A Mutational Analysis of the Structural Basis for Transcriptional Activation and Monomer-Monomer Interaction in the TyrR System of *Escherichia coli* K-12†

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In response to the binding of tyrosine or phenylalanine, the TyrR protein (513 amino acids) activates certain promoters and represses others. In a previous study (J. Cui and R. L. Somerville, J. Bacteriol. 175:303-306, 1993), it was shown that promoter activation was selectively abolished in mutant proteins lacking amino acid residues 2 to 9. An additional series of constructs that encoded mutant TyrR proteins having deletions or point mutations near the N terminus were analyzed. Residues Arg-2 and Leu-3 were shown to be critical for the activation of the *mtr* promoter. In confirmation of previous findings, none of the activation-defective mutant TyrR proteins had lost significant repression function. The TyrR protein was shown by chemical cross-linking to be dimeric. The polypeptide segments critical for dimer formation in vivo were identified by evaluating the negative dominance phenotypes of a series of mutant proteins, all defective in DNA binding, lacking progressively greater numbers of amino acid residues from either the N terminus or the C terminus. Amino acid residues 194 to 438 were found to contain all of the essential dimerization determinants.

The TyrR (tyrosine repressor) protein of *Escherichia coli* (513 amino acid residues) (8) regulates the expression of nine genes known as the TyrR regulon (for a review, see Pittard and Davidson [23]). These genes specify proteins that are involved in either the biosynthesis or the transport of aromatic amino acids. The structural gene for the TyrR protein lies at coordinate 1395.5 (29.85 min) of the recently recalibrated *E. coli* map (9, 26), at great distances from the other genes of the TyrR regulon. Seven of the genes of the TyrR regulon are repressed by the TyrR protein (23), one gene (*mtr*) is activated (15, 27), and another (*tyrP*) is regulated either positively or negatively, depending on whether phenylalanine or tyrosine is present (18, 32). In general, tyrosine serves as the cofactor for repression and phenylalanine is the cofactor for activation (23). However, in the TyrR-mediated activation of the *mtr* promoter, either tyrosine or phenylalanine functions as the cofactor (15, 27). The detailed mechanisms for TyrR-mediated repression and activation are unknown.

There are two clearly identifiable structural domains within the TyrR protein: an N-terminal domain (residues 1 to 190) and a second domain (residues 191 to 467). The remainder (residues 468 to 513) may not exist as a discrete domain (11). A presumptive helix-turn-helix DNA-binding motif (residues 482 to 502) lies near the C-terminal end of the TyrR protein (23).

The N-terminal domain of the TyrR protein bears no homology to any other protein whose amino acid sequence has been reported. In a previous study, it was demonstrated that some or all of the nine amino acid residues at the N terminus of the TyrR protein are absolutely required for the activation of the *mtr* promoter. The same residues were dispensable for the repression of the *aroF* promoter (10).

Within the second domain of the TyrR protein lies a polypeptide segment (residues 207 to 425) that has a high

degree of amino acid sequence identity with the central domains of a family of activators specific for the σ^{54} form of RNA polymerase (29). However, the genes of the TyrR regulon are transcribed by the σ^{70} form of RNA polymerase (23). The second domain of the TyrR protein and the central regions of the σ^{54} activators contain an ATP-binding site(s) (2, 11, 24). The binding of ATP and its subsequent hydrolysis are required for transcriptional enhancement by at least one of the σ^{54} activators, the NtrC protein (3, 24). Whether the same holds true for TyrR-mediated activation has not been established. In contrast, an amino acid switch in the presumptive ATP-binding site of the TyrR protein abolished its repression function without affecting the activation function (1, 23).

In the present study, the structural elements at the N terminus of the TyrR protein required for activation were localized to the second and third amino acid residues. These two residues (Arg and Leu) were each individually replaced by six other amino acids. A phenotypic analysis of cells producing the mutant proteins suggested that Leu-3 is critically important to the ability of the TyrR protein to exercise its activation function. Segment 194 to 438, in the second domain of the TyrR protein, was found by genetic means to be essential for a negative dominant phenotype to be observed. This suggests that this region of the protein plays a critical role in the in vivo assembly of a functional TyrR protein.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. The bacterial strains, bacteriophages, and plasmids used in this study are described in Table 1.

