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DNA-Binding Properties of the Transcription Activator (OmpR) for the Upstream Sequences of *ompF* in *Escherichia coli* Are Altered by *envZ* Mutations and Medium Osmolarity

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Expression in *Escherichia coli* of the genes that encode the major outer membrane porin proteins (OmpF and OmpC) is regulated by the transcription activator protein OmpR and the receptorlike protein EnvZ, which is located in the inner membrane. Using synthesized oligonucleotide fragments containing the OmpR-binding site of *ompF*, we show that soluble extracts and partially purified OmpR derived from both the parent strain grown in nutrient broth plus 20% sucrose and the *envZ11* strain grown in nutrient broth produced high-affinity DNA-binding activity, whereas soluble extracts from the parent strain grown in nutrient broth produced low-affinity binding. We also show that the soluble extracts from the *envZ22*(Am) strain grown in nutrient broth did not produce detectable bound forms of the *ompF* fragments, but low levels of DNA binding were detected with soluble extracts of the *envZ22* strain grown in nutrient broth plus sucrose. In addition, the time course of the repression of OmpF synthesis produced by a shift to high-osmolarity growth medium was correlated with an increase in the DNA-binding affinity of soluble extracts to the *ompF* fragment. These results provide evidence that *envZ* function influences the DNA-binding activity of OmpR and suggest that high-affinity binding of OmpR to the upstream sequences of *ompF* is correlated with the repression of OmpF production.

The pores in the outer membrane of *Escherichia coli* K-12 through which small hydrophilic molecules diffuse passively are formed by the porin proteins OmpF and OmpC (22). The genes encoding OmpF and OmpC are located at 21 and 48 min, respectively, on the *E. coli* chromosome (30, 31). In the *E. coli* strains most commonly used to study osmoregulation, both OmpF and OmpC are expressed when the bacterium is grown at 37°C in minimal medium, whereas OmpC is preferentially expressed and OmpF is repressed when the cells are grown in high-osmolarity medium (4, 14, 32). Numerous genetic studies have shown that the *ompB* operon, which consists of *ompR* and *envZ*, regulates porin expression; it is located at 75 min on the chromosome (7, 8, 28). The DNA nucleotide sequence of this locus has been determined (2, 20, 35). The *ompB* locus encodes two gene products, OmpR and EnvZ, which are apparently produced from a single polycistronic mRNA (20).

OmpR is a DNA-binding protein consisting of 239 amino acid residues that activates transcription of *ompF* and *ompC* binding to sequences approximately 90 base pairs upstream of the transcription initiation site of these genes (5, 9, 11, 13, 18, 23). EnvZ, a molecule consisting of 450 amino acid residues, is an inner membrane protein whose membrane topology (3) is similar to that of the chemotactic transducer proteins Tar (27) and Tsr (1). The periplasmic domain of EnvZ is composed of 115 amino acid residues, and the cytoplasmic domain contains approximately 270 amino acid residues (3).

To date, three *envZ* null mutant strains have been isolated. The *envZ22*(Am) strain was initially shown to exhibit an $OmpF^{+/-}$ $OmpC^{-}$ phenotype (6). Further characterization of this strain indicated that OmpC production could be induced in the presence of 0.3 M NaCl (33) or 20% sucrose (4) and that OmpF production was not effectively repressed by the high-osmolarity growth conditions (4, 33). From genetic analysis of the *envZ22* strain, OmpR function was shown to be unaltered by the amber mutation in *envZ*. In an *envZ* deletion strain (AT142) grown in minimal medium, OmpC was barely produced and OmpF production was noticeably lower than in the parent strain (16). As in the *envZ22* strain, growth in the presence of 20% sucrose induced OmpC production but did not result in efficient repression of OmpF production. Recently, an *envZ*:Tn*10* insertion strain was isolated in which OmpC production was severely reduced and the levels of OmpF were significantly lower than in the parent strain (27). Taken together, these findings indicate that *envZ* is required for expression of *ompC* in intermediate-osmolarity medium and for efficient repression of OmpF production during growth in high-osmolarity medium. Furthermore, *envZ* appears to enhance or potentiate the expression of *ompF* during growth in minimal medium and nutrient broth (4).

In another *envZ* mutant strain, MH1461 (*envZ11*), *ompC* is expressed constitutively and *ompF* expression is greatly reduced (8, 9). The *envZ11* allele was shown to contain a Thr-247-to-Arg conversion in the cytoplasmic domain of this molecule (16). Recently, a suppressor mutation was isolated in OmpR (Leu-16 to Gln) which restored normal porin expression to the *envZ11* strain but did not alter the functional properties of OmpR itself (16). This result provides genetic evidence that EnvZ and OmpR functionally interact.

