Isolated Operator Binding and Ligand Response Domains of the 
TyrR Protein of Haemophilus influenzae Associate to Reconstitute 
Functional Repressor*

(Received for publication, July 13, 1998, and in revised form, September 18, 1998)

Shimin Zhao and Ronald L. Somerville‡

From the Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907-1153

Highly purified preparations of the TyrR protein of Haemophilus influenzae Rd undergo specific and limited proteolytic cleavage during storage at 4 °C to generate two fragments of 28 and 8 kDa. Under non-denaturating conditions, the two fragments remain tightly associated. Nicked TyrR is identical to full-length TyrR in its operator binding characteristics. The 8-kDa fragment containing amino acid residues 258–318 was separated from the 28-kDa fragment (residues 1–257) by gel filtration chromatography in the presence of 4 M urea. Upon renaturation, this fragment bound to operator with an affinity similar to that of full-length TyrR but was unresponsive to ligands that normally modulate operator binding (γ-S-ATP and L-tyrosine). It was not possible to renature the urea-treated 28-kDa fragment. Highly purified soluble preparations of truncated TyrR containing residues 1–257 were obtained after the over-expression of a shortened form of the tyrR gene via a specific plasmid construct. By several criteria, this species had native secondary and tertiary structure. The 28-kDa fragment was unable to bind to operator but could reconstitute nicked TyrR when added to the renatured 8-kDa fragment, as shown by physical properties and responsiveness to cofactors in operator binding. When either the 28- or 8-kDa species was expressed in vivo, there was no detectable operator binding, as evaluated using a lacZ reporter system driven by the repressible araF promoter. When the two fragments were co-expressed in a common cytoplasm, an operator-binding species was formed, as demonstrated through partial restoration of repression capability.

The tyrR gene of Haemophilus influenzae, which has been identified during the determination of the complete genomic sequence of this organism (1), has been cloned and used as the basis of an expression system for the TyrR protein (2). The TyrR protein of H. influenzae was shown to bind to operator DNA both in vitro and in vivo. It is predicted that the TyrR protein of H. influenzae controls a group of genes in H. influenzae similar to those controlled by the TyrR protein of Escherichia coli (2).

Limited trypsin digestion of the TyrR protein of E. coli (513 amino acids) generated two stable subfragments, a 22-kDa N-terminal domain and a 31-kDa central domain (3). The C-terminal segment of E. coli TyrR was completely digested under conditions of limited trypsin digestion. The central domain of the TyrR protein of E. coli has a high degree of sequence similarity to the analogous segments of a family of activators specific for the σ54 form of RNA polymerase (4, 5). The central domain also contains one or more segments that are important for the self-association of TyrR monomers plus at least one ATP binding site and a tyrosine binding site.

The ligand binding sites are important for regulating the interaction of TyrR with its DNA targets. Tyrosine binds to the TyrR protein after it associates with ATP. When the TyrR protein of E. coli binds both ATP and tyrosine, there is an increase in operator affinity. The binding of ATP alone has no effect on operator affinity (3, 6, 7). When the TyrR protein of H. influenzae binds both ATP and tyrosine, its operator binding ability is enhanced. However, one observes a decrease in operator binding with this protein in the presence of ATP alone (2).

The C terminus of the TyrR protein of E. coli contains a helix-turn-helix motif, which is believed to be responsible for DNA binding (7). The TyrR protein of H. influenzae (318 amino acids) has a high degree of sequence similarity to the central and C-terminal domains of the TyrR protein of E. coli (2) but lacks a counterpart to the N-terminal domain, a region that is critical for positive regulation (8). Not surprisingly, the TyrR protein of H. influenzae is unable to activate transcription from promoters that are subject to stimulation by the TyrR protein of E. coli (2).

Despite our current level of understanding of the TyrR protein of E. coli and several related proteins in the NtrC superfamily, little structural information is available for this group of transcription factors. How these proteins are organized and how their diverse regulatory functions are exerted remain unclear. Here we report that the TyrR protein of H. influenzae can be proteolytically cleaved at the boundary between two functional domains. Conditions were established for obtaining each species in pure and native form. The two separated domains reassociate to generate a species whose properties were similar to full-length TyrR protein.

EXPERIMENTAL PROCEDURES

Strains, Bacteriophages, and Plasmids

The bacterial strains, bacteriophages, and plasmids used in this study are described in Table I.

