OmpR is a transcription factor in Escherichia coli whose function is modulated by phosphorylation in the presence of phosphorylated EnvZ, a transmembrane protein histidine kinase involved in osmosensing. Using a protein S-OmpR hybrid protein, we demonstrated that six OmpR molecules bind tandemly to the −100 to −39 sequence of ompF. This sequence consists of three 20-base pair units: F1, F2, and F3, each of which is bound by two OmpR proteins. Polymerase chain reaction selection of nine randomized base pairs within the F1 sequence revealed highly conserved C residues spaced 10 base pairs apart. Further mutational analysis of conserved bases indicated that two OmpR molecules bind tandemly to two direct repeats. Mobility shift assays showed that cooperative interactions play a role in enhancing binding of OmpR to lower affinity F2 and F3 sites. Activation and repression of ompF expression are thus regulated by a total of eight OmpR molecules, including two molecules that bind to a distal site (−380 to −361).

When exposed to changes in environmental osmolarity, Escherichia coli responds by altering the expression of OmpF and OmpC, two porin proteins present in the outer membrane (for review, see Csonka and Hanson (1991)). OmpF and OmpC each exist as homotrimeric proteins that span the outer membrane and serve as channels for the passive diffusion of small molecules between the external media and the periplasmic space (Nikaido and Vaara, 1987). Under low osmolarity conditions, OmpF is present in the outer membrane at significantly higher levels than OmpC. Upon a shift to high osmolarity, OmpF is no longer expressed, while the level of OmpC is increased (Kawaji et al., 1979).

Expression of the ompF and ompC genes is dependent upon two proteins, EnvZ and OmpR (Sarma and Reeves, 1977; Hall and Silhavy, 1981a, 1981b; Mizuno and Mizushima, 1986; Slauch et al., 1987; Slauch et al., 1988). OmpR is phosphorylated at Asp55 (Baker and Weiss, 1990; Forst et al., 1989a; Ito et al., 1990; Aiba et al., 1990). The level of phosphorylated OmpR present in the cell appears to be controlled through the regulation of EnvZ phosphorylation activity (Yan and Inouye, 1991; Russo and Silhavy, 1991; Yang and Inouye, 1993; Jin and Inouye, 1993). At low osmolarity, EnvZ phosphorylation activity maintains a low concentration of phosphorylated OmpR. Upon a shift to high osmolarity, the phosphorylation activity of EnvZ is decreased, allowing an increase in the level of phosphorylated OmpR. Phosphorylation of OmpR enhances DNA-binding activity (Aiba et al., 1989b; Aiba and Mizuno, 1990). OmpR binds to sequences upstream of the ompF and ompC promoters to regulate the transcription of these genes. In vivo and in vitro footprinting has demonstrated that OmpR binds to the −100 to −80 and −74 to −40 sequences of ompF (Maeda and Mizzuno, 1990), and to the −100 to −40 (Maeda and Mizzuno, 1988; Maeda et al., 1988; Mizzuno and Mizushima, 1986; Mizzuno et al., 1988; Tsung et al., 1989) and −380 to −360 sequences of ompF (Rampersaud et al., 1994; Huang et al., 1994). Phosphorylated OmpR binds to the upstream regulatory region of ompC to activate ompC expression. However, in the absence of OmpR, OmpR acts as both an activator (during low osmolarity conditions) and repressor (during high osmolarity conditions) of transcription (Slauch and Silhavy, 1989; Forst et al., 1989b). Since low osmolarity conditions are correlated with low levels of phosphorylated OmpR, it would be expected that under these conditions, only high affinity sites are bound by OmpR. In contrast, under high osmolarity conditions when there is a higher level of phosphorylated OmpR in the cell, OmpR will bind to both high and low affinity sites. It is of interest to understand how OmpR carries out this dual function. Identifying high and low affinity sites will aid in identification of activator and repressor sites.