In this study, we used soluble extracts and partially purified fractions of OmpR to examine the influence of *envZ* and high-osmolarity growth conditions on the DNA-binding properties of OmpR, using DNA fragments containing the upstream OmpR-binding sequence of *ompF*. We demonstrate that OmpR produced in cells grown in high-osmolarity medium and in the *envZ11* mutant strain bound much more tightly to the OmpR-binding region of *ompF* than did OmpR

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TABLE 1. *E. coli* strains

Strain	Relevant properties	Reference
MC4100	F⁻ Δ*lacU169 araD rpsL relA thi flbB*	9
SG477	MC4100 *envZ22*	6
MH1461	MC4100 *envZ11*	9
MH1160	MC4100 *ompB101* (*ompR1*)	9

from the parent strain grown in intermediate-osmolarity medium.

MATERIALS AND METHODS

Bacteria. The strains of *E. coli* used are shown in Table 1.

Media and growth conditions. Nutrient broth (Difco Laboratories, Detroit, Mich.) was used for growth of cells at 37°C. For high-osmolarity conditions, sucrose was added to a final concentration of 20% (wt/vol). M9 minimal medium contained 160 mM Na_2HPO_4, 80 mM KH_2PO_4, 40 mM NaCl, 72 mM NH_4Cl, 0.8 mM $MgSO_4$, 0.4% glucose, 2 μg of thiamine per ml, and 0.008% biotin. Sucrose was added as indicated to a final concentration of 20% to achieve high-osmolarity medium.

Preparation of soluble extracts and electrophoresis procedures. Cells grown at 37°C in the indicated growth medium (50 ml) were harvested during mid-log-phase growth and washed with 20 mM sodium phosphate buffer (pH 7.1). After sonication in 400 μl of sodium phosphate buffer (4°C), the cell extracts were centrifuged (4°C) for 14 min at 393,000 × *g* (TL-100 ultracentrifuge; Beckman Instruments, Inc., Fullerton, Calif.), and the supernatants were collected and stored at −20°C. Soluble extracts were either electrophoresed on a sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gel system (15% acrylamide–0.0625% bisacrylamide) as described previously (4) or used in the DNA-binding assay.

Western blot (immunoblot) analysis. After electrophoresis, the gels were equilibrated in transfer buffer (48 mM Tris, 38 mM glycine, 1.3 mM SDS, 20% methanol) for 60 min at room temperature and electrotransferred onto nitrocellulose as described previously (4). Immunodetection of OmpR was accomplished by using polyclonal anti-OmpR antiserum and either alkaline phosphatase-conjugated goat anti-rabbit antibody (Promega Biotech, Milwaukee, Wis.) or ^{125}I-labeled protein A (Amersham Corp., Arlington Heights, Ill.). Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.).

DNA-binding assay. The binding assay was performed at 24°C in a final volume of 16 μl containing 10 mM Tris hydrochloride (pH 7.4), 1 mM EDTA, 50 mM KCl, 5% glycerol, 0.05% Nonidet P-40, 240 ng of poly(dI-dC) (Pharmacia, Inc., Piscataway, N.J.), and approximately 0.025 pmol of ^{32}P-labeled oligonucleotide fragment. The indicated amount of protein, added either in soluble extract form or as partially purified OmpR, was incubated with the DNA for 20 min, loaded onto a low-ionic-strength 5% polyacrylamide gel (23), and electrophoresed for 3 to 4 h. Gels were dried and exposed for autoradiography. The synthetic oligonucleotides used for creating the DNA-binding fragments were annealed and subsequently radiolabeled with [α-^{32}P]TTP by the Klenow fragment. The DNA fragments were gel purified and finally suspended in 100 to 400 μl of sterile distilled water. Fragment I-IIb-III was produced by ligating equimolar amounts of the individual annealed fragments for 4 h at 20°C. The ligated fragments were radiolabeled with [α-^{32}P]TTP and gel purified as described above.

Partial purification of OmpR. To partially purify OmpR from the parent strain, MC4100, an *ompB*-containing plasmid (pAT428) was introduced into the cells. This increased the amount of OmpR in the cells by a factor of approximately 10-fold, as judged by Western blot analysis (data not shown), and did not affect the steady-state levels of OmpF production in cells grown in either nutrient broth or nutrient broth plus 20% sucrose (4, 12). Cells were harvested during log-phase growth, washed once with 20 mM $NaPO_4$ (pH 7.1), and ruptured in a French press. After centrifugation (100,000 × *g* for 2 h), the soluble fraction was dialyzed against 50 mM Tris hydrochloride (pH 7.8)–5% glycerol–2 mM β-mercaptoethanol. The dialyzed soluble protein was applied to a DEAE-cellulose column and eluted with a linear gradient of 0 to 120 mM NaCl. The entire gradient was monitored by Western blot analysis and DNA-binding activity. A single peak of OmpR eluted at a salt concentration of approximately 70 mM NaCl. OmpR-containing fractions were concentrated by ultrafiltration (Diaflo PM-10) and applied to a Sephacryl 200 column equilibrated in 50 mM Tris hydrochloride (pH 7.4)–150 mM NaCl–2 mM β-mercaptoethanol. OmpR was partially purified approximately 15-fold by this procedure. The same procedure was used to partially purify OmpR from strain MH1461 (*envZ11*). This OmpR was enriched approximately 135-fold. The differences in purification of OmpR from MC4100 (15-fold) and the *envZ11* strain (135-fold) reflect the presence of an *ompR*-bearing multicopy plasmid contained in MC4100.