Materials

Phosphocellulose P-11, DEAE-Sepharose CL-6B, Sephacryl S-200, and hydroxylapatite were purchased from Whatman, Sigma Chemical Co., Pharmacia Biotech, and Bio-Rad, respectively.

Buffer A contained 50 mM Tris (pH 7.5) with 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride.

Buffer B contained 50 mM Na₂HPO₄/NaH₂PO₄ (pH 6.5) with 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride.

Buffer C contained 50 mM Na₂HPO₄/NaH₂PO₄ (pH 7.5) with 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride.

* This work was supported by National Institutes of Health Grant GM22131. This is Journal Paper No. 15873 from the Purdue University Agricultural Experiment Station. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed. Tel.: 765-494-1614; Fax: 765-494-7897; E-mail: somerville@biochem.purdue.edu.

This paper is available on line at http://www.jbc.org
Overexpression and Purification of TyrR Protein of H. influenzae

Plasmid pZZ, a derivative of pET-3a containing the coding sequence of the TyrR protein of H. influenzae under the control of the T7 promoter, was used to overexpress the TyrR protein (2). The procedures for overexpression and purification were modified as described below.

Overexpression—Plasmid pZZ was introduced into E. coli BL21(DE3), selecting ampicillin resistance. A single colony was picked and grown in 10 ml of L broth supplemented with 50 µg/ml ampicillin overnight at 37 °C with shaking. The saturated culture was then transferred into 1 liter of L broth supplemented with 50 µg/ml ampicillin. In a 4-liter Erlenmeyer flask, the culture was incubated at 37 °C on a rotary shaker. After the A₆₀₀ of the culture reached 1.0, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM, and the culture was incubated for another 4 h at 37 °C. Cells were harvested by centrifugation at 5,000 rpm for 20 min (Sorvall RC2B).

Purification—All steps were carried out at 4 °C. Cell paste (40 g) was suspended in Buffer A (200 ml). The cells were broken by three passes through a French pressure cell (Amino) operated at 1,500 p.s.i. Cell debris was removed by centrifugation at 17,000 rpm (J21C; Beckman) for 60 min. The supernatant was loaded directly onto a DEAE-Sepharose CL-6B column (3 × 20 cm). The column was washed with Buffer A until the A₂₈₀ was less than 0.1 and then developed using a linear gradient from 0 to 1.0 M NaCl in 500 ml of Buffer A. Individual fractions were analyzed by SDS-PAGE.¹ The TyrR protein eluted at a salt concentration of about 0.4 M. Fractions containing the TyrR protein were pooled. At this stage, the TyrR protein was about 70% pure (Fig. 1, lane 3). The pooled protein fractions, which were identified by SDS-PAGE, were pooled. At this stage, the TyrR protein was around 97% (Fig. 1, lane 4). The pooled protein fractions were precipitated with ammonium sulfate (final concentration, 50% saturation at 25 °C). The resulting precipitate was dissolved in 30 ml of Buffer B and dialyzed against Buffer B (1 liter, three changes). The dialyzed protein was loaded onto a phosphocellulose P-11 column (3 × 20 cm) that was pre-equilibrated with Buffer B. The column was washed with Buffer B until the A₂₈₀ fell below 0.1. Protein was eluted using a 0.1–1.0 M linear gradient of NaCl in 500 ml of Buffer B. TyrR was detected by SDS-PAGE, and the peak fractions were analyzed by SDS-PAGE. The TyrR protein eluted at a salt concentration of about 0.4 M. Fractions containing the TyrR protein were pooled. At this stage, the TyrR protein was about 97% (Fig. 1, lane 5).

Purification of DNA Binding Domain

Sufficient urea was added to 15 mg of proteolytically nicked TyrR (5 ml) to bring the concentration to 4 M (in Buffer C, pH 7.5). The sample was then loaded onto a Sephacryl S-200 (high resolution; Pharmacia) gel filtration column (2.8 × 120 cm). Buffer C containing 4 M urea was used as the mobile phase. This procedure generated two well-separated peptide peaks at 28 and 8 kDa, respectively (Fig. 2B). The 28-kDa fragment was discarded. The 8-kDa fragment, which contained the DNA binding domain, was renatured by dialysis against Buffer C (five buffer changes).