In the studies presented here, an OmpR fusion protein was designed and used for mobility shift assays to determine the exact number of OmpR molecules that bind to sequences upstream of the ompF promoter. Subsequently we were able to identify individual OmpR binding sites and use a random sequence selection/PCR amplification method to identify critical base pairs within the OmpR binding site. Finally, mobility shift assays were used to demonstrate that cooperative interactions between OmpR proteins bound to the DNA increase the ability
of OmpR to bind to low affinity sites. In this paper, we demonstrate that six OmpR molecules cooperatively bind to the -100 to -39 sequence of ompF. Taken with previously published data regarding the distal -380 to -361 binding site, a total of eight OmpR molecules are involved in the positive and negative regulation of ompF expression.

**Experimental Procedures**

Plasmids and Oligonucleotides—pPR010 is a pt7-7 derivative that contains the fusion gene that encodes the PrS2-OmpR protein. The wild-type ompR gene was obtained from the plasmid pTB020, which harbors the ompR gene. PCR was used to generate a fragment containing the wild-type ompR gene with a NdeI site located at the 5'-end where the first methionine of OmpR is encoded. The 3'-PCR primer annealed to 3'-end of ompR and provided a BamHI site. The PCR fragment was digested with NdeI and BamHI and ligated into the T7 expression vector pET7-7, which had been linearized by a BamHI/NdeI digest, to create the pt7ompR plasmid. To create the PrS5-OmpR fusion gene, plasmid pUC19 was used. This is a pUC9 derivative that contains a fragment encoding the amino-terminal half of protein S. This gene was obtained through PCR amplification of the protein S gene such that NdeI sites were created at the 5'-end where the first methionine is encoded and at the 3'-end where arginine 92 is encoded. The PCR product was sequenced to confirm no mutations had been created by the PCR. The NdeI fragment was cloned into the pt7ompR plasmid digested with NdeI allowed the ligation of two tandem protein S PCR products (encoding protein S amino acids 1-92 followed by a histidine residue introduced by the 3'-PCR primer) to the 5'-end of the ompR gene. The plasmid was named pPR010.

The construct pCF001 contains the upstream regulatory regions of both the ompC and ompF genes, cloned into pGEM3zf(+)(Promega). The ompF upstream regulatory sequence was obtained from the plasmid pAR094 (Rampersaud et al., 1994). The ompC regulatory sequence was obtained from the plasmid pGR241 (Noricka et al., 1986). The fused sequence was created by a two-step PCR reaction. To amplify the ompF sequence, a 3'-PCR primer (AGAAGTTCCAGGCCATCTC) was annealed to 199 - 187 sequence of ompC and also created an EcoRI site. The 3'-PCR primer (AAGGTCCTAATCCAAACGAATTAGA) was annealed to the -44 to -27 sequence of the ompF gene and created a BamHI site. To amplify the ompF sequence, a 5'-PCR primer (GATTGGATCCAGTCTTTAATTATTAC) was annealed to the -112 to -95 sequence of ompF and created a BamHI site. The corresponding 3'-PCR primer (TCAGGCTTGCAGTTCT) was annealed to the -2 to +12 sequence of ompF. The correct sequence was confirmed by DNA sequencing. The 3'-PCR primer binding site was sequenced on an Applied Biosystems DNA synthesizer. Complimentary strands were annealed to create double-stranded DNA sequences for mobility shift assays.

**Mobility Shift Assays—** DNA fragments containing the ompF regulatory sequences were labeled by first digesting the pCF001 plasmid with HindIII followed by incubation with Klenow in the presence of [α-32P]dATP. The DNA was then digested with BamHI, and the labeled fragment was gel-purified. Synthetic DNA molecules were labeled using T4 polynucleotide kinase and [γ-32P]ATP.

Binding reactions were carried out in 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM MgCl2, 5% glycerol, 0.0025% Nonidet P-40, and 200 ng of poly(dI-dC). First, OmpR was phosphorylated in vitro using ATP and the purified catalytic cytoplasmic domain of EnvZ (aminoglycoside-23-Gly) referred to as EnvZIC (Delgado et al., 1993). OmpR was incubated with 7 mM EnvZIC in the binding buffer with 60 mM ATP at 25°C for 10 min. Appropriate amounts of purified OmpR or PrS5-OmpR were then added and allowed to incubate for another 15 min at 25°C. Labeled DNA was added to each reaction, and the final reaction volume was 15 μl. Binding reactions were incubated at 4°C for 30 min and then loaded onto a 5% acrylamide/bisacrylamide (40:1.2) gel. The gel was run at 4°C in 1x TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and run at 120 V. The dried gel was exposed to autoradiography film overnight at -80°C.