RESULTS

Western blot analysis of OmpR. In an attempt to understand the effect of *envZ* on OmpR function, in particular the repression of *ompF* expression, we examined the levels and electrophoretic properties of OmpR contained in soluble extracts derived from the parent strain MC4100, the *envZ* missense strain MH1461 (*envZ11*), and the *envZ* nonsense strain SG477 (*envZ22*). The strains listed in Table 1 were grown either in nutrient broth or in nutrient broth plus 20% sucrose, which is a condition that represses OmpF production in the parent strain. We found that the electrophoretic mobilities on SDS-PAGE gels were identical for OmpR in all of the extracts examined (Fig. 1), whereas the steady-state levels of OmpR varied to some degree. For example, the intracellular levels of OmpR increased in MC4100 grown in the presence of sucrose (Fig. 1, lanes 5 and 6), whereas there was no apparent change in the OmpR levels in MH1461 (lanes 7 and 8) and SG477 (lanes 9 and 10) when the cells were grown in the high-osmolarity medium. A more quantitative analysis was performed by using ^{125}I-conjugated protein A. Data derived from scans of autoradiographs taken from SDS-PAGE gels containing extracts obtained from MC4100 grown in either nutrient broth or nutrient broth plus 20% sucrose indicated that there was a twofold increase in the levels of OmpR in MC4100 grown in the presence of sucrose (Table 2). We also found that the levels of OmpR in the *envZ11* strain grown in nutrient broth were approximately fourfold greater than those of the parent strain. However, the intracellular levels of OmpR do not appear to play a principal role in the osmofluctuation of *ompF* and *ompC* expression, since an approximately 10-fold increase in the intracellular OmpR levels produced by a multicopy *ompB*-containing plasmid did not alter the pattern of porin protein production in the parent strain (S. Forst, unpublished observations). We have previously shown that the isoelectric points of OmpR in soluble extracts from the

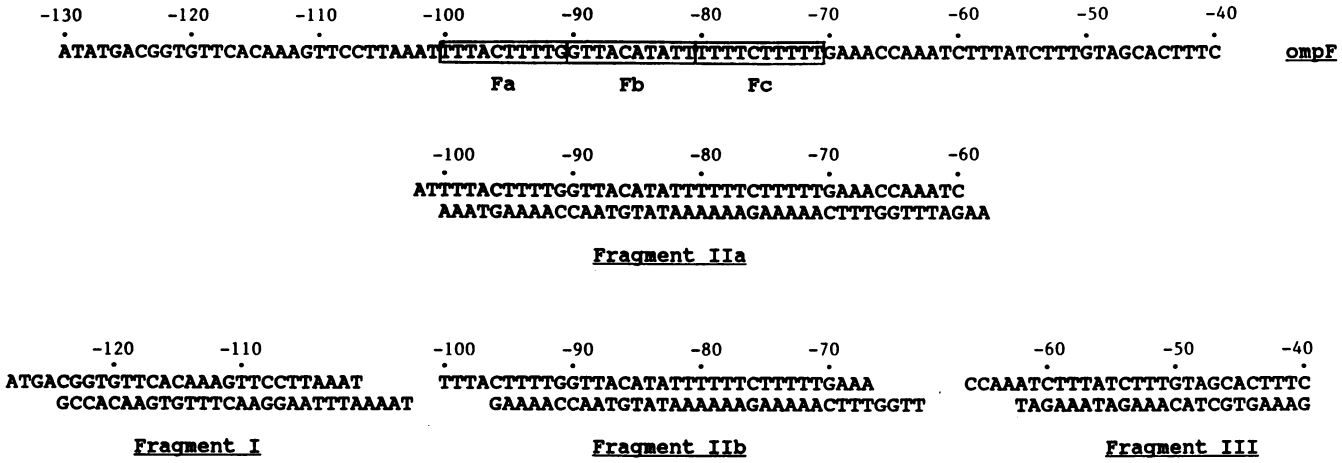

FIG. 1. Oligonucleotide fragments of the OmpR-binding site of *ompF*. Fa, Fb, and Fc represent the consensus sequences for OmpR binding to the upstream sequences of *ompF* (22), which are located between 70 and 100 base pairs upstream relative to the transcription initiation site (+1).

parent strain and the *envZ22* strain grown in either M9 medium or M9 medium plus 20% sucrose are identical (pI 6.8; 4). Similarly, OmpR contained in the *envZ11* extracts had an isoelectric point of 6.8 (data not shown).

Western blot analysis also indicated that the levels of OmpR in the *OmpR1* strain (Fig. 1, lanes 3 and 4), which contains an in-frame deletion from Glu-164 to Arg-182 (21), were too low to be detected by this method. Therefore, the lack of *ompF* and *ompC* expression in this strain could have resulted from either the production of an unstable protein or the low-level production of a functionally inactive molecule.