Overexpression and Purification of the 28-kDa N-terminal Fragment

Plasmid construction pZZ257, which is identical to pZZ, except that it encodes only the first 257 amino acids of the TyrR protein of H. influenzae, was constructed by introducing two consecutive UAA (ochre) codons after amino acid 257 and deleting the remainder of the TyrR gene. A polymerase chain reaction method was used to synthesize the desired DNA fragment. The fragment was inserted into pET3a that had been cleaved with Ndel and BamHI as described previously (2).

Overexpression—pZZ257 was introduced into E. coli BL21(DE3), selecting ampicillin resistance. To overexpress the 28-kDa fragment, a single colony was inoculated into 1 liter of L broth supplemented with 50 µg/ml ampicillin. The culture was grown at 37 °C with shaking. The saturated culture was then transferred into 1 liter of Buffer B (three changes). The sample was loaded directly onto a Phosphocellulose P-11 column (3 × 20 cm) that was pre-equilibrated with Buffer B. The column was washed with Buffer B until the A₂₈₀ fell below 0.1. The resulting solution was clarified by centrifugation at 12,000 rpm for 30 min. The pellet was discarded. The supernatant from the previous step was loaded directly onto a DEAE-Sepharose CL-6B column (3 × 20 cm) that had been pre-equilibrated with Buffer B. The flowthrough material containing the 28-kDa fragment was pooled. About 95% of the desired protein was recovered after this step. The 28-kDa fragment constituted about 80% of the total protein recovered (Fig. 3, lane 4). Pooled fractions from the previous step were treated with ammonium sulfate (final concentration, 50% saturation at 25 °C). The resulting precipitate was dissolved in 30 ml of Buffer B and dialyzed against 1 liter of Buffer B (three changes). The sample was loaded onto a Phosphocellulose P-11 column (3 × 20 cm). The flowthrough fractions were collected. About 85% of the total 28-kDa fragment was recovered after this step, with a purity of about 90% (Fig. 3, lane 5). The 28-kDa species was precipitated by 50% saturated ammonium sulfate.

Reconstitution of a Protein Preparation with DNA Binding Properties Similar to Nicked TyrR

Equimolar amounts of purified, renatured DNA binding fragment (61-mer) were mixed with the purified 28-kDa fragment. The mixture was stored at 4 °C for 24 h in Buffer C.

¹ The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; bp, base pair(s).
TyrR protein of *Haemophilus influenzae*: purification and proteolytic cleavage. The TyrR protein and its cleavage products were analyzed by 10% SDS-PAGE. The various species were visualized by staining with Coomassie Blue. Arrows and numbers refer to molecular masses. Lane 1, whole cell lysate; lane 2, supernatant after French press and centrifugation; lane 3, TyrR after passage through DEAE-Sepharose CL-6B; lane 4, TyrR after passage over a phosphocellulose P-11 column (sample missing); lane 5, TyrR after hydroxylapatite column purification; lane 6, TyrR incubated at 4 °C for 3 weeks.

Operator Protection Assay

pUC-aroF is a derivative of pUC19 that carries a 318-bp *Bam*HI-*Eco*RI fragment containing the aroF promoter-operator region. The aroF promoter has three operators, one of which contains a RsaI site. On complete digestion by RsaI, three fragments of 467, 627, and 1899 bp are produced. If the TyrR protein binds to the aroF promoter, the RsaI site within one of the TyrR operators will be protected. As a result, the digestion products will consist of two DNA fragments of 627 and 2366 bp. Operator protection is reflected by the presence of a DNA fragment of 2366 bp. Appropriate dilutions of TyrR protein or protein fragments and pUC-aroF (5 pmol) were mixed in Buffer C in the presence of 0.5 mg/ml bovine serum albumin in a volume of 20 μl. After incubation at 37 °C for 30 min, 2 μl of 10× New England Biolabs buffer 1 (10 mM bis-tris propane-HCl, 10 mM MgCl₂, 1 mM dithiothreitol, pH 7.0) and 1 μl of RsaI (10,000 units/ml) were added. After incubation at 37 °C for 3 h, the digestion products were analyzed by horizontal gel electrophoresis on 1.1% agarose.