Expression of the PrS5-OmpR Fusion Protein—The PrS5-OmpR protein was expressed using the T7 expression system. BL21(DE3) cells were transformed with pRPR010 and grown until mid-log phase at 37°C in Luria Broth containing 100 μg/ml ampicillin. Expression of PrS5-OmpR was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside, and the cells were grown for another 2.5 h. PrS5-OmpR was purified by the same protocol used for wild-type OmpR purification (Forst et al., 1988a, 1988b). The final PrS5-OmpR protein was greater than 90% pure and was stored as a 1 mg/ml stock in 20% glycerol at -20°C.

Random Selection and Amplification of OmpR Binding Sites—For the initial randomized template, a single-stranded DNA molecule was synthesized. The first template used contained 20 randomized nucleotides and had the sequence ATAGGTTCGGCCGGC (N20)ATATGGAATTCCTACAG, where N20 represents a random 20-mer nucleotide sequence composed of equimolar amounts of G, A, T, and C. Its corresponding PCR primers were ATAGGTTCGGCCGGC (5') and GCGGATCCATAA (3'). A second template had the sequence GACGGATCCTTTATCTTTG (N9)TGCTAGGAGATTCAC, where N9 represents a random 9-mer nucleotide sequence composed of equimolar amounts of G, A, T, and C. The corresponding PCR primers were GACGGATCCTTTATCTTTG (5') and GTGAGAATTCCTACAG (3'). For the initial shift, the single-stranded DNA oligonucleotide containing the random bases was made into a double-stranded DNA template using Klenow in the presence of an end-labeled PCR primer. Approximately 100 pmol of DNA and 350 ng of OmpR were used for the initial binding reaction. The binding reactions were run as described above. The wet gel was exposed for 5 h at 4°C. Gel above the free DNA was cut out, crushed, and soaked overnight at 37°C in elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, pH 8.0, and 0.1% SDS). The eluted DNA was ethanol precipitated and resuspended in 25 μl of 0.1 x TE buffer. Five μl of this DNA was used as template for a PCR reaction. The PCR reaction was 95°C for 1 min, 45°C for 2 min, 72°C for 1 s, cycled 29 times. The PCR product was gel-purified before subsequent gel shifts.

**Results**

Construction of an OmpR Fusion Protein—OmpR DNA-binding activity has been extensively characterized through footprinting and mobility shift assays. In vivo and in vitro footprinting studies have shown that OmpR binds to the -100 to -40 sequence of ompC (Maeda and Mizzuno, 1990) and to the -380 to -360 (Rampersaud et al., 1994; Huang et al., 1994) and -100 to -40 sequence of ompF (Mizzuno et al., 1988). However, it is not known how many OmpR molecules bind to the upstream region of the ompF and ompC promoters to differentially regulate the transcription of these genes in response to changes in environmental osmolarity. Therefore, an OmpR hybrid protein was constructed for use in mobility shift assays to determine the number of OmpR molecules binding to the upstream region of the ompF promoter.

OmpR was fused to protein S, a protein present on the spore coat of a Gram-negative soil bacterium, Myxococcus xanthus (Inouye et al., 1979, 1981). Protein S has a high β-sheet content (90%) and is known to be very stable in solution (Bagby et al., 1994). Two tandem copies of a DNA fragment encoding the amino-terminal 92 amino acid residues of protein S were fused to the 5'-end of the ompR gene to encode a hybrid protein with a molecular mass of 47 kDa, which is 20 kDa larger than OmpR. The purpose of fusing protein S to OmpR was to increase the size of OmpR without affecting OmpR DNA-binding activity. Thus, in a mobility shift assay, the addition of PrS2-OmpR to DNA-binding reactions should simply result in the production of slower mobility bands relative to the mobility of the DNA fragment bound by the intact OmpR molecule. The T7 expression system was used to express this hybrid protein, referred to as PrS2-OmpR (Fig. 1A).