DNA-binding assays with soluble extracts. To examine the possibility that the OmpR proteins contained in the various soluble extracts were functionally distinguishable, the DNA-binding activities of the soluble extracts were assessed by a gel retardation assay. Radiolabeled synthetic oligonucleotide fragments containing the upstream OmpR-binding region of *ompF* were used for this analysis. The OmpR-binding site of *ompF* had been previously identified by in vitro DNase I footprinting and, more recently, by an in vivo footprinting approach (K. Tsung, R. Brissette, and M. Inouye, J. Biol. Chem., in press). Fragment IIa (Fig. 2) contains the 10-base-pair repeated consensus sequences Fa, Fb, and Fc (previously designated Ia, Ib, and Ic; 23) as well as the sequence TGAAAC (−71 to −66), which is part of the 10-base-pair repeated sequences found at positions −89 to −80 and −68 to −59 of OmpR-binding site of *ompC* (15). Fragment IIb contains essentially the same sequences except that it was designed to enable ligation to the upstream (fragment I) and downstream (fragment III) sequences. In our initial studies, we used fragment IIb and the soluble extracts derived from the *envZ11* strain, which, as described below, produced high-affinity DNA-binding relative to that of the parent strain. The specificity of the DNA-binding activity of the soluble extract is shown in Fig. 3. The soluble extract from *envZ11* produced a mobility shift of fragment IIb (lane 3), whereas the extract from the *ompR1* strain, which did not contain detectable levels of OmpR, did not exhibit DNA-binding activity (lane 2). Furthermore, the DNA-binding activity of the *envZ11* extracts could be blocked by preincubation of the extract with anti-OmpR serum (lane 4) but not by preincubation with anti-EnvZ (lane 5) and anti-OmpA (lane 6) sera. DNA binding was specific for the OmpR-binding sequences, since there was no detectable binding to either fragment I (lane 8) or fragment III (lane 10), but DNA binding was observed for the OmpR-binding sequence of *ompC* (fragment IV, lane 12). Therefore, by using short oligonucleotide fragments containing the OmpR-binding sequence, we were able to study the OmpR-dependent DNA-binding activity of soluble extracts.

We next examined the DNA-binding activity of soluble

TABLE 2. Amount of OmpR protein in soluble extracts

Strain	Medium	OmpR concn (μg/mg of soluble extract)[a]
MC4100	Nutrient broth	1.0
MC4100	Nutrient broth + 20% sucrose	2.3
envZ11	Nutrient broth	3.7
envZ11	Nutrient broth + 20% sucrose	3.9

[a] Values were derived from Western blot analysis of soluble extracts from the indicated cells. OmpR protein was detected by using anti-OmpR serum and ^{125}I-conjugated protein A. Increasing amounts (three doses) of purified OmpR and each of the individual extracts were used for the calculation.

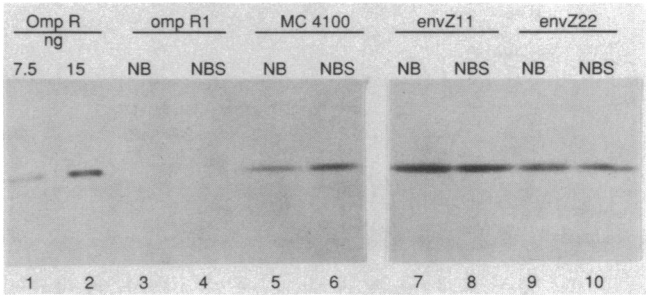

FIG. 2. Western blot analysis of OmpR. Soluble extracts derived from the parent (MC4100) and *envZ* strains were applied to an SDS-PAGE gel, and OmpR was detected as described in Materials and Methods. Purified OmpR was applied to lanes 1 (7.5 ng) and 2 (15 ng). Soluble protein (60 μg) from the *ompR1* (lanes 3 and 4), MC4100 (lanes 5 and 6), *envZ11* (lanes 7 and 8), and *envZ22* (lanes 9 and 10) strains grown in either nutrient broth (NB) or nutrient broth plus 20% sucrose (NBS) was electrophoresed and electrotransferred onto nitrocellulose paper, and OmpR in each extract was detected by alkaline phosphatase-conjugated secondary antibody.