RESULTS

Proteolytic Nicking of Purified TyrR Protein—TyrR protein of *H. influenzae* exists as a homodimer in solution. Each monomer has a molecular mass of 36 kDa (2). When purified TyrR protein was stored at 4 °C for 1 week, two additional species of about 28 and 8 kDa, respectively, became detectable (data not shown). After 3 weeks, more than 95% of the TyrR protein had undergone cleavage (Fig. 1, lane 6). Further incubation at 4 °C for 2 more weeks did not lead to further breakdown (data not shown). Sequential Edman degradation analysis showed that the N-terminal amino acid sequence of the 28-kDa fragment was TISKFN, which exactly matches the N terminus of the mature TyrR protein of *H. influenzae* (2). The N-terminal amino acid sequence of the 8-kDa fragment was SAVISL, which matches that of residues 258–273 of the TyrR protein of *H. influenzae*. Protease inhibitors such as phenylmethylsulfonyl fluoride had no effect on the cleavage of TyrR. Ligands of TyrR, namely γ-S-ATP and γ-tirosine, altered the rate of TyrR cleavage. In the presence of 1 mM γ-S-ATP, a nonhydrolyzable analog of ATP, the rate of cleavage of TyrR was accelerated by 50%. γ-tirosine (0.3 mM) slowed the rate of cleavage by 30% (data not shown). To investigate whether the TyrR protein of *H. influenzae* is capable of self-cleavage, this protein was purified according to a previously described protocol (2). The TyrR protein purified in the alternative fashion also generated the same two fragments after incubation at 4 °C.

Isolation of 28- and 8-kDa Fragments—Nicked TyrR was indistinguishable from full-length TyrR in its ability to bind to the aroF operator (Table I). To study the operator binding ability of the 28- and 8-kDa fragments in more detail, various approaches to the separation of these two fragments were explored. Ion exchange chromatography on either DEAE-Sepharose CL-6B or phosphocellulose P-11 matrices failed to separate the two fragments. Gel filtration chromatography on Sephacyr S-200 at neutral pH was also evaluated. When undenatured nicked TyrR was loaded, the two fragments always eluted together (Fig. 2A). A similar result was obtained when gel filtration was carried out in 3 M NaCl (data not shown). However, if nicked TyrR was pretreated in 4 M urea, and Buffer C containing 4 M urea was used as the mobile phase for gel filtration chromatography, the two fragments could be separated (Fig. 2B). The 28-kDa fragment eluted first, as expected; the 8-kDa fragment emerged later. In attempts to renature the two fragments, fractions containing each fragment were pooled and dialyzed against Buffer C. The 8-kDa fragment remained in solution after dialysis, whereas the 28-kDa fragment precipitated when the urea was removed.

Overexpression and Purification of the 28-kDa Species—To obtain the 28-kDa fragment in native form, a truncated gene that encoded amino acids 1–257 of TyrR was constructed. The resulting construct led to high-level accumulation of the 28-kDa fragment in an expression system based on the T7 promoter. Of special interest was the fact that this species was produced in *E. coli* BL21(DE3) in the absence of induction. The

FIG. 1. TyrR protein of *H. influenzae*: purification and proteolytic cleavage. The various species were visualized by staining with Coomassie Blue. Arrows and numbers refer to molecular masses. Lane 1, whole cell lysate; lane 2, supernatant after French press and centrifugation; lane 3, TyrR after passage through DEAE-Sepharose CL-6B; lane 4, TyrR after passage over a phosphocellulose P-11 column (sample missing); lane 5, TyrR after hydroxylapatite column purification; lane 6, TyrR incubated at 4 °C for 3 weeks.

FIG. 2. Separation of 8- and 28-kDa fragments. Samples were taken from the column (Sephacryl S-200; 2 × 130 cm) fractions (each fraction, 2.5 ml) and analyzed by 10% SDS-PAGE. Peptides were visualized by staining with Coomassie Blue. Numbers at the top refer to fraction numbers. Arrows and numbers refer to the molecular masses. A, separation under non-denaturing conditions. B, separation in the presence of 4 M urea. C, refractionation of reconstituted 8- and 28-kDa fragments in 50 mM phosphate buffer, pH 7.5.
basis of this observation is not understood.

After the cells were broken, the 28-kDa fragment remained in soluble form (Fig. 3, lane 1) and was readily purified. Streptomycin precipitation of cellular nucleic acids failed to co-precipitate the 28-kDa species (Fig. 3, lane 3), which is consistent with the absence of a DNA binding motif on this fragment. Ion exchange matrices such as DEAE-Sepharose CL-6B and phosphocellulose P-11 failed to bind the 28-kDa domain under conditions that led to the retention of full-length TyrR (Fig. 3, lanes 4 and 5), which is consistent with a change in pI caused by the removal of the DNA binding motif. However, the 28-kDa species, like full-length TyrR, was retained effectively by hydroxylapatite (Fig. 3, lane 6).