Media. Liquid minimal medium containing glucose (0.2%), vitamin B₁ (1 mg/liter), biotin (0.1 mg/liter), and salt mix E described by Vogel and Bonner (31). LB medium (19) contained Bacto yeast extract (5 g/liter), Bacto Tryptone (10 g/liter), sodium chloride (8 g/liter), and glucose (1 g/liter). Solid medium contained 1.5% Bacto Agar (Difco Laborato-

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TABLE 1. *E. coli* strains, phages, and plasmids

Strain, phage, or plasmid	Relevant genotype or description	Reference or source
Strains		
SP1312	<i>zah-735::Tn10 Δ(argF-lac)U169</i>	15
SP1313	As SP1312, but <i>Δ(tyrR)</i>	15
SP1322	As SP1312, but <i>trpR::Ωkan</i>	15
SP1323	As SP1313, but <i>trpR::Ωkan</i>	15
Plasmids		
pET3a	T7 vector	25
pJC100	pET3a- <i>tyrR</i> ⁺	28
pJC110	pUC118- <i>tyrR</i> (1-481)	This work
pJC111	pUC118- <i>tyrR</i> (1-438)	This work
pJC112	pUC118- <i>tyrR</i> (1-366)	This work
pJC113	pUC118- <i>tyrR</i> (1-303)	This work
pJC114	pUC118- <i>tyrR</i> (1-399)	This work
pJC115	pUC118- <i>tyrR</i> (44-481)	This work
pJC121	pUC118- <i>tyrR</i> (94-481)	This work
pJC124	pUC118- <i>tyrR</i> (144-481)	This work
pJC125	pUC118- <i>tyrR</i> (194-481)	This work
pJC130	pUC118- <i>tyrR</i> (220-481)	This work
pJC131	pET3a- <i>tyrRΔ4</i>	This work
pJC132	pET3a- <i>tyrRΔ8</i>	This work
pJC133	pET3a- <i>tyrR</i> (R2A)	This work
pJC134	pET3a- <i>tyrR</i> (L3A)	This work
pJC135	pET3a- <i>tyrR</i> (E4A)	This work
pJC137	pET3a- <i>tyrR</i> (R2K)	This work
pJC138	pET3a- <i>tyrR</i> (R2E)	This work
pJC139	pET3a- <i>tyrR</i> (R2P)	This work
pJC140	pET3a- <i>tyrR</i> (R2W)	This work
pJC141	pET3a- <i>tyrR</i> (R2L)	This work
pJC142	pET3a- <i>tyrR</i> (L3K)	This work
pJC143	pET3a- <i>tyrR</i> (L3E)	This work
pJC144	pET3a- <i>tyrR</i> (L3P)	This work
pJC145	pET3a- <i>tyrR</i> (L3W)	This work
pJC146	pET3a- <i>tyrR</i> (L3I)	This work
pUC118	Cloning vector	30
Phages		
λJC1	<i>aroF'-lacZ'</i> transcription-translation fusion	28
λSLW20	<i>mtr'-lacZ'</i> transcription-translation fusion	15

ries). The following compounds were included when appropriate: tyrosine (1 mM), phenylalanine (1 mM), and ampicillin (100 mg/liter).

DNA preparation. Plasmid DNAs were isolated by the alkaline lysis procedure described by Ish-Horowitz and Burke (17). Cells were transformed by the method described by Cohen et al. (7).

Chemicals and reagents. Restriction endonucleases were purchased from New England Biolabs, Promega, and Stratagene and were used according to the manufacturers' directions. T4 DNA ligase was purchased from Promega. The *Taq* polymerase was obtained from U.S. Biochemicals. The reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and for Western blotting (immunoblotting) were purchased from Bio-Rad. Dimethyl pimelimidate was purchased from Pierce.

Synthesis of mutationally altered *tyrR* DNA by PCR. Specific polymerase chain reaction (PCR) primers were designed to introduce specific restriction endonuclease cleavage sites and to permit either the precise replacement of amino acid residues or an alteration of the boundaries of the *tyrR*-specific coding region. The template DNA for synthesis of

tyrR fragments was Kohara phage 259 (9). The amplification conditions were as follows: 94°C for 1 min, 52°C for 2 min, and 72°C for 2 min. A total of 25 cycles in each case were run. The DNA fragments generated by PCR were freed of protein by treatment with phenol and precipitation with ethanol. Appropriate restriction endonuclease digestions were then carried out on the redissolved DNA as described below.

Construction of plasmids. (i) **pET3a derivatives.** Each of the PCR-amplified *tyrR* fragments contained an in-frame *NdeI* site near the 5' end of the coding region and a *BamHI* site at the 3' end. The PCR fragments and pET3a (25) were separately cut with *NdeI* and *BamHI*, purified with Gene-clean (Bio 101), and then mixed together and treated with T4 DNA ligase. The plasmids of this group (Table 2) were used to study the activation function of the TyrR protein.

(ii) **pUC118 derivatives.** Each of the PCR-amplified *tyrR* fragments contained an *EcoRI* site at the 5' end and a *BamHI* site at the 3' end. The synthetic DNA fragments were digested with *EcoRI* and *BamHI* and then separately ligated with pUC118, which had been similarly cut. This group of plasmids was used in negative dominance studies (see Table 4).

Enzyme assays. β-Galactosidase was assayed by the procedure described by Miller (20), with cells grown to mid-log phase in liquid minimal medium containing tyrosine (1 mM) and ampicillin (100 mg/liter).