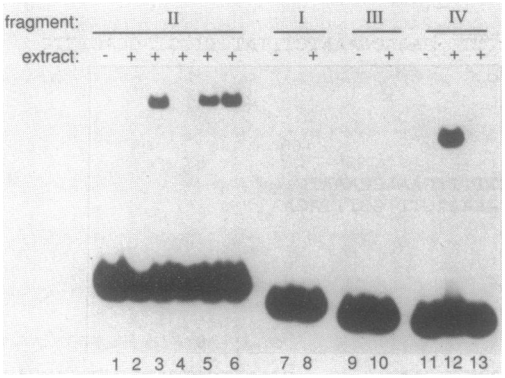

FIG. 3. DNA-binding gel retardation assay, using soluble extracts from the *envZ11* strain. ^{32}P-labeled DNA fragments were used in the DNA-binding assay as described in Materials and Methods. *envZ11* soluble extract (1 μg) was added to the reactions with fragments II (lanes 3 to 6), I (lane 8), III (lane 10), and IV (lane 12). *ompR1* soluble extract (20 μg) was added to the reactions with fragments II (lane 2) and IV (lane 13). *envZ11* extracts were preincubated for 60 min at 4°C with anti-OmpR (lane 4), anti-EnvZ (lane 5), and anti-OmpA (lane 6) antisera before addition of the DNA fragment. −, No extract added. Sequences of the DNA fragments are shown in Fig. 1. Fragment IV (not shown) represents the sequence of *ompC* between −84 and −104 (23).

extracts obtained from the parent strain and the *envZ* mutant strains grown in either nutrient broth or nutrient broth plus 20% sucrose. Experiments indicated that the overall DNA-binding affinity of soluble extracts for fragment IIb was noticeably lower than that for fragment IIa, which is 7 nucleotides longer and contains additional sequences, primarily at the 3′ end (data not shown). Therefore, we used fragment IIa for analysis of the DNA-binding activity of various soluble extracts (Fig. 4). The level of DNA binding of the extracts derived from MC4100 grown in nutrient broth was very low; 30 μg of total protein (~30 ng of OmpR) was required to detect bound forms a and b of fragment IIa. In

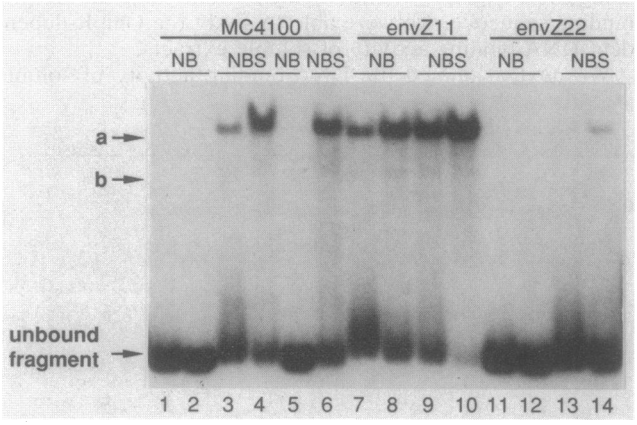

FIG. 4. DNA-binding gel retardation assay of soluble extracts from strains MC4100, *envZ11*, and *envZ22*. ^{32}P-labeled fragment IIa was incubated with the indicated soluble extracts as described in Materials and Methods. The following amounts of soluble extracts were added to the assay: 3 μg (lanes 3, 7, 9, and 13), 6 μg (lanes 4, 8, and 10), 15 μg (lanes 1 and 11), and 30 μg (lanes 2, 5, and 12). The soluble extracts used in lanes 5 and 6 were extensively dialyzed against 20 mM sodium phosphate (pH 7.4) before addition to the assay. NB and NBS, Extracts from cells grown in nutrient broth and nutrient broth plus 20% sucrose, respectively.

TABLE 3. Relative DNA-binding affinities of soluble extracts for fragment IIa

Strain	Medium	Relative DNA-binding affinity[a]
MC4100	Nutrient broth	1
MC4100	Nutrient broth + 20% sucrose	270
envZ11	Nutrient broth	230
envZ11	Nutrient broth + 20% sucrose	460
envZ22	Nutrient broth	ND
envZ22	Nutrient broth + 20% sucrose	30[b]

[a] Values were derived from the results of scanning the autoradiograph shown in Fig. 4 and expressing the amount of bound form of fragment IIa (form a) as a fraction of the total added fragment IIa. Each value was calculated for the amount of OmpR added in each extract (Table 2) and normalized to the value obtained for MC4100 (NB), which was set at 1. ND, Bound forms of fragment IIa not detected.

[b] Approximate value based on the data presented in Fig. 2 in which the amount of OmpR in the *envZ22* extracts (grown in nutrient broth plus 20% sucrose) was approximately 50% that found in the *envZ11* extracts (grown in nutrient broth plus 20% sucrose).

marked contrast, the DNA binding produced by extracts derived from MC4100 grown in the presence of sucrose was much higher; only 3 μg of total protein (~7 ng of OmpR) was required to produce readily detectable bound form a of fragment IIa. Extensive dialysis of these extracts produced the same levels of DNA-binding activity as were found in the undialyzed extracts (Fig. 4, compare lanes 4 and 6). Thus, the dramatic differences in DNA-binding activity we observed were not due to dialyzable inhibitory or stimulatory components in the extracts and presumably reflect changes in the binding affinity of OmpR.