Previous studies have shown that OmpR binds to the -100 to -40 region of ompF. The BamHI-HindIII fragment of pCF001, containing the -100 to -40 region of ompF and referred to as 1001, was used for mobility shift assays. The gel retardation analysis of this fragment with increasing amounts of phosphorylated OmpR is shown in Fig. 1B. A total of 3 bands, labeled a, b, and c, represent binding by different numbers of
OmpR molecules. Band a represents the DNA fragment bound by the minimum number of OmpR molecules under the binding conditions used in the present study. Higher concentrations of OmpR resulted in the binding of all of the DNA with exclusive formation of band c, suggesting that this band represents the DNA fragment bound by the maximum number of OmpR molecules. The existence of three bands indicates that there are three distinct OmpR-binding units in the 100/1 fragment. A similar gel shift pattern consisting of three bands, a, b, and c, is observed when this DNA fragment is incubated with increasing concentrations of PrS2-OmpR (Fig. 1C). These three bound complexes correspond to the three complexes formed when using intact OmpR (Fig. 1C, far right lane), but the mobility of the DNA bound by PrS2-OmpR is slower due to its higher molecular weight. Formation of the complexes required PrS2-OmpR concentrations similar to those of intact OmpR, indicating that fusion of protein S to OmpR did not affect OmpR DNA-binding activity. The PrS2-OmpR-DNA complexes appear as doublets, possibly due to conformational effects imparted by the protein S domain. In vitro copper phenanthroline footprinting in the presence of PrS2-OmpR resulted in a protection pattern identical to that observed in the presence of the intact OmpR (data not shown). This provides further evidence that both OmpR and PrS2-OmpR bind to the 100/1 fragment in an identical manner.

Six OmpR Molecules Bind to the −100 to −40 region of ompF—Incubation of the 100/1 fragment in the presence of 100 nM OmpR resulted in the formation of a single band c. Since this band represents binding by the maximum number of OmpR molecules, we next determined the number of OmpR molecules bound to the DNA in this complex. To do this, 100 nM OmpR was incubated with increasing concentrations of PrS2-OmpR from 5 to 200 nM prior to the addition of the 100/1 DNA fragment. The DNA-binding reactions were then analyzed by gel electrophoresis. As shown in Fig. 2A, a total of seven bound complexes are observed over the range of PrS2-OmpR concentrations. As illustrated to the right of the gel shown in Fig. 2A, this result can be most adequately explained by assuming that there are six OmpR binding sites on the fragment and that both OmpR and PrS2-OmpR have equal affinities to each site. The band with the slowest mobility represents complete displacement of OmpR by PrS2-OmpR. Equal concentrations of OmpR and PrS2-OmpR in the binding reaction result predominantly in the complex formed by binding of three OmpR proteins and three PrS2-OmpR proteins (Fig. 2A, 100 nM OmpR with 100 nM PrS2-OmpR). It is interesting to note that protein S, linked to the N-terminal end of OmpR, appears not to interfere with binding of either OmpR or PrS2-OmpR molecules to the DNA fragment.

Footprinting of the ompF upstream regulatory sequences in the presence of the OmpR2 protein, a DNA-binding mutant, results in protection of the −100 to −60 sequence, but no protection of the −60 to −40 sequence (Mizuno et al., 1988; Tsung et al., 1989). Also, binding studies previously done in this laboratory using a synthetic DNA fragment containing the −100 to −64 sequence of ompF, demonstrated that phosphorylated OmpR can bind this DNA (Ramoversaud et al., 1989). Mobility shift assays of OmpR and PrS2-OmpR with the synthetic DNA fragment containing the −100 to −64 fragment are shown in Fig. 2B. A total of five bands are formed, indicating that four OmpR molecules bind to this fragment. In the presence of increasing concentrations of intact OmpR only (Fig. 2B), two binding events are observed as shown by the presence of two complexes.
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The concentrations of OmpR and PrS2-OmpR are indicated for each lane. The binding reactionswere incubated on ice for 30 min and loaded onto a 5% acrylamide gel and run at 4°C in 1 x TE buffer. To the right of each gel is a diagrammatic representation of the number of OmpR and PrS2-OmpR proteins present in each bound complex. × symbolizes PrS2-OmpR and ○ symbolizes OmpR.