Preparation of antibodies directed against the TyrR protein. A sample of TyrR protein (over 95% pure) (11) was subjected to preparative electrophoresis on SDS-PAGE (10% polyacrylamide). The position of migration of the TyrR protein corresponded to a monomer of 58 kDa (11). After being stained with Coomassie blue (0.1%) for 10 min and then destained with doubly distilled H₂O for 20 min, the TyrR band was cut out of the gel and eluted with an ELUTRAP apparatus (Schleicher & Schuell, Keene, N.H.), according to the supplier's instructions.

Initially, 0.4 mg of TyrR protein prepared as described above was thoroughly mixed with an equal volume of Freund's complete adjuvant and injected intramuscularly into a rabbit. At 3-week intervals, the rabbit was given booster injections another four times with 0.1 mg of the TyrR protein with Freund's incomplete adjuvant. Blood was taken from an ear vein a week after the fifth injection. Blood was allowed to clot at 4°C overnight. Serum was collected by centrifugation at 5,000 × *g* for 10 min, aliquoted, and stored at -20°C.

Immunoblotting analysis. Appropriate amounts of cells were pelleted by low-speed centrifugation and resuspended in 2× loading buffer (11). Following electrophoresis (SDS-PAGE [10% polyacrylamide]), standard procedures were used to transfer the separated proteins to a polyvinylidene difluoride membrane and develop the blot. BLOTTO (5% nonfat powdered milk, 0.02% merthiolate in phosphate-buffered saline) was used as a blocking agent. The primary polyclonal anti-TyrR antibody was a 1:10,000 dilution of high-titer antiserum (see above). Peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad) was employed as the second antibody (1:3,000 dilution). Staining was carried out with H₂O₂ and 4-chloronaphthol according to standard procedures (14).

Chemical cross-linking. The cross-linking experiments were carried out at room temperature (25°C) in 0.2 M triethanolamine buffer at pH 8.0 with 1 mg of TyrR protein and 2 mg of dimethyl pimelimidate per ml. Samples were removed at different times and frozen at -20°C. Before

TABLE 2. β -Galactosidase levels in *E. coli tyrR* strains with plasmids encoding mutant TyrR proteins and with *lacZ* reporter systems appropriate for their functional analysis

Plasmid	Residue deleted or changed	β -Galactosidase level ^{a,b}		% of wild-type level (activation function) ^c
		SP1313 (Δ JC1) (<i>aroF-lacZ</i>)	SP1323 (Δ SLW20) (<i>mtr-lacZ</i>)	
pJC131	Δ 4	18	500	0
pJC132	Δ 8	18	450	0
pJC133	R2A	7	1,130	14
pJC134	L3A	7	933	9
pJC135	E4A	7	4,100	78
pJC137	R2K	7	2,680	47
pJC138	R2E	48	1,260	17
pJC139	R2P	7	954	10
pJC140	R2W	16	4,130	79
pJC141	R2L	7	3,350	62
pJC142	L3K	6	525	0
pJC143	L3E	7	514	0
pJC144	L3P	8	500	0
pJC145	L3W	7	1,060	12
pJC146	L3I	10	4,790	93
pJC100	None	7	5,090	100
pET3a	Vector	2,704	501	0

^a β -Galactosidase assays were performed on permeabilized cells grown from fresh inocula to mid-log phase. The values are reported in Miller's units (20); each value shown is the result of at least four independent assays. The standard deviations were no greater than 15%.

^b The cells were grown in minimal ampicillin-tyrosine medium.

^c The values listed refer to activation proficiency in the *mtr-lacZ* reporter system relative to strains harboring pJC100. In making this calculation, the value corresponding to basal-level expression (cells carrying the pET3a vector) was subtracted from each of the observed β -galactosidase activities.

electrophoresis, an equal volume of $2\times$ loading buffer (11) was added, and the mixtures were heated at 100°C for 3 min.

RESULTS

Systems for the analysis of TyrR functions. As previously described (28), the T7 vector pET3a (25) is useful in structure-function studies of DNA-binding regulatory proteins. It is frequently found that the overexpression of such proteins is harmful to the cell. This tends to occur when the regulatory gene is present within a multicopy plasmid. However, in pET3a, in the absence of T7 RNA polymerase, the expression of cloned genes is close to the haploid level (28). A series of DNA fragments carrying TyrR coding sequences were inserted at the *Nde*I (5' end) and *Bam*HI (3' end) sites of pET3a. The amino acid sequences of the mutant TyrR proteins expressed from such constructs were identical to that of the wild-type TyrR protein, except for the designated changes. Of necessity, all mutant proteins, like the wild-type protein, began with Met. The plasmids were introduced into *tyrR* background strains carrying either an *aroF-lacZ* reporter system or an *mtr-lacZ* reporter system. From three to six independent isolates of each construct, all derived from the same PCR, were retained. Each group of isolates was independently grown and assayed in quadruplicate for regulatory phenotype through the use of two β -galactosidase reporter systems.