Soluble extracts derived from the *envZ11* strain produced high-affinity DNA binding to fragment IIa (Fig. 4, lanes 7 to 10). With 3 μg of total soluble protein (~11 ng of OmpR) derived from the *envZ11* strain grown in nutrient broth, a significant amount of bound form a of fragment IIa was observed. Growth in the presence of sucrose further enhanced the DNA-binding activity of the *envZ11* extracts. In contrast, the extract derived from the *envZ22* strain, in which *ompF* expression is not effectively repressed, showed a very different pattern. In the absence of *envZ* function, the DNA-binding activity of soluble extracts obtained from nutrient broth-grown cells was below the levels of detection of the gel retardation assay, whereas the DNA binding produced by 30 μg of total protein derived from cells grown in the presence of sucrose was noticeable, albeit at levels well below that observed for the parent strain.

On the basis of the amount of bound forms of fragment IIa and the amount of OmpR contained in the various soluble extracts, we determined a relative binding affinity for the different extracts tested (Table 3). OmpR produced in the parent strain grown in the presence of 20% sucrose showed a 270-fold-higher DNA-binding activity, and the *envZ11* allele stimulated DNA-binding activity approximately 230-fold relative to the level found in the parent strain in the absence of sucrose. In addition, 20% sucrose enhanced DNA-binding activity in the *envZ22* strain to a level that was approximately 10% that observed for the parent strain grown in the presence of sucrose. This *envZ*-independent aspect of the sucrose-induced enhancement of DNA binding may be due to cross-talk between OmpR and the proteins in *E. coli* that share structural similarity with EnvZ (26).

Partial purification of OmpR. The results obtained with the soluble extracts raised the possibility that the differences in

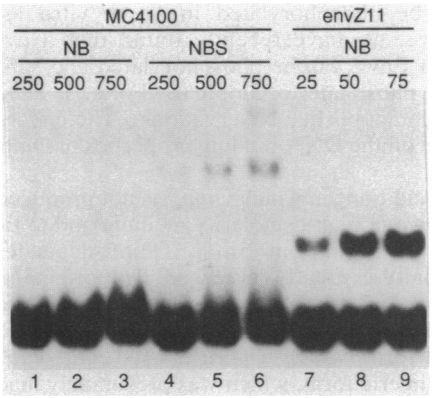

FIG. 5. DNA-binding gel retardation assay of partially purified OmpR. The OmpR protein was partially purified from the indicated cells as described in Materials and Methods. ^{32}P-labeled fragment I-IIb-III was incubated with the amount (in nanograms) of OmpR indicated above each lane. The amount of OmpR added was determined by Western blot analysis.

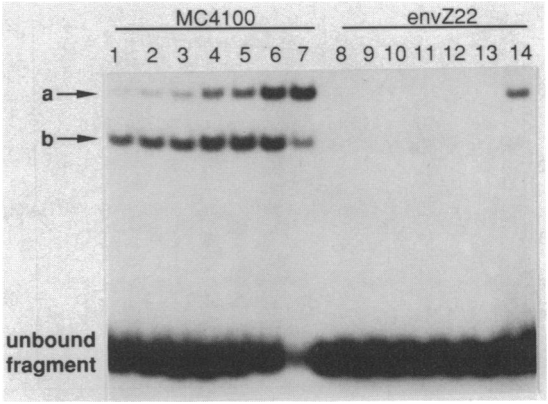

FIG. 6. DNA-binding gel retardation assay of soluble extracts derived from cells during a shift to high osmolarity. Soluble extracts (15 μg) from cells grown in either M9 minimal medium (lanes 1 and 8) or the same medium plus 20% sucrose (lanes 7 and 14) were incubated with ^{32}P-labeled fragment IIa. The shift to high osmolarity (20% sucrose, final concentration) was accomplished as described previously (4), and cells were harvested 5 min (lanes 2 and 9), 10 min (lanes 3 and 10), 20 min (lanes 4 and 11), 30 min (lanes 5 and 12), and 60 min (lanes 6 and 13) after the addition of sucrose. At each time point, 20 ml of culture was rapidly adjusted to 20 mM sodium azide (final concentration) and quickly chilled, and the cells were pelleted and frozen at −20°C. Soluble extracts were prepared as described in Materials and Methods.