Finally, a 20-bp fragment representing the ompF -100 to -81 sequence was used in a mobility shift assay with OmpR and PrS2-OmpR. With increasing concentrations of phosphorylated OmpR alone, only a single bound complex is formed (Fig. 2C). The addition of increasing concentrations of PrS2-OmpR to OmpR leads to the formation of three complexes (Fig. 2C), indicating that the 20-mer is bound by two OmpR molecules.

The results obtained above indicate that two OmpR molecules bind to each of the three 20-bp sequences in the region downstream of -100 and that a total of six OmpR molecules bind to the region from -100 to -40. The binding of the 100/1 fragment using increasing concentrations of OmpR resulted in the step-wise formation of three distinct complexes. These results suggest that OmpR binds to the DNA "two-at-a-time" and that bands a, b, and c in Fig. 1B correspond to two, four, and six OmpR molecules binding to the 100/1 fragment. There are not sufficient data at present to indicate that OmpR forms dimers prior to binding DNA. Binding by two OmpR molecules may occur in a highly cooperative manner, such that formation of a complex with an OmpR monomer was not detected by the mobility shift assay. It is evident that there are three OmpR binding units in the -100 to -40 sequence of ompF. Each consists of approximately 20 bp, to which two OmpR molecules bind. These units are assigned F1, F2, and F3 and determined to be from -100 to -81, -80 to -61, and -58 to -39, respectively, on the basis of the results above and a consensus sequence as described below (see Fig. 3).

Determination of an OmpR Consensus Sequence—Binding of a 20-mer sequence by two OmpR molecules as shown above poses the question of whether the proteins bind to two direct repeats or two inverted repeats. When the 20-bp OmpR binding sequences F1, F2, and F3 are aligned with another upstream sequence of the ompF promoter (−380 to −361 and assigned F4; Rampersaud et al. (1994) Huang et al. (1994)) and an OmpR-binding sequence of the ompC promoter (−98 to −89 and assigned C1; data not shown), a consensus sequence can be found as shown in Fig. 4. Interestingly, the 5'-half of the consensus sequence is very T-rich; eight out of 10 bases are T. The only non-T bases are A and C at the fourth and fifth positions from the 5'-end, respectively, and the same AC sequence is repeated in the 3'-half. It should also be noted that the T at position 2, the C residue at position 5, and the AC sequence at positions 14 and 15, are the only residues completely conserved in the five OmpR binding sequences (Fig. 4).

Random selection and PCR amplification (Oliphant and Struhl, 1988) was used to determine a consensus sequence for OmpR binding. Initially, an oligonucleotide was designed for OmpR binding in which 20 bp were randomized. After repeated attempts failed, a new PCR template was designed that contained the 5'-11 bp of the F1 sequence. The final PCR template thus used was 46 bp in length, having the following sequence: GACGGATCCTTTTACTTTTGG(N9)CTGTAGGAATTCTCAC. The omf-100 to -90 sequence (underlined) is followed by nine randomized bases (N9) on the 3'-side. After four cycles of binding and PCR amplification, 5% of the DNA was retarded in the mobility shift gel electrophoresis. Essentially 100% of the DNA was retarded after six cycles of binding and PCR amplification. This shifted DNA was eluted, PCR-amplified in the presence of labeled fragment, and used in a mobility shift assay in the presence of 200 nM OmpR and increasing concentrations of PrS2-OmpR. As shown in Fig. 5, the results demonstrate that the selected sequence is bound by two OmpR molecules as judged from the formation of three bands.