The *mtr-lacZ* reporter system (Table 1) was used to evaluate the activation function of the TyrR protein derivatives. The wild-type TyrR protein stimulates the *mtr* promoter 8- to 10-fold (15). The *aroF-lacZ* reporter system was used to evaluate the repression function of the TyrR protein derivatives. The wild-type TyrR protein represses the *aroF* promoter over 20-fold (6, 21).

Importance of amino acid residues Arg-2 and Leu-3 for activation function. It was previously shown that the TyrR Δ 9 protein, missing amino acid residues 2 to 9 from the N

terminus, was completely devoid of activation function (10). To locate with greater precision the region of the TyrR protein required for activation of the *mtr* promoter, two new deletion mutants were constructed. The TyrR Δ 8 mutant protein (Fig. 1), like TyrR Δ 9, was totally incapable of activating the *mtr* promoter (Table 2). Another mutant protein, TyrR Δ 4, lacking amino acid residues Arg-2, Leu-3, and Glu-4 (Fig. 1), was also defective in promoter activation (Table 2). The repression functions of the TyrR Δ 4 and TyrR Δ 8 mutant proteins were about the same as that of the wild type (Table 2). The *aroF*-specific β -galactosidase activities in cells containing these two proteins were slightly higher than those in cells containing the wild-type form of

FIG. 1. Deletion mutants (Δ 4 and Δ 8) and point mutants of the TyrR protein constructed and analyzed in the present study. The capital letters refer to the first 10 amino acid residues of the wild-type TyrR protein. The two horizontal lines below the amino acid sequence indicate the end points of the Δ 4 and Δ 8 deletions, respectively. The amino acid switches shown above the wild-type sequence refer to changes that did not abolish *mtr* promoter activation. The amino acid switches shown below the wild-type sequence were associated with activation-defective mutants. +, activation active; -, activation defective.

FIG. 2. Analysis by immunoblotting of protein from *E. coli* $\Delta(tyrR)$ cells producing truncated TyrR proteins and mutant TyrR proteins with single amino acid switches. Plasmid-bearing derivatives of strain SP1323 (Δ SLW20) were grown to stationary phase in LB-ampicillin medium and harvested by centrifugation. The plasmids that were present in each case are described in Table 1. The TyrR⁺ sample came from cells carrying pJC100. The TyrR⁻ sample came from cells carrying pET3a. Each TyrR_{ref} lane was loaded with 50 ng of purified TyrR protein. Each lane of an SDS gel (10% polyacrylamide) was loaded with protein from the equivalent of about 10⁷ cells (for detailed procedures, see Materials and Methods). MW, molecular weight.

the TyrR protein. This result is probably attributable to a slightly lower intracellular level of these mutant proteins (Fig. 2A). These data point clearly to an important role for some or all of amino acid residues 2 to 4 in the activation function of the TyrR protein.

To pinpoint which of these three amino acids are determinants of the activation function, an approach patterned after the technique of alanine scanning (12) was employed. Amino acid residues 2, 3, and 4 were individually changed to alanine. The ability of the resulting mutant proteins to enhance the expression of β -galactosidase from an *mtr-lacZ* reporter system was tested. Mutant protein E4A was 78% as active as the wild-type protein (Table 2). However, mutant proteins R2A and L3A were only 14 and 9% active, respectively (Table 2). Each alanine-substituted protein was fully functional in the repression of the *aroF* promoter (Table 2). By Western blotting analysis, cells containing each of the alanine-substituted proteins were indistinguishable in protein level from cells containing the wild-type TyrR protein (Fig. 2A). These data suggest that amino acid residues Arg-2

and Leu-3 play important roles in the activation of the *mtr* promoter.

Next, amino acid residues Arg-2 and Leu-3 were changed to five other amino acids with very dissimilar side chain groups to evaluate which chemical functionalities are essential for the activation function. The five amino acids that were selected were either positively charged (Arg or Lys), negatively charged (Glu), an α -helix breaker (Pro), bulky (Trp), or hydrophobic (Leu or Ile). When Arg-2 was changed to Lys, the activation activity of the mutant protein was 47% of that of the wild-type TyrR protein (Table 2). The mutant proteins R2W and R2L retained 78 and 62% of the activation activity, respectively, of the wild-type TyrR protein. However, mutant proteins R2E and R2P showed only 17 and 10% of the activation function (Table 2). Each of the mutant proteins having amino acid switches in the second position was expressed at about the same level as the wild-type TyrR protein, except for R2E (Fig. 2B). The intracellular level of the R2E protein, as estimated by immunoblotting, was threefold lower than those of the others. If the level of the R2E protein was the same as those of the others, its predicted activation activity would probably be higher than the observed 17%. Evidently, any of the amino acid residues tested that had large side chains was able to effectively substitute for Arg-2.