Properties of the Purified 28-kDa Species—To investigate whether the 28-kDa form of TyrR had a secondary and tertiary structure similar to that of the full-length TyrR protein, two experiments were carried out. First, circular dichroism studies of the purified 28-kDa fragment at different temperatures showed that this species had the same heat stability as the full-length H. influenzae TyrR in terms of α-helical content (Fig. 4). It was possible to estimate the α-helical content of each species at different temperatures from the height of the negative peak at 220 nm. Because the temperature at which the α-helical content was half-maximal was almost identical for both the full-length protein and the 28-kDa fragment, it is concluded that each species has the same heat stability (Fig. 4).

Second, the purified 28-kDa species was shown to form heterodimers with full-length TyrR. This abolished the operator binding ability of the full-length protein (Fig. 5).

In Vitro Reconstitution of the 8- and 28-kDa Species—To confirm that the 28-kDa species can physically associate with the 8-kDa fragment in vitro, the purified 28-kDa species was mixed with an equimolar amount of the purified 8-kDa species. After incubation at 37 °C for 30 min, the mixture was subjected to native Sephacryl S-200 gel filtration chromatography (30 cm; pH 7.5). It was found that the elution of the 8-kDa species coincided with that of the 28-kDa species (Fig. 2C). This result supports the idea that these two fragments can associate in vitro.

Operator Binding Properties of the 8-kDa Domain, the 28-kDa Domain, Nicked TyrR, and Reconstituted Mixtures—The full-length TyrR protein of H. influenzae efficiently binds to a target within the aroF operon. DNA binding ability is inhibited by γ-S-ATP and L-tyrosine (2). Proteolytically nicked TyrR was identical to uncleaved TyrR in operator binding ability and response to cofactors (Fig. 6A; Table I). The purified 28-kDa fragment failed to bind to the aroF operator (Fig. 6B). The renatured 8-kDa fragment, which contains the DNA binding motif of the TyrR protein, engaged the aroF operator with the same apparent affinity as full-length TyrR. The binding of the 8-kDa fragment to operator was unaffected by γ-S-ATP and L-tyrosine (Fig. 6C; Table II). When the 28-kDa fragment was added to a mixture containing the 8-kDa fragment, the operator binding properties were unaffected, but this mixture now responded to γ-S-ATP and L-tyrosine by binding to the operator in a less effective manner (Fig. 6D).

Association In Vivo between the 28- and 8-kDa Species—The ability of the separated fragments of TyrR to reassociate in vitro raised the question of whether a similar form of reconstitution might occur in vivo. To address this possibility, we used a sensitive aroF reporter system based on the Δ(tyrR) strain.
there was clear-cut evidence for repression. The reporter enzyme levels were reduced by a factor of 3.5 from 1,500 to 420 Miller units. To explore the structural specificity of in vivo reassociation, plasmid constructs encoding the 8-kDa species with (His)$_6$ extensions at either the N-terminal or C-terminal ends were evaluated. Significant reductions in the level of reporter enzyme were not observed in either case, even when the ends of the histidine-tagged species were raised via isopropyl-1-thio-β-d-galactopyranoside induction.

It is concluded that reconstitution of operator-binding forms of TyrR can occur in vivo. However, either this process is inefficient or the reconstituted species are subject to degradation or turnover, thereby preventing reductions in promoter activity to control (TyrR$^*$) levels.

**DISCUSSION**

The results presented here suggest that the TyrR protein of *H. influenzae* is organized in the form of two domains of 28 and 8 kDa. The two species remain stable in solution and readily reassociate, indicating a distinct domain structure for the TyrR protein of *H. influenzae* and a strong interaction between the two domains. The fact that the TyrR protein of *H. influenzae* can be resolved into two separate functionally active domains provides new insight into the structure-function relationships of this protein. Because the TyrR protein of *H. influenzae* bears significant sequence similarity to the TyrR protein of *E. coli* and the other members of the NtrC superfamily of transcription factors, studies of the TyrR protein of *H. influenzae* have the potential to contribute to our understanding of structure-function relationships of other members of the NtrC superfamily of transcription factors.