The PCR products were subcloned and 20 clones were sequenced. The results are shown in Fig. 6. Most striking are the 100% conserved ACA sequence at positions 3, 4, and 5 of the N9 sequence, and the highly conserved TTACA sequence at positions 1–5. It should be noted that the C at position 4 occurs 10 bp downstream of the C in the template, which represents the C at position -96 within the F1 box. Both C residues correspond to the C residues that are fully conserved in all five OmpR binding sequences shown in Fig. 3. Therefore, it is reasonable to assume that the C residue in the N9 sequence was selected by the distance from the C residue in the portion of the sequence held constant. This accurate determination of the distance can be explained by sequential binding of two OmpR molecules to the 20-mer sequence containing N9; an OmpR molecule first recognizes the motif in the 5'-half, TTAC,
The second G residue (G11) is highly conserved (in three of the
second of two G residues gives a palindromic nature to F1. However, in the F1 sequence, the second of two G residues gives a palindromic nature to F1. The second G residue (G11) is highly conserved (in three of the five sites; see Fig. 4) and the GT sequence (residues 11 and 12 of F1) is palindromic to AC (residues 14 and 15 of F1). When G11 is changed to T (G11 → T; see Fig. 7A), OmpR was still able to bind the sequences (Fig. 7B), albeit weaker than the wild-type 20-mer sequence (Fig. 7B), which exists in a 10-bp subunit sequence; one is the 5'-half and the other is the 3'-half. The 20-mer fragment was not selected in the 3'-half. The results described above support the notion that two OmpR molecules bind tandemly to two direct repeats, each of 10 bp in the 5'-half of the F1 site, which were present in the initial binding template. The highly conserved residues that were selected are in boldface, and a consensus sequence is indicated at the bottom.

Next we analyzed the role of the two fully conserved C residues by individually substituting them with T. Interestingly, the change of C to T at positions 5 and 15 of the 20-mer caused a decrease in binding to approximately 10% of the wild-type 20-mer sequence (Fig. 7B), albeit weaker than the wild-type F1 sequence (Fig. 7B). This result indicates that G11 is dispensable, and thus that OmpR does not bind inverted repeat sequences.

The results described above support the notion that two OmpR molecules bind tandemly to two direct repeats, each of which exists in a 10-bp subunit sequence; one is the 5'-half (which we will call subunit a), and the other is the 3'-half (which we will call subunit b). Examination of all five binding sequences shown in Fig. 4 reveals an interesting feature; 33 Ts out of a total of 50 bases (66%) are found within subunit a, while only 15 Ts out of a total of 50 bases (30%) are found within subunit b. To examine the significance of this finding, the order of subunits was reversed by placing subunit b of F1 as the 5'-half and subunit a of F2 as the 3'-half. The 20-mer fragment thus encompasses the ompF upstream sequence from −90 to −71 (90/71 fragment, Fig. 7A). This fragment was not bound by OmpR (data not shown). Since the substitution of G11...
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### DISCUSSION

The present results unambiguously demonstrated that six OmpR molecules bind to the -100 to -39 region of ompF. We determined that this region contains three individual binding sites, 20 base pairs in length, which are each bound by two OmpR proteins. The binding sites were designated F1, F2, and F3, representing the -100 to -81, -80 to -61, and -58 to -39 sequences of ompF, respectively (Fig. 3). Recent data have shown a far upstream OmpR binding site located at the -380 to -361 sequence of ompF (Huang et al., 1994; Rampersaud et al., 1994), which also shares sequence identity with F1, F2, and F3. Based on the results reported in this paper, we defined this distal site as F4. Although the number of OmpR molecules bound to this site was not determined due to poor binding to this sequence by OmpR, it is reasonable to assume that two OmpR proteins bind to this 20-base pair motif. Similarly, the ompC -98 to -79 sequence is another independent binding site bound by two OmpR molecules (data not shown) and assigned C1.

Although two OmpR molecules bind to a 20-base pair seq-

| 20mer | Sequence |
|-------|----------|
| wt | TTTAGCTTGTCTGATACATATT AAAAGAAAAACAAATGTAATA |
| CST | TTTTTTTTGTCTGATACATATT AAAAGAAAAACAAATGTAATA |
| C15T | TTTACTTCTTCTGATACATATT AAAAGAAAAACAAATGTAATA |
| G11T | TTTACTTCTTCTGATACATATT AAAAGAAAAACAAATGTAATA |
| 90/71 | GTTTAAGTTTTTCTTTTTCAGGAAAAA |

Fig. 7. Mutating the conserved residues of the 20-base pair OmpR binding motif decreases binding by OmpR. Twenty-base pair oligonucleotides were synthesized and annealed to form double-stranded DNA molecules for OmpR binding assays. The sequences are shown in A, wt is the wild-type F1 sequence. Numbers above this sequence indicate the positions of the residues within the 20-bp sites as discussed in the text. Boldface residues indicate the point mutations within the mutated 20-bp sequences. 90/70(T11G) is the ompF -90 to -71 sequence in which the T at position -80 is changed to a G. The mobility shift assays are shown in B. The 20-mers were labeled with [γ-32P]ATP and T4 kinase and incubated with or without in vitro phosphorylated OmpR (100 nM).