DNA-binding activity may be due to changes in the affinity of OmpR for the upstream sequences of *ompF*. To further examine this possibility, we partially purified OmpR from MC4100 grown in either nutrient broth or nutrient broth plus 20% sucrose and from the *envZ11* strain grown in nutrient broth, as described in Materials and Methods. In the partially purified fractions, OmpR constituted approximately 30 to 50% of the total protein. The different OmpR molecules eluted from the DEAE-cellulose column at the same salt concentration, and only a single peak of OmpR was detected, as judged by Western blot analysis and DNA-binding activity. Each of the different OmpR molecules eluted from the gel filtration column at an apparent molecular weight of 30,000, which indicated that they existed as monomers during the purification. We found that the partially pure OmpR from both the parent and *envZ11* strains could no longer bind to fragment IIa even at the levels of approximately 1 μg of OmpR, in contrast to the much lower levels of OmpR required when the protein was present in the soluble extracts. This finding suggests either that other proteins or factors in the extract helped to stabilize the binding of OmpR to the smaller oligonucleotide fragment or that the affinity of OmpR for the DNA fragment was altered during purification. Therefore, we attempted to use a longer fragment to detect the DNA-binding activity of OmpR. When we used the ligated fragment I-IIb-III to perform the gel retardation assay, the partially pure OmpR from the parent was able to bind the DNA fragment (Fig. 5). Although the partially purified OmpR from MC4100 grown in nutrient broth did not produce detectable bound forms of fragment I-IIb-III (lanes 1 to 3), the DNA-binding activity of the partially pure OmpR from MC4100 grown in the presence of 20% sucrose became detectable at the lower levels of added OmpR (lane 4) and progressively increased as more OmpR was added (lanes 5 and 6). These results support the idea that the differences in DNA-binding activity observed with the soluble extracts may have resulted from functional changes in OmpR. In contrast to the OmpR from the parent, partially purified OmpR derived from the *envZ11* strain still exhibited very high binding activity; as little as 25 ng was required to detect bound forms of fragment I-IIb-III (lane 7). The bound form of fragment I-IIb-III produced by the partially pure OmpR from *envZ11* was retarded much less than was the bound form produced by the OmpR from MC4100 (e.g., lane 6). The differences in DNA-binding activity, and the degree of retardation of the bound DNA fragment, between the OmpR of the parent strain grown in 20% sucrose and the *envZ11* strain were not apparent when fragment IIa was used in the gel retardation assay (Table 3) but were readily detectable with the longer DNA fragment (I-IIb-III). These results suggest that OmpR may interact with the flanking sequences contributed by fragment I, fragment III, or both and that the OmpR of the *envZ11* strain binds differently to the *ompF* upstream sequence than does the OmpR of the parent strain.

DNA-binding activity during a shift in osmolarity. To further examine the correlation between the increase in DNA-binding activity of soluble extracts and the repression of OmpF production stimulated by high osmolarity, we prepared extracts from MC4100 grown in M9 minimal medium as well as at various time points after the addition of sucrose to the growth medium (Fig. 6). The DNA-binding activity of soluble extracts obtained from cells grown in minimal medium resulted in retardation of significant levels of the bound form b of fragment IIa, whereas smaller amounts of bound form a were present. The most dramatic increase in DNA-binding activity, as measured by the increased formation of bound form a, occurred between 10 and 20 min. The increase in the levels of bound form a was closely correlated with the repression of de novo synthesis of OmpF (Fig. 7) that was determined by pulse-labeling OmpF at each time point. It should be noted that bound form b also increased during the shift to high osmolarity. Also, the *envZ22* extracts did not produce detectable bound forms of fragment IIa unless the cells were grown under steady-state conditions in the presence of 20% sucrose. Unlike the extracts derived from cells grown in nutrient broth, the M9 minimal medium-grown extracts produced pronounced amounts of bound form b. The molecular details and functional significance of the formation of two OmpR bound forms of the *ompF* fragments are not clear at this time.

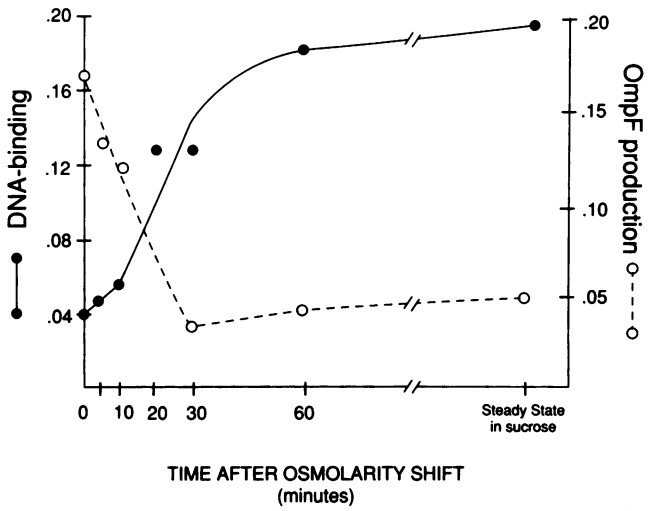

FIG. 7. Correlation of increased DNA-binding affinity and decreased OmpF production MC4100 during a shift to high osmolarity. The DNA-binding values were calculated by scanning the autoradiograph shown in Fig. 6 and expressing the amount of bound fragment IIa (form a) as a fraction of the total added fragment IIa. The values for OmpF production were derived from the previously published pulse-labeling experiments (4) and represent the rate of production of OmpF expressed as a fraction of the total outer membrane protein (OmpF, -C, and -A) produced during the pulse-labeling.

