml of L-broth supplemented with ampicillin (50 μg/ml) to midlogarithmic phase. DNA footprinting (G-specific banding) was carried out as described. (*A*) Methylation protection patterns of the bottom or antisense strands from promoters 5, 14, 1, and 10. (*B*) Methylation protection patterns of the top (sense) strand from promoter 10. Fa, Fb, Fc, and Cd are indicated by brackets and have been shown to be DNA sequence motifs known to be protected by OmpR *in vivo* (11). The G residue in the −35 region protected from methylation is indicated by dots. Lane 1, the G residue at positions −15 and +5, which are also protected, are indicated by dots. Lane 2, enhanced cleavages of A residues at positions +15, +25, and +35 are indicated by open arrows. Methylation was carried out in both the *ompR*$^+$ and *ompR*$^-$ cells, which are indicated by + and −, respectively, on the top of each lane. The numbers on the top of the lanes indicate the promoter numbers (see Fig. 1). The −35 and −10 regions are marked by vertical bars.

However, clear OmpR-dependent protection by RNA polymerase at the −35 regions can be observed for promoter 1 as well as for promoter 10. Both promoters share the consensus −35 sequence, and promoter 1 contains the *ompC* −10 region, while promoter 10 contains the *lpp* P-5 −10 region (16). Transcription from promoter 1 was stimulated ≈10-fold by OmpR (Fig. 2*A*), while transcription from promoter 10 was repressed ≈5-fold by OmpR (Fig. 2*B*). Nevertheless, in both cases the same G residue in the −35 region (complementary to the C residue of TTGACA) was protected (Fig. 3*A*, lanes 5, 7, and 8 vs. lane 6, indicated by dots), which is most likely due to RNA polymerase binding for the following reasons: First, the protected G residue is in the RNA polymerase recognition region. Second, *in vitro* DNA footprinting of the promoters with the same −35 sequence as promoters 1 and 10 has shown the same protection pattern by RNA polymerase (18). Third, there is no protection at this G residue (complementary to the $\overset{*}{C}$ residue of CTGA$\overset{*}{C}$G) for promoters 5 and 14 in the presence of OmpR, while the clear protection is observed even in the absence of OmpR for promoter 10 (compare lane 8 to lane 6 in Fig. 3*A*). These facts eliminate the possibility of OmpR binding at this position. Finally, *in vivo* DNA footprinting on the other strand of promoter 10 (Fig. 3*B*, lanes 1 and 2) showed three more OmpR-dependent protections in the RNA polymerase binding region (−35, −15, and +5, indicated by dots).

These observations strongly suggest that OmpR stabilizes the binding of RNA polymerase to the promoters. Since the OmpR-dependent enhancement of RNA polymerase binding was found for both promoter 1, an OmpR-stimulated promoter, and promoter 10, an OmpR-inhibited promoter, both the positive and negative effects of OmpR are likely to be caused by the same interaction between OmpR and RNA polymerase. In the case of a weak promoter, this interaction is essential for RNA polymerase to transcribe the otherwise inactive promoter and results in transcriptional activation. In the case of a strong promoter, which does not require OmpR for transcription, the same interaction becomes inhibitory for RNA polymerase to initiate transcription.

In addition to the protections described above, four hypermethylation sites were found on promoter 10: In the presence of OmpR (Fig. 3*B*, lane 1), a hypermethylated band appeared at the +4 position (a T residue). In the absence of OmpR, three A residues (at positions +15, +25, and +35) were hypermethylated (lane 2), while these A residues were not hypermethylated in the presence of OmpR (lane 1). These hypermethylations are most likely due to the change of DNA conformation in this region. Such a change is possibly due to the isomerization from a closed promoter in the presence of OmpR to an open promoter complex in the absence of OmpR. The methylation protection by RNA polymerase was also observed in the DNA footprinting on promoter 19, which contains the consensus −35 sequence and the −10 sequence from the *araBAD* promoter (data not shown). This indicates that a consensus sequence in the −35 region but not in the −10 region is essential for the methylation protection by RNA polymerase.