with T in F1 resulted in a substantial reduction of OmpR binding (see Fig. 7B), we examined whether the substitution of the T residue with G at position 11 of the 90/71 fragment recovers OmpR binding ability. The resultant fragment (Fig. 7A, 90/71(T11G)) was poorly bound by OmpR (Fig. 7B). These results indicate that the T-richness in the 5’-half of the OmpR-binding 20-mer fragments plays an important role in the coordinate binding of two OmpR molecules to the fragment.

OmpR Binds Cooperatively to ompF Regulatory Sequences—Rampersaud et al. (1994) proposed that OmpR binds to the upstream regulatory sequences in a hierarchical manner, showing that the distal -380 to -360 site (F4; see Fig. 3) is bound only after prior binding to the -100 to -40 sequence. This work suggested that OmpR-OmpR interactions enhance binding of OmpR to this far upstream F4 sequence. Therefore, we further examined the role of OmpR-OmpR interactions in enhancing binding to the F1, F2, and F3 sequences. In mobility shift assays, the 100/1 fragment is completely bound in the presence of 50 nM OmpR (Fig. 8). However, when the F1 box is removed, the resultant fragment (referred to in Fig. 8 as 80/1) is bound poorly by OmpR. Concentrations up to 200 nM OmpR failed to shift all of the labeled DNA. When the 100/1 fragment was incubated in the presence of increasing concentrations of OmpR, three complexes are formed, representing binding by two, four, and six OmpR molecules (Fig. 8, ompF 100/1; also Fig. 1B). However, when the 80/1 fragment containing only the 100 to 80/1 fragment was incubated in the presence of increasing concentrations of OmpR, only a single complex is formed (Fig. 8, ompF 80/1). It was found that this single complex contains four OmpR molecules as determined by a gel shift with PrS2-OmpR (data not shown). This indicates that OmpR will bind both F2 and F3 sequences at the same time, but cannot form a stable complex by binding to only one of the sites. Thus, OmpR-OmpR interactions are essential for OmpR binding to lower affinity F2 and F3 sequences, and OmpR molecules bound to F2 must interact with OmpR molecules bound to F3 in order to form a stable OmpR-DNA complex.

This cooperative binding can be further supported by mutational analysis of the 100/1 fragment containing either the C96T or the C86T mutation, which corresponds to C10T and C15T in Fig. 7A, respectively. The C86T to T mutation completely eliminated OmpR binding to the F1 20-mer (Fig. 7B, lane marked C15T). The mutated sequence was bound by six OmpR molecules as determined by gel shift analysis with PrS2-OmpR (data not shown). However, unlike the wild-type ompF sequence in which incubation with increasing OmpR concentrations leads to the formation of three bands, incubation of increasing OmpR concentrations with the mutated sequences produced only two complexes, b and c (Fig. 9). Gel shift assays with PrS2-OmpR showed that this mutated fragment is bound by six proteins (data not shown). This suggests that although OmpR can bind to the mutated sequence at -96 or -86, this binding requires OmpR binding to the downstream F2-F3 region.
sequence, each 20-mer is considered a single site since binding of a single OmpR molecule was never detected. Three complexes observed in mobility shift assays with OmpR and the DNA fragment containing the F1, F2, and F3 sequences (Fig. 1B) were thus formed by binding of two (to F1), four (to F1 and F2), or six OmpR (to F1, F2, and F3) proteins. Currently, there are not sufficient data to suggest that OmpR dimerizes in solution prior to binding DNA. Methods such as gel filtration and light scattering detect only OmpR monomers.  

2 Co-precipitation of OmpR (not fused to protein S) with PrS2-OmpR bound to a single OmpR molecule was never detected. Three complexes (bound by six OmpR proteins) and c (bound by six OmpR proteins) were formed with the mutated fragment, as demonstrated by binding assays using PrS2-OmpR (data not shown).

To rC86 T mutation was more detrimental to binding than the C15 to T in a 20-base pair binding motif. Introduction of the C96 A mutation within this 20-base pair motif makes it unlikely that OmpR proteins bind to an inverted repeat structure.