Proteolytic cleavage occurred without adding any additional reagents to the purified preparations of the TyrR protein. The basis of this cleavage is not understood. Two possibilities can be proposed. First, the cleavage could be catalyzed by a protease of *E. coli*. This hypothetical protease, which is present in trace amounts, may have been co-purified with the TyrR protein of *H. influenzae* by each of the two methods that were used. If this were the case, the enzyme that cleaves the TyrR protein must be a currently unknown protease of *E. coli*, because the cleavage site in TyrR, which is between a glutamine-serine peptide bond, matches no currently known protease target site in *E. coli*. Second, the cleavage may be a self-digestion process. If this were the case, the cleavage of the TyrR protein of *H. influenzae* could have important regulatory implications. For example, it could be either a way to regulate the amount of TyrR protein itself (via turnover) or a way to generate a separate functionally active form of TyrR that has a different role than undamaged TyrR in vivo. The fact that none of the common protease inhibitors affected the cleavage process but known cofactors of the TyrR protein did alter the rate of cleavage is consistent with a self-cleavage mechanism. However, none of the available facts conclusively prove a self-cleavage mechanism. For example, the effects of cofactors on proteolytic cleavage could be explained by assuming that the cofactors alter the conformation of TyrR so as to either facilitate or hinder proteolytic cleavage. Similarly, the ineffectiveness of common protease inhibitors could indicate that the enzyme that cleaved TyrR is not a serine protease. Additional detailed studies need to be carried out to elucidate the mechanism of the cleavage process.

Most prokaryotic repressors must form dimers to bind to operator DNA targets. For the TyrR protein of *E. coli*, the central domain is responsible for dimerization and ligand binding, whereas the C terminus of TyrR containing the helix-turn-helix motif is mainly responsible for DNA recognition (6). Given the high degree of sequence similarity between the TyrR pro-

---

**Table II**

Operator binding constants for TyrR of *E. coli*, TyrR of *H. influenzae* and its fragments

| Protein species | No additions | + γ-S-ATP | + γ-S-ATP + L-tyrosine |
|-----------------|--------------|-----------|------------------------|
| *E. coli* TyrR  | 0.11         | 8.0×10$^{-3}$ | 2.2×10$^{-2}$          |
| *H. influenzae* TyrR (full length) | 7.8×10$^{-3}$ | 1.2 | 5.2 |
| 8 kDa | 8.0×10$^{-3}$ | 8.0×10$^{-3}$ | 8.6×10$^{-3}$ |
| 8 kDa + 28 kDa (nicked TyrR) | 8.0×10$^{-3}$ | 1.0 | 5.03 |
| 8 kDa + 28 kDa (reconstituted TyrR) | 7.8×10$^{-3}$ | 0.1 | 0.7 |
| 28 kDa | >216 | >216 | >216 |

---

**FIG. 6. Operator protection assays.** For each assay, TyrR or fragments (5 μM) and the amount of operator (5 μg) were kept constant. γ-S-ATP was added to each reaction as follows: 0 mM, lane 1; 0.25 mM, lane 2; 0.50 mM, lane 3; 0.75 mM, lane 4; 1.0 mM, lane 5; 1.25 mM, lane 6. Lane 0 was the Φ x174 HaeIII DNA standard, and arrows with numbers refer to the corresponding band DNA size (bp). A, nicked TyrR. B, the purified and renatured 8-kDa fragment. C, the purified 28-kDa species. D, the reconstituted 8- and 28-kDa species. For a more complete description of this assay, see “Experimental Procedures.”
proteins of *E. coli* and *H. influenzae*, it is reasonable to suppose that the TyrR protein of *H. influenzae* is also composed of a dimerization/ligand binding domain (the 28-kDa domain) and a DNA binding domain (8-kDa domain). The finding that the 8-kDa domain alone can bind to an operator target with an affinity similar to that of the full-length TyrR protein of *H. influenzae* was unexpected. Two possible explanations can be considered. First, the dimerization of the TyrR protein of *H. influenzae* may not be solely determined by the 28-kDa domain. The 8-kDa domain containing the helix-turn-helix DNA binding motif may be able to dimerize on its own. However, the structural elements responsible for the dimerization within the 8-kDa domain need to be defined. Second, it is possible that the 8-kDa species can bind to target DNA as a monomer. This is less likely, because the binding of full-length TyrR to operator was inhibited by the 28-kDa fragment, both in vitro and in vivo, presumably as a consequence of the formation of inactive heterodimers (Fig. 5; Table III). This observation strongly supports the notion that dimer formation precedes the binding of the TyrR protein to its DNA target.