When Leu-3 of the TyrR protein was changed to Lys, Glu, or Pro, the activation function was completely lost (Table 2). The L3W mutant protein retained 12% of the wild-type activation function (Table 2). However, the L3I mutant protein had 93% of the wild-type activation function (Table 2). Except for the conservative change to isoleucine, the amino acid at the third position of the TyrR protein could not be replaced by a representative of any of the other groups of amino acids without loss of activation function. Each mutant TyrR protein having substitutions at residue 3 was expressed at levels comparable to those of the wild type (Fig. 2C). The dramatic reductions in activity observed upon replacement of residue Leu-3 of the TyrR protein suggest that hydrophobic interactions involving this amino acid play a role in the activation function. These proposed hydrophobic interactions might involve another part of the TyrR protein or a different protein, such as RNA polymerase.

The repression function of each of the TyrR point mutants was also tested. Of the 10 mutants that were constructed, 9 showed repression activity similar to that of the wild type. In cells expressing these proteins, there was virtually full ability to repress the expression of β -galactosidase from an *aroF-lacZ* reporter system (Table 2). In cells containing the R2E mutant protein, repression was sixfold less effective than in cells containing the wild-type protein (Table 2). This difference is likely to be attributable to the relatively lower amount of this protein (Fig. 2B).

Activation-defective mutant TyrR proteins interfere with the ability of the wild-type protein to execute the activation function. To address the question of whether any other properties of the activation-defective mutants had changed, the ability of the mutant proteins to interfere with promoter activation by the wild-type TyrR protein was tested. Plasmids carrying genes specifying the TyrR Δ 4, TyrR Δ 8, L3E, L3K, L3P, and L3W mutant proteins were separately transformed into the *tyrR*⁺ strain SP1322(Δ SLW20) (Table 1). In this strain, a single-copy *mtr-lacZ* reporter system was present on the chromosome in prophage form. In the absence of any plasmid-encoded mutant TyrR proteins, the *mtr* promoter was about eightfold activated by the wild-type TyrR protein (Table 3). In *tyrR*⁺ cells containing the TyrR Δ 4

TABLE 3. β -Galactosidase levels in *E. coli tyrR*⁺ strains with plasmids encoding truncated and point mutant TyrR proteins

Plasmid	Residue deleted or changed	β -Galactosidase level with SP1322 (λ SLW20) (<i>mtr-lacZ</i>) ^{a,b}
pJC131	$\Delta 4$	2,280
pJC132	$\Delta 8$	2,310
pJC142	L3K	926
pJC143	L3E	1,140
pJC144	L3P	1,030
pJC145	L3W	1,770
pET3a ^c	Positive control	4,280
pET3a ^d	Negative control	560

^a See footnote a of Table 2.^b See footnote b of Table 2.^c The assay was done with the *tyrR*⁺ strain SP1322 (λ SLW20) carrying plasmid vector pET3a.^d This assay was carried out with the *tyrR* strain SP1323 (λ SLW20) carrying plasmid vector pET3a.

and TyrR $\Delta 8$ mutant proteins, the activation of the *mtr* promoter was fourfold (Table 3). In *tyrR*⁺ cells containing the L3K, L3E, and L3P mutant proteins, the activation of the *mtr* promoter was only twofold, compared with *tyrR* cells (Table 3). The mutant protein L3W itself had 12% activation function relative to that of the wild-type TyrR protein (Table 2). In *tyrR*⁺ cells containing the L3W mutant protein, the level of β -galactosidase from the *mtr-lacZ* reporter system was only threefold higher than the level in *tyrR* cells (Table 3). From these data, it is clear that each of the mutant proteins that was tested interfered with the activation function of the wild-type TyrR protein. The apparent functional interference imposed by the TyrR mutant proteins could either reflect the formation of nonfunctional heterodimers with wild-type monomers or the nonproductive occupancy of binding sites (TyrR boxes) upstream of the *mtr* promoter. Regardless of the mechanism, these data suggest that the mutant proteins retained at least two properties distinct from the activation of the *mtr* promoter, namely, essentially full ability to repress (Table 2) and at least partial ability to interfere with the wild-type TyrR activation function (Table 3).

The TyrR protein exists as a dimer in solution. As a DNA-binding protein that engages target sequences that are interrupted palindromes, the TyrR protein is predicted to be a dimer (23). Earlier physical studies have suggested that the TyrR protein is a dimer (2). To directly confirm the dimeric nature of the TyrR protein, chemical cross-linking experiments were carried out. The monomeric subunits of highly purified TyrR protein were readily converted, in a time-dependent manner, to stable species with twice the molecular weight of the monomer upon incubation with the cross-linking agent dimethyl pimelimidate (Fig. 3). This cross-linking reagent reacts with ϵ -amino groups of lysine residues present within oligomeric proteins, provided that the amino groups are separated by no more than 9.2 Å (0.92 nm). The apparent heterogeneity in molecular weight of the observed TyrR dimers following SDS-PAGE probably reflects either cross-linking between lysine located at widely different positions within the TyrR monomers or an intramolecular cross-linking event within an already cross-linked dimer. This result, plus the fact that no species with sizes equivalent to that of a trimer or with larger sizes were observed, strongly suggests that the TyrR protein is a homodimer under normal conditions.