DISCUSSION

In this study, we show that *envZ* function affects the DNA-binding activity of OmpR-containing extracts to the *ompF* fragments; the presence of the *envZ11* allele enhanced binding, whereas the lack of *envZ* function (*envZ22* strain) resulted in greatly reduced binding. These results raise the question of whether EnvZ directly affects OmpR activity. Several lines of evidence suggest that EnvZ function is mediated via OmpR. First, the *envZ11* phenotype is suppressed by a point mutation in OmpR, which provides genetic evidence that OmpR and EnvZ functionally interact (16). Second, the *envZ473* allele, which produces a porin protein expression pattern essentially identical to that of *envZ11*, requires the presence of a functional OmpR to express its phenotype (29). Third, we provide biochemical evidence showing that partially purified OmpR from the *envZ11* strain possesses high-affinity DNA-binding properties and that soluble extracts of the *envZ22* strain exhibit markedly lower OmpR-dependent DNA-binding activity than does the parent strain. Thus, EnvZ appears to promote optimal DNA-binding activity of OmpR to fragment IIa (Fig. 4 and 6). Taken together, these findings support the idea that EnvZ directly modifies OmpR. In another procaryotic regulatory system which senses the availability of nitrogen in the extracellular environment, phosphorylation of an aspartic acid residue (34) of the transcription activator protein NtrC by the sensory protein NtrB, which are structurally similar to OmpR and EnvZ, respectively (26), activates transcription of the *glnA* gene. The phosphorylation of NtrC by NtrB was also recently shown to increase the affinity of the activator for the upstream NtrC-binding sites of *nifLA* (17). Since OmpR and EnvZ belong to this family of two-component regulatory systems, a similar phosphorylation pathway may exist for the differential expression of *ompF* and *ompC*. This possibility appears to be likely, since EnvZ has been shown to be phosphorylated in an in vitro assay (10). Furthermore, we have recently found that a cytoplasmic fragment of EnvZ can be phosphorylated and subsequently transfer its phosphate to OmpR (S. Forst, J. Delgado, and M. Inouye, unpublished observations). The effects of phosphorylation on the DNA-binding properties of OmpR are not yet known.

Under conditions in which OmpF is not produced, such as in cells grown in high-osmolarity medium and in the *envZ11* strain grown in nutrient broth, OmpR-dependent DNA-binding affinity is greatly increased. The original model for the decrease in *ompF* expression proposed that during high-osmolarity growth EnvZ converted OmpR from a monomeric form, which was responsible for *ompF* expression, to a dimeric form, which was presumably inactive with respect to *ompF* (8, 9). Our data do not support the original model but rather indicate that high-affinity binding to the OmpR-binding sites is responsible for the active repression of *ompF* expression. Thus, in cells grown in sucrose and in the *envZ11* strain, at least a portion of the OmpR molecules may exist in a high-affinity form that binds tightly to the upstream OmpR-binding sequence of *ompF*, resulting in repression. Whether the change to the higher-affinity binding activity reflects a change in the binding affinity of a portion or all of the OmpR molecules in the cell is an important aspect of the molecular mechanism of *ompF* repression which we are presently investigating. Recently, a form of OmpR that apparently represses *ompF* expression was proposed to exist in the *envZ473* strain (29). Additional suggestive evidence is derived from genetic analysis of the transcriptional control of *ompF* expression by *ompB* in which, by diploid analysis, strains containing both a wild-type *envZ* and an *envZ11* allele were found to exhibit a codominant phenotype (8), which suggests that the *envZ11* allele converted at least a portion of the OmpR molecules into a repressor form. Further support for the correlation between high-affinity binding and *ompF* repression has been obtained by using in vivo footprinting of the OmpR-binding sequences of *ompF*; in these studies, greater protection of the OmpR binding sequences was noticed in the *envZ11* strain than in the parent strain (Tsung et al., in preparation).

Whether sequences other than the OmpR-binding region (−100 to −70) are involved in the repression of *ompF* expression is not clear. It is possible that the region of *ompF* between −60 and −40 plays a role in repression, since OmpR purified from the *ompR2* strain, in which OmpF is constitutively produced, did not protect this sequence from DNase I digestion, whereas OmpR purified from the parent and *ompR3* strains did afford protection (19). DNA regions located both upstream and downstream of the OmpR-binding sequence have also been suggested to play a role in the osmoregulation of *ompF* (24, 25).

Although high-affinity binding is produced by the OmpR-containing extracts and by partially purified OmpR derived both from the parent strain grown in the presence of sucrose and from the *envZ11* strain grown in nutrient broth, our data suggest that the underlying mechanism for the high-affinity binding may be different for the sucrose-induced affinity change than for that found in the *envZ11* strain. One reason for this speculation is that the DNA-binding affinity of the partially purified OmpR from the *envZ11* strain is much greater than that derived from the parent strain grown in sucrose, and the bound fragment resulting from the *envZ11*-derived OmpR was retarded less than were the fragments from the parent-derived OmpR. It is also important to note that the *envZ11* allele produces a pleiotropic phenotype that

includes suppression of LamB, PhoA, and MalE production (8), whereas sucrose does not reduce expression of these genes and, in fact, has been shown to stimulate the expression of *phoA* (33).

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