The binding of cofactors has long been known to alter the DNA binding ability of TyrR protein (3). How the signal generated by the binding of cofactors in the central domain of TyrR is transmitted to the DNA binding domain to regulate transcription remains unclear. The ability of the 28- and 8-kDa domains to reassociate to yield a species of TyrR that can mimic the cofactor response of full-length TyrR promises to provide a useful tool for addressing this question.

REFERENCES

1. Fleischmann, R. D., Adams, M. D., White, O., Clayton, R. A., Kirkness, E. F., Kerlavage, A. R., Bult, C. J., Tomb, J.-F., Dougherty, B. A., Merrick, J. M., Mckenney, K., Sutton, G., Fitzhugh, W., Fields, C., Gocayne, J. D., Scott, J., Shiver, R., Liu, L.-I., Gledke, A., Kelley, J. M., Weidman, J. F., Phillips, C. A., Spriggs, T., Hedblom, E., Cotton, M. D., Utterback, T. R., Hanna, M. C., Nguyen, D. T., Saudek, D. M., Brandon, R. C., Fine, L. D., Fritchman, J. L., Fuhrmann, J. L., Geoghagen, N. S. M., Gnehm, C. L., McDonald, L. A., Small, K. V., Fraser, C. M., Smith, H. O., and Venter, J. C. (1995) *Science* 269, 496–512

2. Zhu, Q., Zhao, S., and Somerville, R. L. (1997) *Protein Expression Purif.* 10, 237–246

3. Cui, J., and Somerville, R. L. (1991) *J. Bacteriol.* 173, 1767–1776

4. Pittard, A. J., and Davidson, B. E. (1991) *Mol. Microbiol.* 5, 1585–1592

5. Cornish, E. C., Argyropoulos, V. P., Pittard, J., and Davidson, B. (1986) *J. Biol. Chem.* 261, 403–410

6. Yang, J., Ganesan, S., Sarsero, J., and Pittard, A. J. (1993) *J. Bacteriol.* 175, 500–504

7. Argaet, V. P., Wilson, T. J., and Davidson, B. E. (1994) *J. Biol. Chem.* 269, 5171–5178

8. Cui, J., and Somerville, R. L. (1993) *J. Bacteriol.* 175, 303–306

9. Miller, J. H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

10. Somerville, R. L., Shieh, T. S., Hagewood, B., and Cui, J. (1991) *Biochem. Biophys. Res. Commun.* 181, 1056–1062

11. Heatwole, V. M., and Somerville, R. L. (1991) *J. Bacteriol.* 173, 3601–3604

**TABLE III**

| Plasmids present | *H. influenzae* TyrR species produced | β-Galactosidase |
|------------------|--------------------------------------|----------------|
|                  |                                      | SP1312(lJC1) (tyR<sup>+</sup>) | SP1313(lJC1) (<tyR<sup>-</sup>) |
| None             | None                                 | 54                          | 1500                     |
| pZZ              | TyrR (full length)                   | 34                          | 60                       |
| pSZ110           | 28 kDa                               | 1720                        | 1420                     |
| pSZ108           | 8 kDa                                | 50                          | 1470                     |
| pSZ108 + pSZ109  | 8 kDa + 28 kDa                       | 376                         | 420                      |
| pSZ110           | 8 kDa(C-His)                         | 66                          | 1520                     |
| pSZ110 + pSZ109  | 8 kDa(C-His) + 28 kDa                | 607                         | 1570                     |
| pSZ110 + pSZ109(1PTG) | 8 kDa(C-His) + 28 kDa             | 511                         | 1160                     |
| pSZ111           | 8 kDa(N-His)                         | 61                          | 1490                     |
| pSZ111 + pSZ109  | 8 kDa(N-His) + 28 kDa                | 1280                        | 1450                     |
| pSZ111 + pSZ109(1PTG) | 8 kDa(N-His) + 28 kDa          | 1100                        | 1150                     |

β-Galactosidase levels in *E. coli* strains carrying different TyrR species

Cells were grown in minimal ampicillin (50 μg/ml)-tyrosine (0.5 mM) medium. The values are reported in Miller's units (9). Each value shown above is the result of at least three independent assays.