Negative dominance: truncated mutant proteins reduce or

FIG. 3. Chemical cross-linking of TyrR monomers detected on an SDS gel (8% polyacrylamide). The incubation times are indicated on the top. The migration positions of molecular weight markers are indicated at the right. The positions of TyrR monomers and dimers are indicated by the arrows on the left. For details, see Materials and Methods.

abolish the ability of the wild-type TyrR protein to repress the *aroF* promoter. Having established that the TyrR protein is dimeric, it became of interest to determine which regions of this protein are critical for association between monomers. To identify such segments of the polypeptide chain, a genetic approach was employed. The principle of this experimental strategy is that excess inactive mutant TyrR proteins are expected to act as a sink for wild-type monomers, provided that the mutant protein in question is capable of dimerization. It is further assumed that TyrR⁺/TyrR⁻ heterodimers are nonfunctional in repression. The greater the dimerization ability is, the higher are the β -galactosidase values for *tyrR*⁺ cells carrying an *aroF-lacZ* reporter system. A series of mutant *tyrR* genes carrying either N-terminal or C-terminal deletions were constructed (Fig. 4). All of the mutant genes were inserted into pUC118 (Table 1) via *Eco*RI (5' end) and *Bam*HI (3' end). Each mutant construct was introduced into strain SP1312 (λ JC1) (Table 1). This *tyrR*⁺ host contains an *aroF-lacZ* single-copy reporter system on its chromosome. The levels of β -galactosidase in cells sampled during log-phase growth were measured. The levels of the mutant TyrR proteins expressed within the cells were about 50 times greater than that of haploid *tyrR*⁺ cells, as determined by immunoblotting analysis (data not shown).

Initially, TyrR(1–481) (TyrR residues 1 to 481) was made in order to settle two points. First, it was important to determine whether the segment of the TyrR protein carrying the predicted helix-turn-helix motif (residues 482 to 502) is absolutely required for dimerization. Second, when overproduced, the TyrR protein is known to be harmful to the cell. Upon deletion of the DNA-binding element, the deleterious effect of high intracellular levels of the TyrR protein was greatly reduced, as shown by near-normal patterns of growth on various media under conditions in which substantial quantities of the C terminally truncated mutant protein were being formed. As shown by studies with *tyrR* tester strains, the TyrR(1–481) mutant protein was defective both in activation and in repression (data not shown).

When a plasmid carrying the *tyrR*(1–481) mutant gene was introduced into a *tyrR*⁺ strain, the β -galactosidase levels of the *aroF* reporter were 44-fold higher than those in controls (Table 4). This result showed that cells expressing the TyrR(1–481) mutant protein were unable to exhibit the TyrR⁺ phenotype. An additional series of four C-terminal deletions were then constructed in order to define with greater precision the polypeptide segment(s) that might be capable of sequestering monomeric TyrR⁺ protein. The TyrR(1–438) mutant protein caused a 36-fold elevation of

FIG. 4. Schematic representation of N- and C-terminal deletion mutants of the TyrR protein and their effect on the negative dominance phenotype. Wild-type TyrR protein is represented at the top. The lightly striped areas represent the N-terminal domain (residues 1 to 190). The darker striped areas represent the second domain (residues 190 to 467). The dark square in the top of the figure represents the DNA-binding element (residues 482 to 502). The numbers on the left refer to the amino acid residues actually present in the deleted constructs. The effects of the truncated TyrR proteins are summarized on the right. +, negative dominance; -, no negative dominance; $\pm$, slight but detectable negative dominance.

β -galactosidase driven by the *aroF* promoter (Table 4). Cells expressing the TyrR(1-399) mutant protein showed a two-fold derepression of the *aroF* promoter (Table 4). However, in cells expressing mutant TyrR proteins, TyrR(1-366) and TyrR(1-303), there was no effect on the TyrR⁺ phenotype (Table 4). The fact that the levels of reporter enzyme in cells expressing the TyrR(1-438) mutant protein were 18-fold higher than the enzyme levels of cells expressing the TyrR(1-399) mutant protein (Table 4) suggests that segment 400 to 438 contains dimerization determinants. The slight negative dominance observed in cells expressing a TyrR(1-399) protein showed that polypeptide segment 1 to 399 retains a very low level of dimerization capability. The negative dominant phenotype was not observed in cells expressing either the TyrR(1-230) or the TyrR(1-187) protein (data not shown). In summary, the maximum deletion from the C terminus of the TyrR protein that retained reasonable ability to exert negative dominance involved amino acid residues 439 and higher. The elimination of additional amino acid residues (at least within segment 399 to 438) resulted in a version of the TyrR protein that was virtually unable to affect the expression of the TyrR⁺ phenotype.