DISCUSSION

The present study clearly demonstrates that OmpR can bind the OBSs without interacting with RNA polymerase. However, RNA polymerase appears to then be able to associate with the OmpR molecules bound to DNA. If there is a −35 and a −10 sequence properly positioned from the OmpR binding site, σ^{70} RNA polymerase is considered to be held on the promoter site by two factors: (*i*) the interaction between RNA polymerase and the −35 and −10 sequences and (*ii*) the interaction with OmpR. The latter interaction stabilizes RNA polymerase binding to weak promoter sequences, which are otherwise unable to function as promoters. Thus, as shown in this study, OmpR is able to activate transcription from a number of weak promoters regardless of their nucleotide sequences.

It appears that the enhancement of RNA polymerase binding to the promoter sequence by OmpR simply stabilized the formation of a closed promoter complex. Thus, the OmpR–RNA polymerase interaction probably functions to inhibit the isomerization of the closed promoter complex to an open promoter complex. This is particularly evident in the case of strong promoters such as promoter 10 when the OBSs are added upstream of the promoters. *In vivo* footprinting indicates that the closed promoter complex for promoter 10 was accumulated in the presence of OmpR (Fig. 3), which agrees well with the fact that the transcription of the gene with promoter 10 was inhibited by OmpR (Fig. 2). These results demonstrate a unique mechanism for a transcriptional activator to function not only as a positive but also as a negative regulatory factor for gene expression. Indeed, it has been proposed that OmpR may function as a transcriptional

repressor for the *ompF* gene in high osmolarity by binding to the most proximal OBS, the Cd box, to the *ompF* −35 sequence (11). It is speculated that the OmpR binding to the Cd box holds RNA polymerase tightly at the promoter site and prevents the isomerization from the closed to the open promoter complex. Transcriptional activators such as the AraC protein (19) and the λcI protein (3) are known to function as repressors as well. However, these proteins are considered to block the entry of RNA polymerase to their respective promoters.

Generalized enhancement of RNA polymerase II-dependent transcription by transcriptional activators has been shown in the eukaryotes, while promoter nonspecific activation of transcription has not been demonstrated previously in *E. coli* (1). Among prokaryotic transcriptional activators, the λ repressor (3), the NtrC protein (20), and the cAMP receptor protein (15) have been proposed to interact with RNA polymerase. Whether these activators can enhance transcription in a promoter nonspecific manner as OmpR remains to be determined.

It has been suggested that cAMP receptor protein stimulates the formation of a closed promoter complex (5), while the λ repressor protein (21) and NtrC (20) have been shown to stimulate the isomerization from a closed to an open promoter complex. It is interesting to note that both OmpR and NtrC belong to a family of bacterial regulatory proteins sharing high sequence homologies at their N-terminal domains of ≈120 amino acid residues (22). However, the C-terminal domain of NtrC consisting of ≈350 residues is much larger than that of OmpR, which is ≈120 residues. The C-terminal domain of NtrC is responsible not only for DNA binding but also for ATP-dependent stimulation of promoter complex isomerization (23, 24), while the C-terminal domain of OmpR is considered to be required only for DNA binding (11). Thus, the most important difference between NtrC and OmpR is that NtrC does not stimulate the binding of σ^{54} RNA polymerase to the promoter, while OmpR enhances the binding of σ^{70} RNA polymerase to the promoter but not the isomerization of the RNA polymerase–promoter complex. In fact, a recent study has shown that NtrC could activate other σ^{54} promoters but not the *lac* promoter, which is recognized by σ^{70} (25).

At present, it is not known which subunit(s) of RNA polymerase directly interacts with OmpR. Genetic data, however, suggest that the α subunit is involved in the interaction (17). In a preliminary experiment, overproduction of the α subunit *in vivo* showed an inhibitory effect on the transcriptional activation of the *ompF* and *ompC* genes by OmpR (unpublished result). This inhibitory effect was not observed on the expression of housekeeping genes (as monitored by cell growth rate). Also, it is not observed from the overproduction of the major σ factor (σ^{70}), which is required for the OmpR-mediated transcriptional activation (S. Norioka and M.I., unpublished result).

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