Previously, we had assigned each of the direct repeats in F1 as two binding sites, Fa and Fb, which share the 10-base pair sequence NTTACNTTN (Tsung et al., 1989). It should be noted that Fa is highly T-rich in comparison with Fb. The T-richness in the 5'-halves of the 20-base pair motifs is common in all sequences as shown in Fig. 4 and appears to contribute asymmetry to the 20-base pair motif, since exchanging the position between the two halves resulted in the loss of OmpR binding. Maeda et al. (1991) also proposed an asymmetric consensus motif with the sequence TTTTACCTTTTTGTAACAT. Interestingly, Makino et al. (1989) proposed that the OmpR homologue PhoB binds to an asymmetric 18-base pair sequence, the pho box, which is tandemly repeated upstream of promoters controlled by PhoB (Kimura et al., 1989; Kasahara et al., 1991).

Binding of protein dimers to direct repeats has been reported for AraC (Carra and Schleif, 1993), GABP (Thompson et al., 1991), and Matu2 (Smith and Johnson, 1992). In each case, the authors propose the presence of a flexible linker domain between the dimerization domain and the DNA-binding domain. The flexible linker allows AraC (Carra and Schleif, 1993) and Matu2 (Smith and Johnson, 1992) to bind to the nucleotide sequence repeats in either a direct or inverted orientation, as well as to repeats with variable spacing. In contrast, there appears to be little or no flexibility in the positioning of the two OmpR binding sequences within a 20-base pair motif. One possibility is that the nucleotide sequence of the binding motif confers a strict conformation on the DNA to position the two direct repeats such that two bound OmpR proteins can cooperatively interact for stabilization of the complex. T-rich sequences are known to cause bending of DNA, and bending of the ompF upstream regulatory sequence has been experimentally shown. (Mizuno, 1987; Slauch and Silhavy, 1991).

Note that identification of the fully conserved C residues of an OmpR binding site aided in the more precise identification of the F3 site, which is actually two base pairs removed from the F2 site. This positioning of the F3 site may function to place the bound OmpR proteins on the same face of the DNA helix. Since a full helical turn is 10.5 base pairs in length, the 40 base pairs of the F1 and F2 sites may fall short of four full turns. The location of the F3 site may compensate for this.

Computer alignment of the five OmpR binding sites (Fig. 4) agrees well with the established affinity hierarchy, F1 > C1 > F2 > F3 > F4 (Rampersaud et al., 1994). Comparison of these sequences suggests that F3 and F4 have the most mismatches and thus are likely to have the lowest affinity for OmpR. Both F3 and F4 lack the highly conserved G residue at position 11 of the 20-base pair, F4, which seems to have the lowest affinity for OmpR, also lacks the T-richness in its 5'-halves.

Cooperative interactions also affect the ability of OmpR to bind to the sites upstream of the ompF promoter. Rampersaud et al. (1994) showed that binding of the F4 site depended upon prior binding of OmpR to the downstream region. The present data show that cooperative interactions are also important for binding to the lower affinity F2 and F3 sites. Deletion of the F1 site resulted in a decreased affinity of OmpR for the F2 and F3 sites. It was clear that OmpR was not able to bind to the F2 or F3 sites alone. Clearly, binding of these low affinity sites was enhanced by the presence of OmpR-OmpR interactions.

According to the model proposed above, six OmpR proteins bind tandemly to six direct repeats upstream (100 to 39) of the ompF promoter. In addition to these six OmpR molecules,
two other molecules are bound to F4, making a total of eight molecules involved in the regulation of ompF expression. Multiple transcription factor binding sites upstream of a promoter is also known in other systems (for review, see Collado-Vides et al. (1991)). In the case of ompF, this allows more flexibility and fine-tuning in the regulation of transcription, as more OmpR binding shifts the function of OmpR from activation to repression. Binding is clearly regulated by the concentration of phosphorylated OmpR in the cell. An increase in medium osmolarity is accompanied by an increase in the number of phosphorylated OmpR molecules (Forst et al., 1990), leading to binding of additional, lower affinity sites. In addition, the need for cooperative interactions ensures that binding of proteins to high affinity sites precedes binding to adjacent low affinity sites.

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