When the levels of the proteins were examined by immunoblotting, all of the C terminally truncated TyrR proteins were present at about the same level (Fig. 5A). This suggests that the failure of some deletion mutants to derepress the *aroF* promoter was not attributable to any intrinsic instability of these mutant proteins.

A series of *tyrR* genes in which variable numbers of

FIG. 5. Analysis by immunoblotting of *E. coli* cells producing N and C terminally truncated TyrR proteins. Samples were prepared from plasmid-bearing derivatives of strain SP1312 (Δ JC1) grown to mid-log phase in tyrosine-ampicillin minimal medium. The plasmids that were present are described in Table 1. Each lane of an SDS gel (10% polyacrylamide) was loaded with protein extracted from about 5×10^5 cells, except for the sample representing TyrR(144-481), for which the protein from 2×10^6 cells was loaded. The TyrR_{ref} lane is identical to that described in the legend to Fig. 2. For details, see Materials and Methods. MW, molecular weight.

codons were deleted at the 5' end were synthesized in order to locate the N-terminal boundary of the dimerization-determining segment of the TyrR protein. Each N-terminal deletion mutant was constructed in a way that eliminated the C-terminal DNA-binding elements. It was found that cells expressing either TyrR(44-481), TyrR(94-481), TyrR(144-481), or TyrR(194-481) exhibited negative dominant phenotypes (Table 4). These data indicate that the N-terminal domain of the TyrR protein does not contain elements essential for negating the repressing effect of the TyrR⁺ protein. Cells expressing the TyrR(194-481) mutant protein were about twice as effective in exhibiting the phenotype of negative dominance as cells expressing the other N-terminal deletions (Table 4). This may reflect a superior ability of the TyrR(194-481) mutant protein to fold into a stable state. The structural boundaries of this mutant protein correspond to domain junctions defined in limited proteolysis experiments (11). Cells expressing a shorter deletion protein, TyrR(220-481), were functionally normal with respect to the TyrR⁺ phenotype (Table 4). This suggests that this mutant protein may be incapable of forming heterodimers with the wild-type TyrR protein and that essential dimerization determinants lie within polypeptide segment 194 to 219.

The levels of mutant proteins in cells containing different N terminally truncated alleles were examined by immunoblotting (Fig. 5B). With the exception of the TyrR(144-481) mutant protein, the proteins were present at similar levels

that were comparable to that of wild type. By immunoblotting, the TyrR(144–481) protein appeared to be present at about 25% of the level of the other proteins (Fig. 5B).

These data show that polypeptide segment 194 to 438 contains determinants essential for the phenotype of negative dominance. By implication, this segment is likely to contain elements essential for the dimerization of the TyrR protein. Neither residues 439 to 513 nor residues 1 to 193 appear to contain determinants that are critical for dimer formation.

DISCUSSION

Transcriptional activation by the TyrR protein. The effects on activation function of single amino acid switches and deletions near the N terminus of the TyrR protein are shown schematically in Fig. 1. Amino acid residues Arg-2 and Leu-3 of the TyrR protein are particularly important for activating the *mtr* promoter, but these residues play no significant role in the repression function. There appears to be a requirement for an amino acid residue with a large side chain at position 2. Because the functional role of Leu-3 could not be served by several other amino acids (only Ile was able to substitute), it is likely that some form of hydrophobic interaction may be mechanistically important in TyrR-mediated transcriptional activation.

By computer analysis, the first 43 amino acids of the TyrR protein are predicted to be organized in the form of an α -helix. Arg-2 and Leu-3 are likely to be part of this structure. In limited proteolysis studies (11), it was found that the N-terminal 190 amino acids of the TyrR protein constitute a compactly organized domain structure that exposes no trypsin-sensitive peptide bonds, either in the intact TyrR protein or in the polypeptide containing amino acid residues 1 to 190. Although there are 6 Arg residues within the first 43 amino acids of the TyrR protein (8), none of these potentially susceptible peptide bonds were cleaved by trypsin. In particular, the cleavage of the R2-L3 peptide bond has never been observed. This indicates that the N-terminal end of the TyrR protein is not likely to be a solvent-exposed hydrated random coil but is probably part of a tightly organized structural element. Amino acid residues other than Arg-2 and Leu-3 might also participate in the activation function. It is tempting to speculate that the N-terminal segment of the TyrR protein, in the form of an α -helix, interacts as a unit either with some other region of this protein or with another protein component of the transcriptional apparatus, such as RNA polymerase. Our identification of amino acid residues critical to transcriptional activation of the *mtr* gene is likely to provide useful guidance for future studies of how the TyrR protein fulfills its functional roles.

When overexpressed, the central domain of the NifA protein was capable of promoter activation (5). A similar series of experiments with the TyrR system were carried out. Cells that had been engineered to overexpress TyrR(144–481) and TyrR(194–481) had no ability to activate the *mtr* promoter (data not shown). In order for the NtrC protein to carry out its activation function, Asp-54 must be phosphorylated by NtrB (22). NtrC⁺ cells that had been engineered to overexpress the N-terminal domain of NtrC were phenotypically NtrC[−], because of the function of titration of NtrB (13). However, TyrR⁺ cells that overexpressed the N-terminal domain of the TyrR protein were unaffected with respect to transcription from the *mtr* promoter (data not shown). Although these negative results

TABLE 4. β -Galactosidase levels in *E. coli* *tyrR*⁺ strains with plasmids encoding different forms of truncated TyrR proteins

Plasmid	β -Galactosidase level ^{a,b}		
	Residues retained	SP1312 (Δ JC1) (<i>aroF-lacZ</i>)	Fold derepression
pJC110	1–481	2,210	44
pJC111	1–438	1,780	36
pJC114	1–399	110	2
pJC112	1–366	45	1
pJC113	1–303	64	1
pJC115	44–481	618	12
pJC121	94–481	730	15
pJC124	144–481	610	12
pJC125	194–481	1,310	26
pJC130	220–481	78	1
pUC118 ^c	Positive control	50	1
pUC118 ^d	Negative control	3,190	64

^a See footnote a of Table 2.

^b See footnote b of Table 2.

^c This assay was carried out with the *tyrR*⁺ strain SP1312 (Δ JC1) carrying the plasmid vector pUC118.

^d The β -galactosidase activity was assayed for the *tyrR* strain SP1313 (Δ JC1) carrying plasmid vector pUC118.

must be viewed with caution, given the fact that NifA and NtrC affect transcription only from certain promoters, it is not unreasonable to assume that promoter activation by the TyrR protein may be mechanistically different from NifA- and NtrC-mediated activation.

Dimerization and the expression of negative dominant phenotypes. That the TyrR protein exists as a dimer in solution was shown by chemical cross-linking with the bifunctional alkylating agent dimethyl pimelimidate (Fig. 3). To identify the segment of polypeptide essential for the dimerization of the TyrR protein, a genetic approach that used the principle of negative dominance was taken. On the basis of the ability of certain N or C terminally truncated alleles to functionally abolish repression in a tester strain with a *tyrR*⁺ allele, it was established that amino acid residues 1 to 193 and 439 to 513 were nonessential for dimerization (Fig. 4). The TyrR(1–481) mutant protein was more effective in the negative dominance test than the TyrR(1–438) and TyrR(44–481) mutant proteins, suggesting that segments 439 to 481 and 1 to 43 contribute slightly to dimerization. However, these residues are not essential for this process. The 18-fold difference in negative dominance between TyrR(1–438) and TyrR(1–399) suggests that segment 400 to 438 is very important for dimerization.

A deletion of the N-terminal domain (residues 1 to 190) generated a version of the TyrR protein that retained negative dominance activity (Table 4). This suggests that the N-terminal domain is dispensable for dimer formation. The TyrR(220–481) mutant protein was completely devoid of negative dominance over TyrR⁺, implying that the polypeptide segment containing amino acid residues 194 to 219 contains important determinants for dimerization.

Taken as a whole, these studies point to segment 194 to 438 as the most likely location of amino acid residues essential for interaction between monomers of the TyrR protein. In addition, the dimerization of the TyrR protein seems to involve multiple sites. From the data described above, it appears that segments 1 to 43 and 439 to 481 play subordinate roles in the formation and/or stabilization of TyrR dimers.

The segment essential for dimerization (194 to 438) coincides with the region (207 to 425) of amino acid sequence

homology between the TyrR protein and the central domains of σ^{54} activators such as NtrC, NifA, and DctD. In the case of the NifA and DctD proteins, the central domains, expressed in vivo as truncated proteins, were capable of promoter activation (5, 16). If our results had predictive value for these systems, the central domains of these proteins are also likely to be dimerization competent.

The use of deletion mutations of *nifA* and *ntrC* to study dimerization has not been reported elsewhere. However, it has been established in these systems that amino acid switches in the ATP-binding sites led to mutant proteins that remained dimerization competent, in that they were able to negate the activating effects of wild-type NifA or NtrC proteins expressed in *trans* (4, 5). This implies that the binding and/or hydrolysis of ATP is not important in monomer-monomer association in this class of proteins and that the ATP site serves some other (probably regulatory) function. It will therefore be of particular interest to explore in detail the consequences of alterations within the predicted ATP-binding site of the TyrR protein. The experimental tests and reporter systems developed in the course of the present study will lend themselves readily to such studies.

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